Quantitative Analysis of Anti–Hepatitis C Virus Antibody–Secreting B Cells in Patients With Chronic Hepatitis C

Takeji Umemura,[1,2] Richard Y.-H. Wang,[1] Cathy Schechterly,[1] J. Wai-Kuo Shih,[1] Kendo Kiyosawa,[2] and Harvey J. Alter[1]

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.

From the [1]Department of Transfusion Medicine, Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, MD; and [2]Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan.

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Address reprint requests to: Harvey J. Alter, M.D., M.A.C.P., Department of Transfusion Medicine, Warren Grant Magnuson Clinical Center, Building 10, Room 1C711, National Institutes of Health, Bethesda, MD 20892-1184. E-mail: halter@cc.nih.gov; fax: 301-402-2965.

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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 (10^5 · 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.

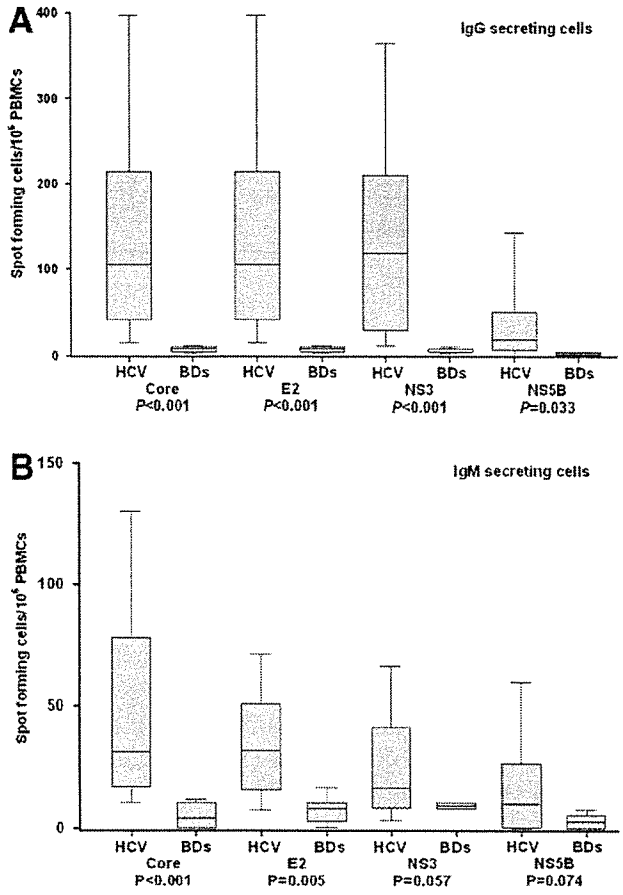

Fig. 1. Detection of anti-HCV antibody-secreting B cells in patients with HCV infection and volunteer blood donors. Boxes represent the IQR of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, 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/

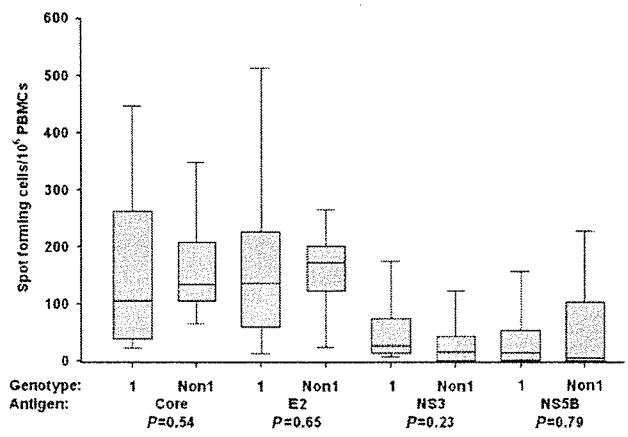

Fig. 2. Detection of anti-HCV IgG-secreting B cells in patients infected with HCV of genotype 1 and nongenotype 1. Boxes represent the IQR of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, 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.

10^6 PBMCs; IQR, 13.3-149.7) than in recovered patients (15.3 SFCs/10^6 PBMCs; IQR, 3.3-142.7; $P = .05$) and normal controls (5.3 SFCs/10^6 PBMCs; IQR, 2.7-8.0; $P < .001$). The median numbers of the sum of anti-IgG–secreting B cells to structural antigens were not significantly higher in patients with chronic hepatitis C (108.3 SFCs/10^6 PBMCs) than in those who recovered (97.4 SFCs/10^6 PBMCs) (Fig. 4A). In contrast, the median numbers of the sum of anti-HCV IgG-secreting B cells to nonstructural antigens were significantly higher in patients with chronic hepatitis C (19.0 SFCs/10^6 PBMCs) than in patients who recovered (4.9 SFCs/10^6 PBMCs; $P = .018$), particularly for NS3 antigen (26.7 vs. 5.3 SFCs/10^6 PBMCs; $P = .032$) (Fig. 4B). Furthermore, patients with chronic hepatitis C had a significantly higher frequency of anti-HCV IgG-secreting B cells to the NS3 antigen than those who recovered (85% vs. 44%; $P = .02$) (Fig. 4C).

The median numbers of the sum of anti-HCV IgM-secreting B cells to all HCV antigens were similar in patients with chronic hepatitis C (22.0 SFCs/10^6 PBMCs; IQR, 8.2-49.3) and recovered patients (20.7 SFCs/10^6 PBMCs; IQR, 12.2-36.7) and were significantly higher than in the controls (8.0 SFCs/10^6 PBMCs; IQR, 0.0-10.7; $P < .001$) (Fig. 4A). When the responses were analyzed for structural and nonstructural antigens, the median numbers of the sum of anti-HCV IgM-secreting B cells were not significantly different in patients with chronic hepatitis C and recovered subjects for either structural antigens (30.7 vs. 31.6 SFCs/10^6 PBMCs) or nonstructural antigens (20.7 vs. 12.7 SFCs/10^6 PBMCs) (Fig. 4A).

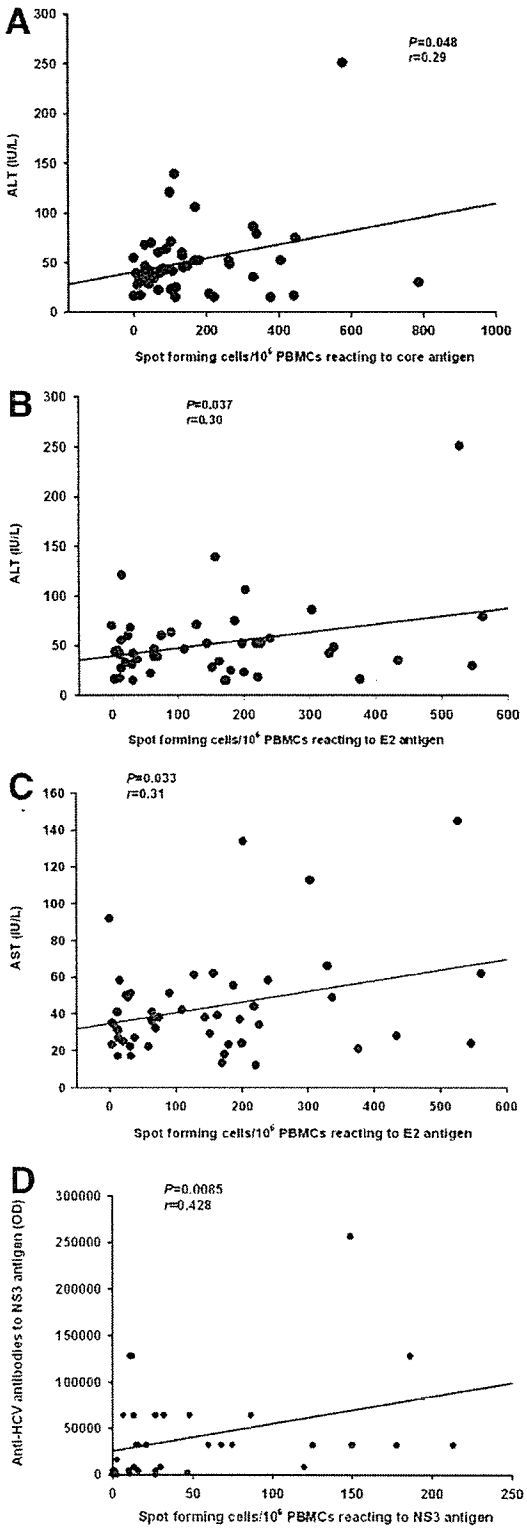

Fig. 3. Correlation of the number of anti-HCV IgG-secreting B cells and clinical characteristics in 48 patients with HCV infection. (A) Frequency of circulating anti-HCV IgG-secreting B cells to core antigen was significantly correlated with the value of ALT ($r = 0.29$, $P = .048$). (B-C) Frequency of circulating anti-HCV IgG-secreting to E2 antigen was correlated with the value of (B) ALT ($r = 0.30$, $P = .037$) and (C) AST ($r = 0.31$, $P = .033$), respectively. (D) Frequency of circulating anti-IgG-secreting B cells to NS3 antigen was correlated with the value of anti-HCV antibodies to NS3 antigen ($r = 0.43$, $P = .0085$). ALT, alanine aminotransferase; PBMCs, peripheral blood mononuclear cells; AST, aspartate aminotransferase; HCV, hepatitis C virus.

Discussion

We developed an ELISpot assay for sensitive quantitative assessment of anti-HCV antibody-secreting B cells in PBMCs from patients with HCV infection and used this technique to analyze the induction of humoral immune responses at the single-cell level. IgG and IgM anti-HCV antibody secreting B cells to core, E2, NS3, and NS5 were detected and quantified in patients with chronic HCV infection and compared with recovered patients and uninfected controls. The key findings were: (1) anti-HCV secreting B-cell responses were greater in chronically infected patients than in recovered patients, suggesting that antibody does not play a major role in recovery from acute

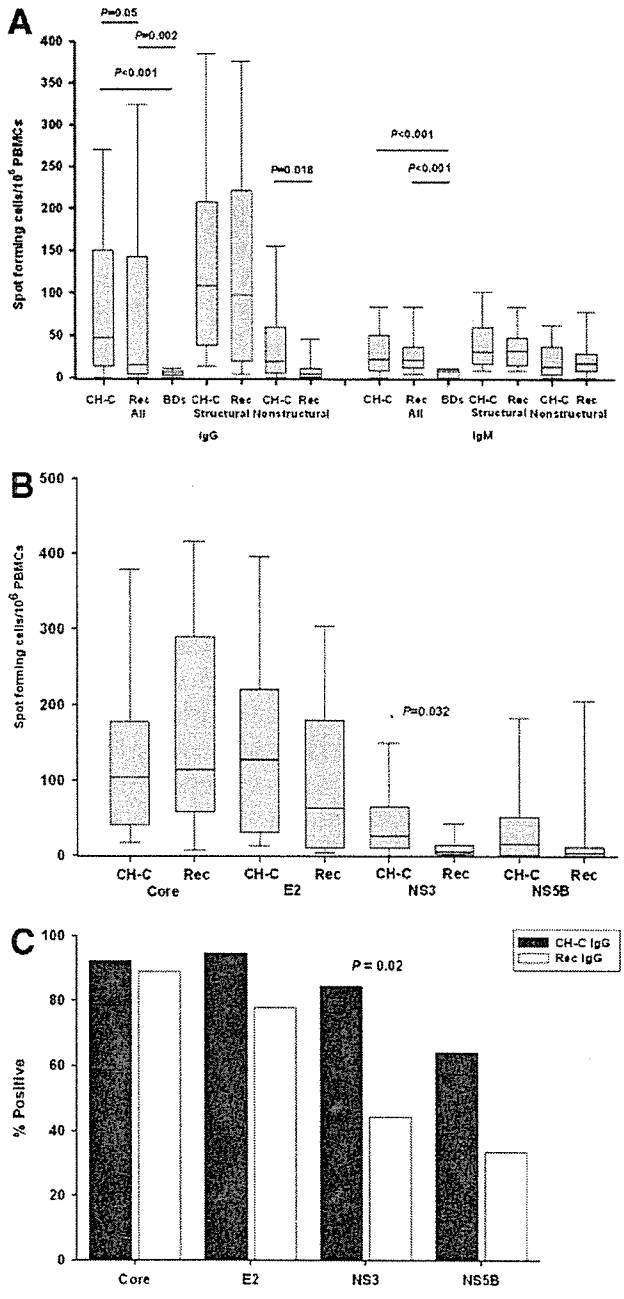

Fig. 4. Detection of anti-HCV antibody-secreting B cells in patients with chronic hepatitis C and in patients who had recovered from HCV. (A) Circulating anti-HCV IgG-secreting B cells were detected in 39 patients with chronic hepatitis C, 9 patients who had recovered from HCV infection, and 11 volunteer blood donors. Circulating anti-HCV IgM-secreting B cells were detected in 34 patients with chronic hepatitis C, 9 patinets who had recovered from HCV infection, and 6 volunteer blood donors. (B) Frequency of circulating anti-HCV IgG-secreting B cells to 4 HCV antigens were detected in 39 patients with chronic hepatitis C and in 9 recovered patients. (C) The prevalence of anti-HCV IgG-secreting B cells in 39 patients with chronic hepatitis C and 9 recovered patients. PBMCs, peripheral blood mononuclear cells; CH-C, chronic hepatitis C; BDs, blood donors; Rec, recovered; IgG, immunoglobulin G; IgM, immunoblobulin M.

HCV infection, as also indicated by recently developed pseudotype assays for HCV-neutralizing antibodies[24,25]; (2) the primary difference between chronically infected and recovered subjects was in the greater reactivity of the former to nonstructural antigens; (3) in chronic infection, HCV antibodies were cross-reactive against genotypes, again consistent with recent findings by neutralizing antibody assays[26,27]; (4) the ELISpot assay can measure IgM as well as IgG responses at the single-cell level, providing a new means to measure the more elusive IgM response; (5) IgM responses were surprisingly well maintained during chronic infection; and (6) IgG responses correlated positively with serum transaminase levels.

In this study, the B-cell ELISpot assay showed high specificity (91% to 100%) and sensitivity (58% to 92%) to all HCV antigens through analysis of the ROC curves and thus achieved high diagnostic accuracy. Although there was a general problem that raw numbers of SFCs were low, statistical analysis and prior publications[28,29] suggest that these small differences are consistent and relevant. Of note, individuals infected with nongenotype 1 variants were strongly positive in this assay, which used only genotype 1–derived antigens. This suggests that genotype 1 contains conserved epitopes that will allow the ELISpot assay to assess humoral immune responses to HCV irrespective of genotype (Fig. 2), with the caveat that we did not assess genotypes 4, 5, and 6, all of which are rare in the United States.

ELISpot assay has been used as a sensitive and specific tool to measure B-cell responses in autoimmune diseases[28,29] and viral infections such as cytomegalovirus,[15] rotavirus,[16] measles virus,[17] and hepatitis B virus,[18,19] as well as to evaluate responses to bacterial[30] and viral vaccines.[18,19] Other reports demonstrate that ELISpot is able to detect and numerate antigen-specific memory B cells in PBMCs after *in vitro* stimulation in both autoimmune diseases and viral infection.[31,32] Thus, the B-cell ELISPOT assay might be a useful tool to detect anti-HCV-specific memory B cells, and to monitor the efficacy of future HCV vaccines.

Interestingly, this study showed a strong correlation between the numbers of anti-HCV IgG-secreting B cells to the core and E2 antigens and the values of serum transaminases. The clinical significance of these observations is unknown, but raises the possibility that antibodies can contribute to liver cell injury. In addition, Ni et al.[33] recently reported that 10 of 36 hepatitis C patient samples showed increased B-cell frequencies that correlated with the degree of hepatic fibrosis. There are insufficient histological data in our study to assess whether the numbers of anti-HCV antibody–secreting B cells correlate with the degree of fibrosis as well as biochemical evidence of inflammation.

The median numbers of the sum of anti-HCV IgG-secreting B cells to nonstructural antigens were significantly higher in patients with chronic hepatitis C than in recovered patients. Similarly, an HCV-specific B-cell response was more frequently detected in patients with chronic hepatitis C than in recovered subjects (92% vs. 56%; $P = .017$) and was directed against a broader range of HCV antigens, particularly to NS3. In contrast, CD4 T-cell responses to NS3 epitopes are greatest in patients who recover from HCV infection.[34,35]

We have also developed and evaluated the ELISpot assay for detecting anti-HCV IgM-secreting B cells. It has been reported that IgM anti-HCV in serum might be predictive of viral clearance in acute hepatitis C or response to interferon therapy.[36-40] However, these results have been controversial and other studies have shown a significant correlation between IgM anti-HCV levels in serum and the recurrence of hepatitis C after liver transplantation.[41,42] In this study, we found that IgM-secreting B cells persisted during chronic infection so that the usefulness of IgM detection for assessing acute versus chronic HCV infection would have to depend on quantitative differences in IgM level rather than the simple presence or absence of IgM antibody. The fact that there are no standardized assays for measuring IgM anti-HCV in serum and the ready detection of IgM-secreting B cells in this study suggests that the ELISpot assay could be used to better define the clinical relevance of IgM antibody in acute and chronic HCV infection.

Overall, this study, as do studies of HCV-specific neutralizing antibodies,[26,27] suggest that the humoral arm of the HCV immune response is not a critical element of spontaneous viral clearance. However, because of the difficulty in obtaining serial acute-phase PBMC collections from recovering subjects, our study does not exclude a role for antibody-mediated viral clearance early in HCV infection. Sequential acute phase ELISpot IgM testing of PBMCs is planned in forthcoming chimpanzee infectivity studies. Nonetheless, studies of neutralizing and antienvelope antibodies that measured serial acute phase serum samples from recovering subjects[26,27] did not show that such antibodies correlated with viral clearance. Rather, it appears in those studies and the current study that antibodies to HCV increase in strength and broadness of reactivity during the course of chronic infection, presumably because of persistent antigenic stimulation. This is in contrast to cell-mediated immunity that is markedly diminished in chronically infected compared with recovered subjects. This dichotomy between the humoral and cellular immune response to HCV is intriguing and suggests T-cell tolerance in the absence of B- cell tolerance.

It is interesting to speculate on the role that antibodies might play in HCV infection. First, it seems reasonable that such antibodies complexed to virus would reduce the level of free virus and diminish transmission to others. This reduction in free virus in addition to lowered viral load might explain the relative rarity of sexual and perinatal transmission during chronic HCV infection. More intriguing is whether such antibodies establish the set point for viral load during chronic infection. It is known that viral loads are highest early in HCV infection prior to the appearance of antibody[43] and that chronically infected patients establish a lower and relatively constant level of viremia.[44] It appears that production and elimination of virus achieve a steady state. This steady state is probably multifactorial in origin, but antibody may play a key role. When patients in a steady state are immunosuppressed at the time of transplantation[45] or when coinfected with human immunodeficiency virus,[46] the viral load increases, supporting an immunological role for viral containment even in the absence of clearance. A deleterious function of anti-HCV is that it serves to drive quasispecies evolution making it increasingly hard for the immune system to achieve viral clearance. Farci et al.[47] have shown in both humans and chimpanzees that the appearance of antibody coincides with increasing viral diversity and complexity and predicts progression to chronic infection.

In conclusion, there is much to explore regarding the function and relevance of IgG and IgM antibodies in HCV infection, and we believe the ELISpot assay, by measuring antibody production at the single-cell level, provides a new and useful tool for understanding the complex interplay between HCV and the host immune response.

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Patients With and Without Loss of Hepatitis B Virus DNA After Hepatitis B e Antigen Seroconversion Have Different Virological Characteristics

Noriko Misawa,[1] Akihiro Matsumoto,[1] Eiji Tanaka,[1]* Akinori Rokuhara,[1] Kaname Yoshizawa,[1] Takeji Umemura,[1] Noboru Maki,[2] Tatsuji Kimura,[2] and Kendo Kiyosawa[1,3]

[1]*Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan*
[2]*Advanced Life Science Institute, Inc., Wako, Japan*
[3]*Shinshu University Graduate School of Medicine, Institute of Organ Transplants, Reconstructive Medicine and Tissue Engineering, Matsumoto, Japan*

The characteristic differences between patients with and without loss of hepatitis B virus (HBV) DNA after achieving hepatitis B e antigen seroconversion were analyzed by comparing changes in HBV DNA and HBV core-related antigen levels during a period from 3 years before to 3 years after the seroconversion. Of the 24 seroconverters, 6 (inactive replication group) showed continuous loss of HBV DNA in serum after the seroconversion and the remaining 18 did not lose HBV DNA (active replication group). The HBV DNA level was similar between the two groups, while the HBV core-related antigen level was significantly lower in the active replication group than in the inactive replication group before the seroconversion. The levels of both HBV DNA and HBV core-related antigen decreased remarkably around the time of seroconversion in the inactive replication group, while these levels did not change or decreased slightly in the active replication group. After the seroconversion, the HBV DNA level was significantly higher in the active replication group than in the inactive replication group, while the HBV core-related antigen level was similarly low between the two groups. Because the serum level of HBV core-related antigen mainly reflects that of HBe antigen, the low level of HBV core-related antigen seen after seroconversion in both groups might have contributed to the occurrence of seroconversion. The precore and core promoter mutations which cause diminished excretion of hepatitis B e antigen were significantly more frequent in the active replication group than in the inactive replication group. It was therefore considered that the seroconversion was caused mainly by a decrease in viral replication in the inactive replication group, and mainly by a decrease in HBe antigen production in the active replication group. ***J. Med. Virol. 78:68–73, 2006.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HBV DNA; seroconversion; HBV core-related antigen; precore mutation; core promoter mutation

INTRODUCTION

A total of 350 million people worldwide are estimated to be carriers of hepatitis B virus (HBV) [Maynard, 1990; Maddrey, 2000]. HBV is important as a causative agent for liver diseases such as chronic hepatitis and hepatocellular carcinoma, especially in Asian countries [Lee, 1997]. In the natural history of chronic HBV infection, seroconversion from hepatitis B e (HBe) antigen to its antibody (anti-HBe) is usually accompanied by a decrease in HBV replication and remission of hepatitis [Realdi et al., 1980; Hoofnagle et al., 1981; Liaw et al., 1983]. Thus, HBe antigen seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who continue to have elevated HBV DNA levels in the serum and active liver disease after the seroconversion [Bonino et al., 1986; Hsu et al., 2002].

Although the detailed mechanisms of HBe antigen seroconversion have not been fully clarified, several mutations in the HBV genome have been reported to be associated with the phenomenon. When the precore (pre-C) and core genes in the HBV genome are

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*Correspondence to: Eiji Tanaka, MD, Second Department of Internal Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan.
E-mail: etanaka@hsp.md.shinshu-u.ac.jp

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transcribed and translated in tandem, HBe antigen is produced and secreted into circulation [Bruss and Gerlich, 1988; Garcia et al., 1988]. The G to A mutation at nucleotide (nt) 1896 in the pre-C region (G1896A), which converts codon 28 for tryptophan to a stop codon, is associated with the loss of HBe antigen [Carman et al., 1989; Okamoto et al., 1990]. The double mutation (A1762T and G1764A) in the core promoter (CP) has been shown to reduce the synthesis of HBe antigen by suppressing the transcription of precore mRNA [Okamoto et al., 1994; Takahashi et al., 1995; Buckword et al., 1996]. Convincing lines of evidence have indicated a close association of HBe antigen seroconversion with the appearance of precore and core promoter mutations [Okamoto et al., 1994; Takahashi et al., 1995; Buckword et al., 1996; Yamaura et al., 2003] as well as the severity of liver disease [Kosaka et al., 1991; Aritomi et al., 1998; Lindh et al., 1998].

A chemiluminescence enzyme immunoassay (CLEIA) was developed previously for the detection of HBV core-related antigen [Kimura et al., 2002; Rokuhara et al., 2003]. The HBV core-related antigen is expressed on HBe and core (HBc) antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical. The HBVcrAg CLEIA measures the serum levels of HBe and HBc antigens simultaneously, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. However, the amount of HBV core-related antigen mainly reflects that of HBe antigen, because the concentration of HBe antigen in serum is much higher than that of HBc antigen [Kimura et al., 2002]. In the present study, the characteristic differences that may exist between patients with and without HBV DNA in serum after HBe antigen seroconversion were examined by comparing chronological changes of HBV DNA and HBV core-related antigen as well as by testing HBV genome mutations associated with the seroconversion.

MATERIALS AND METHODS

Patients

The present study is a retrospective one using stored sera from Japanese patients with chronic hepatitis B seen in Shinshu University Hospital. The clinical database was reviewed to identify all patients who had been followed from January 1985 to June 2001 and also showed seroconversion from HBe antigen to anti-HBe during the follow-up period. A total of 24 patients were recruited in the present study. The 24 patients consisted of 17 men and 7 women with a median age of 39 years. Seroconversion of HBe antigen was defined as disappearance of HBe antigen accompanied by the development of anti-HBe on at least two consecutive visits. All 24 patients met the following three criteria: (1) follow-up was performed for at least 3 years before and after the seroconversion; (2) chronic hepatitis without liver cirrhosis was confirmed by histological examination; and (3) serum samples were available for testing every 6 months during the follow-up period. Of the 24 patients, 12 patients received interferon administration of at most 4 weeks and none received nucleotide analogs such as lamivudine, adefovir, or entecavir during the follow-up period.

Serum concentrations of HBV DNA and HBV core-related antigen were determined every 6 months during the follow-up period, which ran from 3 years before to 3 years after the seroconversion. The presence or absence of the pre-C mutation of A1896 and the double mutation in the CP (T1762/A1764) was determined every year during the follow-up period. The serum samples had been stored at −20°C or below until tested. Written informed consent was obtained from each patient.

Serological Markers for HBV

Conventional HBV markers, including HBe antigen and anti-HBe, were tested using CLEIA kits (Fuji Rebio, Tokyo, Japan). Six major genotypes (A–F) of HBV were determined using the method reported by Mizokami et al. [1999], in which the surface gene sequence amplified by PCR was analyzed by restriction fragment length polymorphism.

The Pre-C and CP mutations were determined on nucleic acids extracted from 100 μl of serum with a DNA/RNA extraction kit (Smitest EX-R and D; Genome Science Laboratories Co., Ltd., Tokyo, Japan). The stop codon mutation in the Pre-C region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest; Genome Science Laboratories). In principle, G1896 in the wild-type HBV and A1896 in the mutants were determined by mini-sequence reactions using labeled nucleotides that are complementary to either the wild-type or mutant. The results were expressed as a percent mutation rate according to the definition by Aritomi et al. [1998]. The sample was judged positive for the pre-C mutation when the mutation rate exceeded 50% in the present study, because the mutation rate steadily increase to 100% afterward once it exceed the rate of 50% [Yamaura et al., 2003]. The double mutation in the CP was detected using an HBV core promoter detection kit (Smitest; Genome Science Laboratories) [Aritomi et al., 1998]. This kit detects T1762/G1764 or A1762/T1764 by a polymerase chain reaction (PCR) with primers specific for either the wild-type or mutant. The results were recorded in three categories, that is, wild, mixed, and mutant types. In the present study, the sample was considered positive for the CP mutation when the results were in the mutant type category. The detection limits of the pre-C and the CP mutation kits are both 1,000 copies/ml according to the manufacturer. The pre-C mutation could be determined in 136 (99%) of 137 samples, which had HBV DNA levels higher than 1,000 copies/ml and in 30 (97%) of 31 samples which had levels lower than 1,000 copies/ml. Similarly, the CP mutation could be determined in 136 (99%) of 137 samples and in 28 (90%) of 31 samples.

The serum concentration of HBV DNA was determined using an Amplicor HBV monitor kit (Roche,

Tokyo, Japan) which had a quantitative range of 2.6–7.6 log copies/ml [Kessler et al., 1998]. Sera containing over 7.0 log copies/ml HBV DNA were diluted 10- or 100-fold in normal human serum and measured again to obtain the end titer.

The serum concentration of HBV core-related antigen was measured using the CLEIA reported previously [Kimura et al., 2002; Rokuhara et al., 2003]. In summary, 100 μl serum was mixed with 50 μl pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70°C for 30 min, 50 μl pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61, and HB114) and filled with 100 μl assay buffer. The mixture was incubated for 2 hr at room temperature and the wells were washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and incubated for 1 hr at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBV core-related antigen concentration was read by comparison to a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBV core-related antigen concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cut-off value was set tentatively at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBV core-related antigen were diluted 10- or 100-fold in normal human serum and measured again to obtain the end titer.

Statistical Analyses

The Mann–Whitney U test was used to analyze continuous variables. The Fisher's exact test was used in the analysis of categorical data. The Manzel Haentel chi-square test was used to evaluate positive rates for the pre-C and CP mutations. The Wilcoxon test was used to analyze the change in the level of HBV DNA and HBV core-related antigen. *P*-values less than 0.05 were considered significant. Statistical analyses were performed using an SPSS 11.5 J statistical software package (SPSS, Inc., Chicago, IL).

RESULTS

Grouping of Seroconverters According to HBV DNA Outcome

The 24 seroconverters enrolled in the present study were classified into two groups according to changes in serum levels of HBV DNA. The HBV DNA level decreased substantially around the time of the seroconversion and then became continuously undetectable in one group (inactive replication group), and the level decreased slightly and did not become continuously undetectable even after the seroconversion in another group (active replication group). In the present study, the former group of patients were defined as those whose HBV DNA levels were lower than 2.6 log copies/ml at each of the time points of 1.5, 2, 2.5, and 3 years after the seroconversion, and the latter group of patients were defined as those whose HBV DNA levels were not. Of the 24 seroconverters, 6 belonged to the inactive replication group and the remaining 18 belonged to the active replication group.

The clinical backgrounds of the active and inactive replication groups are compared in Table I. The median age, gender ratio, and history of interferon therapy did not differ between the two groups. All patients were infected with genotype C HBV. Normalization of serum alanine aminotransferase (ALT) after seroconversion was considered to have occurred in cases in which ALT was normal at each of the time points of 2, 2.5, and 3 years after the seroconversion in the present study. The normalization of ALT was more frequent in the inactive replication group than in the active replication group, but the difference was not statistically significant.

Changes in HBV DNA and HBV Core-Related Antigen Concentration

Changes in the serum level of HBV DNA are compared between the active and inactive replication groups in Figure 1A. At the start-point of the follow-up, the level was distributed within a similarly high range in both groups. In the inactive replication group, the median

TABLE I. Comparison of Clinical Backgrounds Between the Inactive and Active Replication Groups

Characteristics	Inactive replication group n = 6	Active replication group n = 18	*P*
Age at seroconversion (yr)[a]	37 (23-65)	39 (17-64)	>0.2*
Gender (M:F)	4:2	13:5	>0.2**
Genotype C[b]	6 (100%)	18 (100%)	>0.2**
History of interferon therapy[b]	3 (50%)	9 (50%)	>0.2**
ALT normalization[c]	4 (67%)	5 (28%)	0.150**

*Mann–Whitney U test.
**Fisher's exact test.
[a]Data are expressed as the median (range).
[b]Data are expressed as a positive number (percent).
[c]Normalization of serum ALT level after seroconversion (the ALT value was within the normal range at each of the time points of 2, 2.5, and 3 years after the seroconversion).

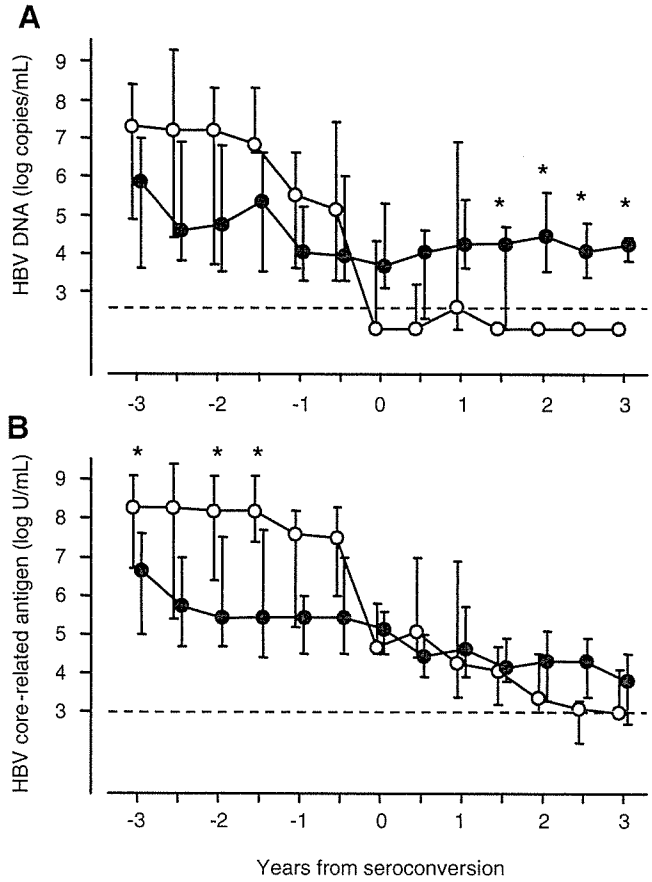

Fig. 1. Comparison of changes in HBV DNA (**A**) and HBV core-related antigen (**B**) levels between the inactive and active replication groups. Data are shown as the median ±25% ranges. The broken lines indicate the detection limits of the HBV DNA and HBV core-related antigen assays, respectively. Open circles indicate inactive replication group and closed circles indicate active replication group. $^*P < 0.05$ between the inactive and active replication groups.

concentration decreased around the time of seroconversion and became continuously undetectable thereafter. In the active replication group, on the other hand, the median concentration tended to decrease around the time of seroconversion, but was not undetectable even at 3 years after seroconversion. The median HBV DNA level in the active replication group was significantly higher than that in the inactive replication group at 1.5 years after the seroconversion and each of the subsequent time points.

Changes in the serum concentration of HBV core-related antigen are compared between the active and inactive replication groups in Figure 1B. The concentration of HBV core-related antigen was significantly higher in the inactive replication group than in the active replication group at the start of the follow-up and at 1.5 and 2 years before the seroconversion point. The median concentration of HBV core-related antigen in the inactive replication group appeared to decrease around the time of seroconversion and reached a level comparable to that in the active replication group. The median HBV core-related antigen level was similar between the inactive and active replication groups at all time points after the seroconversion, and it decreased slowly with time in both groups.

Changes in the log ratio of HBV core-related antigen/HBV DNA concentrations are compared between the inactive and active replication groups in Figure 2. The values of HBV core-related antigen and HBV DNA were substituted by their corresponding detection limit values when they were under the detection limit. The log ratio was similar between the two groups at points before the seroconversion. The log ratio decreased after the seroconversion in the active replication group, but did not change in the inactive replication group. The log ratio of HBV core-related antigen/HBV DNA was significantly lower in the active replication group than in the inactive replication group at all post-seroconversion time points except 1 year.

Comparison of Pre-C and CP Mutations

The positive rates for the pre-C and CP mutations at the time points before and after the seroconversion are compared between the inactive and active replication groups in Figure 3. The pre-C mutation did not appear during the follow-up period in the inactive replication group. On the other hand, the positive rate for the pre-C mutation was around 30% before the seroconversion, and then increased to around 60% after the seroconversion in the active replication group. The difference in the positive rate was significant at the time points of 2 and 3 years after the seroconversion. The positive rate for the CP mutation was less than 40% in the inactive replication group during the follow-up period except at the last time point, while it was over 60% in the active replication group throughout the follow-up period. The difference in the positive rate was statistically significant at the time points of 2 and 3 years before the seroconversion and at 1 and 2 years after it.

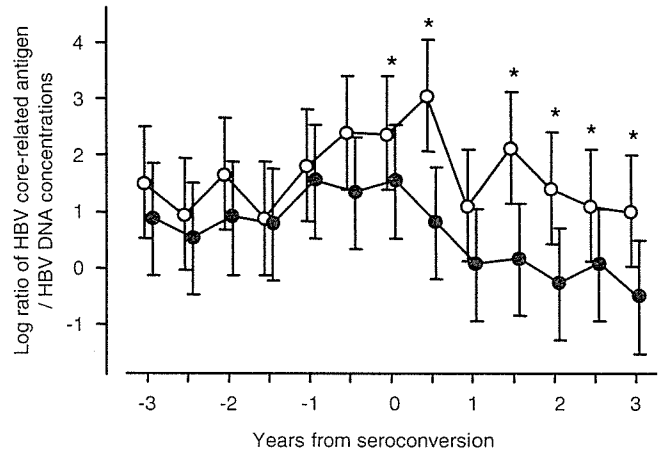

Fig. 2. Comparison of changes in the log ratio of HBV core-related antigen/HBV DNA levels between the inactive and active replication groups. Data are shown as the median ±25% ranges. Open circles indicate inactive replication group and closed circles indicate active replication group. $^*P < 0.05$ between the inactive and active replication groups.

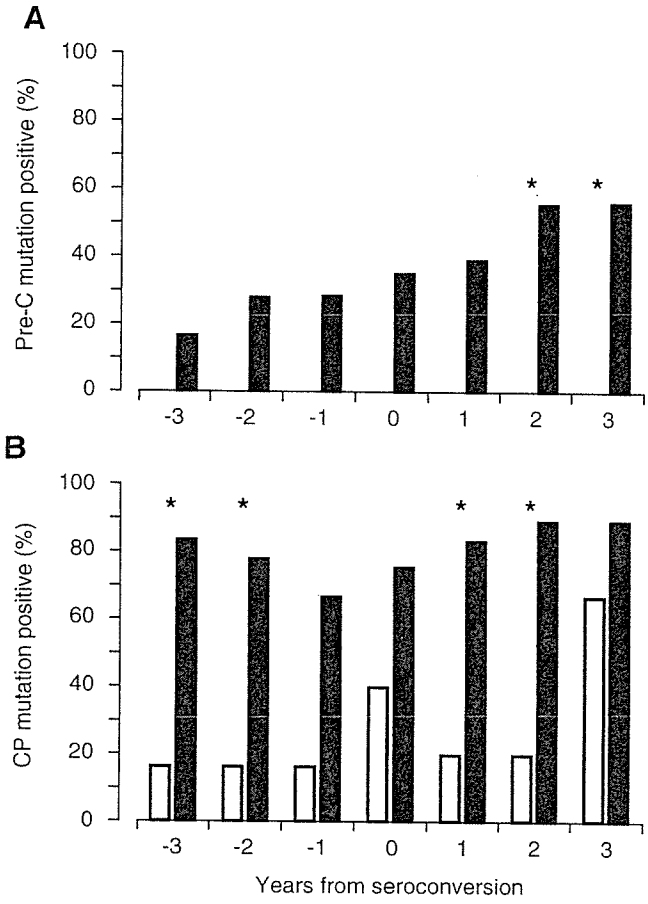

Fig. 3. Comparison of positive rates for the pre-C (**A**) and CP (**B**) mutations between the inactive and active replication groups. Open bars indicate inactive replication group and closed bars indicate active replication group. Number of patients in the inactive replication group is six at each time point except the followings: point 0 year (n = 5) in A, and points 0 year (n = 5), 1 year (n = 5), and 2 years (n = 5) in B. Number of patients in the active replication group is 18 at each time point except the followings: point 0 year (n = 17) in A and point 0 year (n = 17) in B. *$P < 0.05$ between the inactive and active replication groups.

DISCUSSION

Seroconverters were divided tentatively into two groups according to their levels of serum HBV DNA in the present study. It has been reported that older age and female gender are factors predicting occurrence of HBe antigen seroconversion in patients with chronic hepatitis B [Alward et al., 1985; Lok et al., 1987; McMahon et al., 2001]. On the other hand, in the present study, median age and gender distribution were similar between the inactive and active replication groups. A history of interferon treatment was recorded in half of the patients enrolled. The treatment history did not seem to be associated with the loss of HBV DNA after seroconversion, because the history was similarly distributed between the two groups and the duration of interferon therapy was as short as 4 weeks at most. Although the difference was not statistically significant, patients in the inactive replication group tended to show continuous normalization of ALT. Further, none of the six patients in the inactive replication group developed end stage liver diseases such as cirrhosis and hepatocellular carcinoma after the follow-up period, while 4 of the 18 patients in the active replication group developed them (data not shown). High viral load, which is usually associated with active hepatitis, has been reported to be a risk factor for development of hepatocellular carcinoma even in patients with chronic hepatitis B who achieved HBe antigen seroconversion [Ikeda et al., 2003; Ohata et al., 2004]. We could not compare long-term prognosis between patients in the inactive and active replication groups in the present study. However, patients in the active replication group tended to show active hepatitis after the seroconversion and to develop end stage liver diseases. Thus, further analysis of patients whose active viral replication continues after the seroconversion would be of clinical significance.

Analysis of the changes in HBV DNA and HBV core-related antigen revealed a clear contrast between the two. Namely, the HBV DNA level was similar between the two groups, while HBV core-related antigen was significantly lower in the active replication group than in the inactive replication group before seroconversion. The levels of both HBV DNA and HBV core-related antigen decreased remarkably around the time of seroconversion in the inactive replication group, while these levels did not change or decreased slightly in the active replication group. After seroconversion, the HBV DNA level was significantly higher in the active replication group than in the inactive replication group, while the HBV core-related antigen level was similar between the two groups. Because the discrepancy in the log ratio of HBV core-related antigen/ HBV DNA between the two groups first appeared at the time of seroconversion and continued thereafter, the difference between the HBV DNA and HBV core-related antigen changes was suggested to be closely associated with the seroconversion. The results obtained in the present study indicate that the mechanism of seroconversion was different between the two groups.

Because the serum level of HBV core-related antigen mainly reflects that of HBe antigen [Kimura et al., 2002], the low level of HBV core-related antigen seen after seroconversion in both the inactive and active replication groups might have contributed to the occurrence of seroconversion. The pre-C and CP mutations, which were associated with the seroconversion, were frequent in the active replication group and rare in the inactive replication group, at least at around the time of seroconversion. The decrease of HBV core-related antigen excretion seen after seroconversion was thought to have been caused mainly by the decrease of viral replication in the inactive replication group, because viral replication did not resume in this group. On the other hand, the decrease of HBV core-related antigen was thought to have been caused mainly by the appearance of pre-C and/or CP mutations, because active viral replication continued in this group. These results suggested that the two groups had different mechanisms of seroconversion.

It has been reported that the frequency of the pre-C and the CP mutations differs among HBV genotypes. Orito et al. reported that the CP mutation was significantly associated with genotype C [Orito et al., 2001]. Yamaura et al. [2003] reported that the CP mutation was already commonly seen several years before the seroconversion in patients with genotype C. These results are consistent with the present finding that the majority of patients in the active replication group had the CP mutation from the start of follow-up. The fact that patients in the active replication group had a lower level of HBV core-related antigen before the seroconversion may be attributable to the frequent CP mutation seen in this group.

In conclusion, the present study showed that there were different mechanisms of HBe antigen seroconversion between patients in whom HBV viraemia continued after the seroconversion and those in whom it did not. Measurement of HBV core-related antigen in addition to HBV DNA was suggested to be useful in examining specific conditions of chronic hepatitis B.

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Identification of Novel HCV Subgenome Replicating Persistently in Chronic Active Hepatitis C Patients

Shintaro Yagi,[1] Kenich Mori,[1] Eiji Tanaka,[2] Akihiro Matsumoto,[2] Fumiko Sunaga,[1] Kendo Kiyosawa,[2] and Kenjiro Yamaguchi[1]*

[1]*R&D Group, Advanced Life Science Institute, Inc., Saitama, Japan*
[2]*2nd Department of Internal Medicine, Shinshu University School of Medicine, Nagano, Japan*

In an effort to clarify the life cycle of HCV, the HCV genome in liver biopsies taken from chronic active hepatitis C patients undergoing interferon treatment was investigated. Molecular cloning by long distance reverse-transcription polymerase chain reaction (RT-PCR) revealed that the HCV genome in two patients with high viral loads in the liver had in-frame deletions of approximately 2 kb between E1 and NS2, which encode the E1–NS2 fusion protein and six other HCV proteins: core, NS3, NS4A, NS4B, NS5A, and NS5B. Among the remaining 21 chronic active hepatitis C patients, these types of deletion were found in another two patients and in two hepatocellular carcinoma patients. Out-of-frame deletions in the structural region were isolated from the other five patients, but the dominant RT-PCR products were non-truncated genomes. Retrospective analysis of a series of serum samples taken from a patient carrying the subgenome with the in-frame deletion revealed that both the subgenome and the full genome persisted through the 2-year period of investigation, with the subgenome being predominant during this period. Sequence analysis of the isolated cDNA suggested that both the subgenome and the full genome evolved independently. Western blotting analysis of HCV proteins from the HCV subgenome indicated that they were processed in the same way as those from the full genome. HCV subgenomes thus appear to be involved in the HCV life cycle. ***J. Med. Virol. 77:399–413, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HCV; deletion; replication; biopsy

INTRODUCTION

Hepatitis C virus (HCV) is primarily transmitted via blood and blood-derived materials [Alter et al., 1989] and often causes chronic hepatic diseases that progressively worsen to chronic active hepatitis, cirrhosis, and finally to hepatocellular carcinoma (HCC) [Kiyosawa et al., 1990, 1994, 2004; Alter and Seeff, 2000]. Interferon (IFN) and interferon with ribavirin treatment are effective in eradicating HCV from patients [Iino et al., 1994; McHutchison and Fried, 2003], improving liver histological findings, and in prolonging life in patients with hepatitis C [Yoshida et al., 1999; Kasahara et al., 2004]; however, their efficacy is limited.

HCV was first identified as cDNA clones, and was characterized molecularly using cDNA isolates [Choo et al., 1989; Kuo et al., 1989]. The HCV genome is single-stranded RNA of about 9,600 nucleotides with an untranslated region (UTR) at each end, and encodes a polyprotein of about 3,010 amino acids [Choo et al., 1989; Kato et al., 1990], which is processed into 10 proteins by a host peptidase and two HCV proteases [Hijikata et al., 1991, 1993; Grakoui et al., 1993a]; Core, E1, and E2 are structural proteins for virion formation, and NS3, NS4A, NS4B, NS5A, and NS5B are components of the replication machinery for the RNA genome [Houghton et al., 1994]. However, isolation of virion particles has been difficult owing to a lack of in vitro culture systems for HCV.

HCV replication in chimpanzee following intrahepatic injection of an RNA transcript from HCV genomic cDNA proved that a molecular clone could represent a functional HCV genome [Yanagi et al., 1997]. Lohmann

Shintaro Yagi's present address is Laboratory of Cellular Biochemistry, Animal Resource Science, Graduate School of Agricultural and Life Science, The University of Tokyo, Tokyo, Japan.

*Correspondence to: Kenjiro Yamaguchi, Advanced Life Science Institute, Inc., R&D Group, 2-10-23 Maruyamadai, Wako, Saitama, 351-0112, Japan. E-mail: kmori@alsi-i.co.jp

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et al. [1999] established a dicistronic subgenomic RNA that replicates in a hepatoma cell line (Huh7) and consists of the HCV NS protein coding region, the 5′- and 3′-UTR of HCV and a selective marker gene. HCV subgenomic RNA replicon systems are vital to the study of the mechanisms of HCV RNA replication, but there remain problems with regard to viral replication. Information obtained from liver biopsies of hepatitis C patients thus remains important in clarifying the life cycle of HCV.

Histological grading for diagnosis [Perrillo, 1997], immunohistochemical analysis, immuno-staining [Infantolino et al., 1990; Hiramatsu et al., 1992], electron microscopic analysis [Fagan et al., 1992], in-situ hybridization and in-situ reverse transcription polymerase chain reaction (RT-PCR) [Lau et al., 1996; Dries et al., 1999], and quantitation of HCV RNA in liver biopsy specimens [Sakamoto et al., 1994; Nuovo et al., 2002] have all been used to demonstrate HCV replication in liver. However, molecularly characterized data have been limited; HCV RNA isolated from liver was found to be equivalent in size to the well-characterized RNA seen in circulating HCV [Nielsen et al., 2004], thus confirming HCV replication in liver.

In order to obtain data to elucidate the nature of HCV in liver, viral loads and the structure of the HCV genome in patient liver biopsy specimens were examined. A highly sensitive ELISA for quantitation of the HCV core antigen [Aoyagi et al., 1999; Tanaka et al., 2000] and a quantitative RT-PCR system were applied to estimate viral loads in serum and liver biopsies. For structural analysis of the whole genome in specimens, molecular clones were used. Surprisingly, it was found that novel HCV subgenomes were predominant in several patients and, in one patient, these persisted for several years. The nature of these subgenomes are described and discussed in this paper.

MATERIALS AND METHODS

Samples and Antibodies

Serum and liver biopsy specimens were taken from patients undergoing IFN-α treatment at Shinshu University Hospital. Informed consent was obtained from all patients from whom samples were taken. A 7.2 mega-unit dose of IFN-α was administered daily for 2 weeks, followed by three times per week for 22 weeks. Serum samples were collected 1 or 2 months before administration of IFN-α. A series of serum specimens was collected from one patient from before IFN treatment until 1 year after treatment. Liver biopsy specimens from two HCC patients were obtained from surgically removed cancerous liver tissues.

An anti-core monoclonal antibody (5E3) has been described previously [Kashiwakuma et al., 1996]. Anti-E1 monoclonal antibody was raised against recombinant E1 and E1/E2 proteins expressed in Sf-9 cells infected with recombinant baculoviruses (Yamaguchi unpublished). Anti-NS3 rabbit polyclonal antibody was purified from the serum of rabbits immunized with the recombinant NS3 antigen expressed in *E. coli* [Saito et al., 1992]. Anti-mouse and rabbit immunoglobulin antibody conjugated with HRP were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD) and Bio-Rad Laboratories (Tokyo, Japan), respectively. All primers were purchased from Sigma Genosys (Tokyo, Japan) and Texas Genomics Japan (Tokyo, Japan), and sequences are available on request.

Quantitation of HCV Core Antigen

Quantities of HCV core antigen were measured by EIA as described previously [Aoyagi et al., 1999; Kato et al., 2003]. The concentration of core antigen was expressed in fmol/L, and the cut-off value of the assay was set at 7.5 fmol/L. For quantitation of the core antigen in liver, extracts were diluted to 100 μg of liver protein per milliliter with negative control serum before pretreatment of the samples. Samples were heated at 56°C for 30 min with pretreatment solution containing SDS, CHAPS, and Triton X-100, and were then added to wells pre-coated with anti-HCV core antibodies, and reaction buffer was used to fill the wells. Captured core antigen after 1-hr incubation was reacted for 30 min with anti-HCV antibodies conjugated with horseradish peroxidase after stringent washing. Bound enzyme activities were measured using a Fusion plate reader (PerkinElmer, Tokyo, Japan) with a chemiluminescent reagent (SuperSinal Pico ELISA, Pierce, Rockford, IL)

Real-Time PCR Assay for HCV RNA

HCV RNA was recovered from samples by using the QIAamp viral RNA kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions. HCV RNA was reverse-transcribed and amplified using QuantiTect One-Step RT-PCR kit (QIAGEN) with primers. For quantitation of the 5′-UTR, the forward primer, chiba-s (5′-TAGTGGTCTGCGGAACCGGT-3′), and reverse primer, chiba-as (5′-TGCACGGTCTACGAGACCT-3′), yielded fragments corresponding to nucleotides 141–339 of HCV RNA. In the case of the E2 region, HC1986S (5′-TGGTTCGGCTGYACATGGATGAA-3′) and HC2199AS (5′-GGRTAGTGCCARAGCCTGTATGGGTA-3′) primers were used. Reactions were performed with a LightCycler system (Roche Diagnostics K.K., Tokyo, Japan), and fluorescence by SYBR green was monitored after each elongation reaction for real-time monitoring of DNA products during PCR. The amount of HCV RNA was calculated according to the calibration curve produced with serial dilutions of standard RNA synthesized by T7 RNA polymerase (Ambion, Inc., Austin, TX) from plasmids carrying the HCV cDNA isolate (genotype 1b). To examine the specificity of PCR, the melting point of DNA products was analyzed by melting curve analysis using LCDA software (Roche Diagnostics).

Cloning and Analysis of HCV cDNA

HCV cDNA was amplified by long distance RT-PCR (LD-RT-PCR) as described previously [Tellier et al., 1996;

TABLE I. Viral Data of Patients

Patient No.	HCV genotype	Viral loads: Serum Core antigen (fmol/L)	Viral loads: Serum HCV RNA (copies/ml)	Viral loads: Liver biopsy Core antigen (fmol/g protein)	Viral loads: Liver biopsy HCV RNA (copies/g protein)	PCR primer sets for positive results: Non-truncated genome	PCR primer sets for positive results: Truncated genome	PCR primer sets for positive results: Test primer sets
368	1b	17,108.5	1.73×10^5	5,462.4	4.71×10^8	—	j	j
207	1b	12,695.2	1.44×10^5	30,792.3	1.43×10^{10}	NT	NT	NT
204	1b	5,082.4	5.74×10^4	8,779.7	2.22×10^9	i	—	i
274	1b	1,034.4	4.24×10^3	2,651.7	3.56×10^7	a, b, c, d	e	a–e
193	1b	988.8	3.09×10^4	14,519.9	1.07×10^9	a, b, c, d, e	—	a–e
331	1b	922.2	2.03×10^3	2,387.1	2.84×10^8	a, c, d	b, e	a–e
325	1b	623.5	3.82×10^3	10,127.9	7.28×10^7	a, b, c, d, e	d	a–e
288	1b	254.5	1.00×10^1	4,037.9	9.50×10^6	a, b, c, d, e	d, e	a–e
299	1b	166.6	1.14×10^3	1,287.8	5.35×10^7	c, d	—	a–e
295	1b	1.0	5.11×10^1	261.5	2.62×10^7	a, b, c, d, e	b	a–e
171	1b	1,077.3	6.42×10^3	3,781.8	6.91×10^6	c, d	b	a–e
257	1b	12.7	1.06×10^2	568.5	2.78×10^7	d	—	a–e
372	1b	723.7	2.28×10^4	1,784.1	3.35×10^8	a, b, c, d, e	—	a–e
373	1b	597.0	8.31×10^3	33,919.0	2.65×10^9	—	a, c, d	a–e
248	2a	209.3	2.58×10^2	4,417.1	3.70×10^8	—	—	a–e
235	2a	3,616.2	3.66×10^2	7,462.1	1.55×10^9	c	—	a–e
203	2b	95.1	1.46×10^2	5,590.9	1.82×10^9	—	b, d	a–e
178	2b	34.5	8.08×10^1	609.1	4.51×10^7	—	—	a–e
297	2	3,112.7	8.35×10^3	2,883.6	1.14×10^8	—	—	a–e
298	2a	180.0	8.09×10^2	3,015.0	1.76×10^9	b	—	a–e
305	2a	173.6	1.12×10^3	1,782.8	5.96×10^7	b	—	a–e
201	2a	127.6	2.40×10^3	497.6	1.87×10^7	—	—	a–e
357	2	227.2	3.11×10^3	321.9	2.29×10^7	—	—	a–e

NT: not tested.

Yanagi et al., 1998]. HCV cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen K.K., Tokyo, Japan) with HC1b9405R primer (5′-GCCTA-TTGGCCTGGAGTGTTTAGCTC-3′). After RNase H (Invitrogen) treatment at 37°C, a cDNA mixture was subjected to PCR with KlenTaq DNA polymerase (BD Biosciences Clontech, Tokyo, Japan), HClong A1 primer (5′-GCCAGCCCCCTGATGGGGGCGACA-

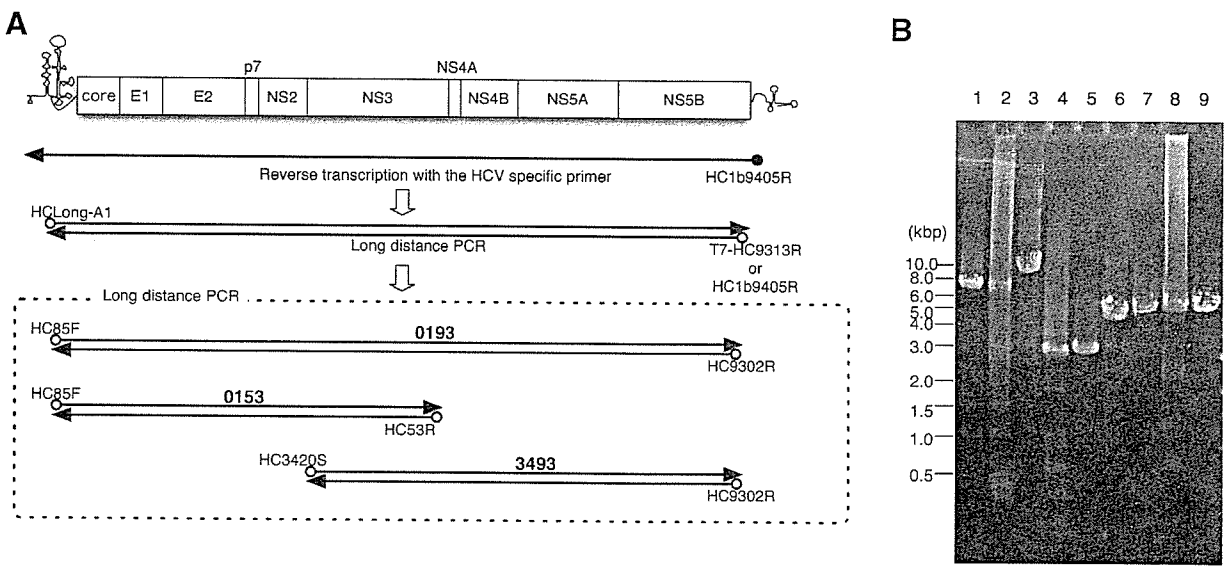

Fig. 1. Molecular cloning of HCV genome by long distance reverse-transcription PCR. **A**: Schematic view of HCV RNA is shown at the top of the figure. HCV cDNA, which was synthesized from total RNA from liver using reverse transcriptase with HCV-specific primers for the 3′-UTR, was amplified by nested PCR with HCV-specific primers. The longest LD-RT-PCR product, 0193, covered 99% (amino acids 1–2,987) of the HCV polyprotein coding sequence of genotype 1b HCV (length: 3,011 amino acids). Fragments obtained by LD-RT-PCR using HC85F and HC9302R, HC85F, and HC53R; and HC3420S and HC9302R were designated 0193, 0153, and 3493, respectively. After agarose gel electrophoresis, LD-RT-PCR products from liver biopsy samples were stained with ethidium bromide. **B**: **Lanes 1–3**, **lanes 4–6**, and **lanes 7–9** represent 0193, 0153, and 3493 fragments from Patient 207 (lanes 1, 4, and 7), 373 (lanes 2, 5, and 8), and control HCV cDNA (lanes 3, 6, and 9), respectively. The positions of markers are indicated at the left side of the image.

CTCCACC-3′) and T7-HC9313R primer (5′-TCTAGTC-GACGGCCAGTGAATTGTAATACGACTCACTCTAG-GGCGGCGGGGTCGGGCWCGNGACABGCTGTGA-3′) or HC1b9405b for 35 cycles of denaturation at 94°C for 20 sec and extension at 68°C for 9 min. Second-round PCR was carried out with the primers, HC85F (5′-ATGGCGTTAGTATGAGTGTCGTGCAGCCT-3′) and HC9302R (5′- TCGGGCACGAGACAGGCTGTGATA-TATGTCT-3′), HC85F and HC53R (5′-GCTTAAGTG-ACGACCTCCAGGTCAGCCGACAT-3′), and HC3420S (5′-GCGCCCATCACGGCCTACTCCCAACAA-3′) and HC9302R, for 20 cycles under the same conditions as first-round PCR. PCR products were purified from the gel using a QIA-quick gel kit (QIAGEN), and were then cloned into the pGEM-T easy vector (Promega K.K., Tokyo, Japan). The cDNA clones, LV207-0193-1, -3, -15, and -6, and LV373-0193-10, LV373-0153-5, and LV373-0153-6 were obtained from liver biopsy samples from Patient 207 and 373, respectively.

Nucleotide sequences of the cloned cDNA fragments were determined using a CEQ-2000 XL analysis system with a DTCS quick start kit and HCV-specific primers according to the manufacturer's instructions. Sequence data were analyzed on Macintosh computers with the

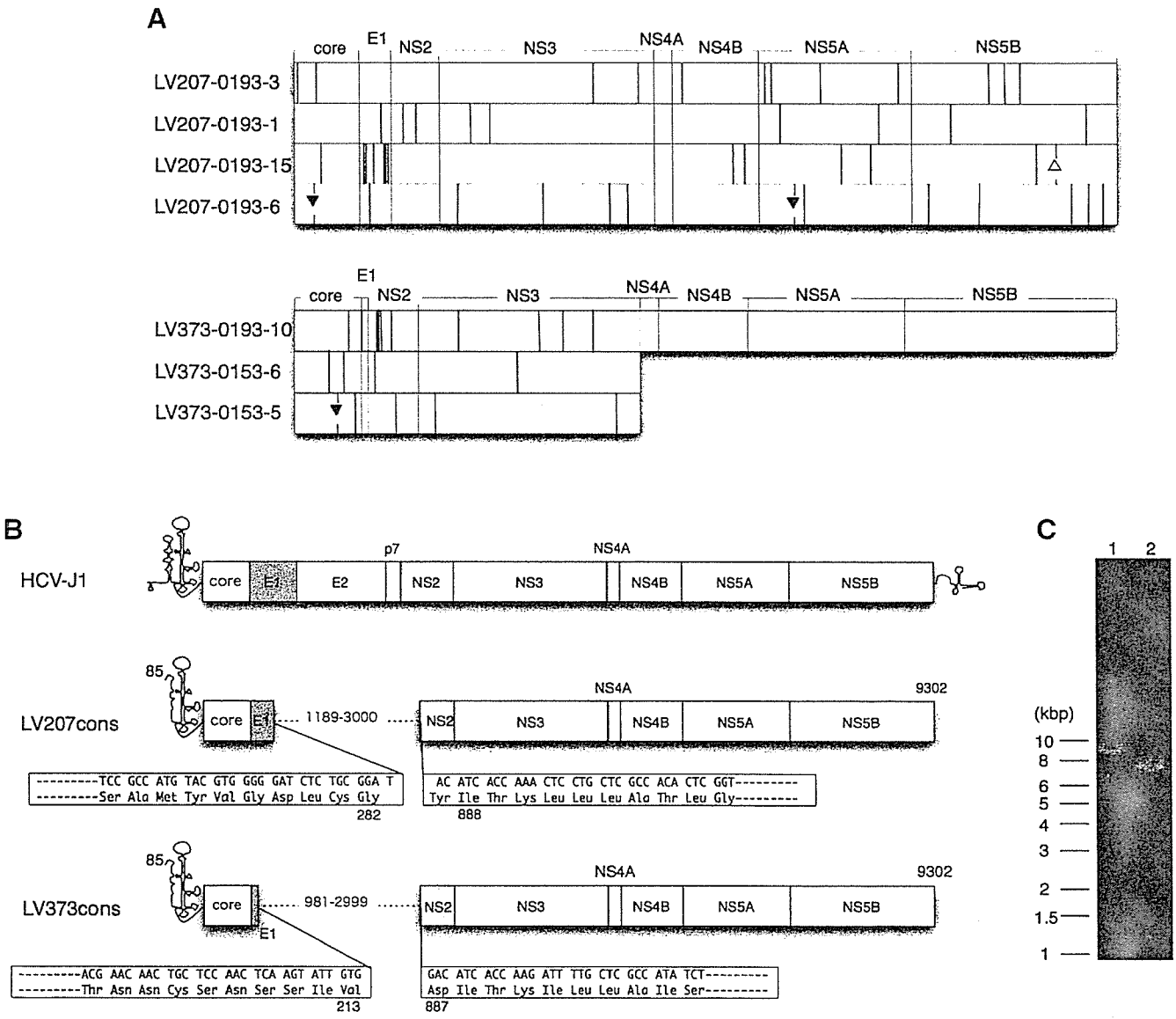

Fig. 2. Schematic presentation of HCV subgenomes from livers containing in-frame deletion. **A**: Comparisons of the deduced amino acid sequences of LD-RT-PCR fragments cloned with pGEM-T Easy are depicted. The cDNA isolates from liver biopsy are designated by the prefix LV followed by Patient numbers and fragment names described in legend of Figure 1. Bold bars indicate the positions of amino acids that differed. Closed triangles show the positions of stop codons, and open triangles indicate base deletions. **B**: Schematic HCV cDNA structures with their nucleotide sequences and deduced amino acid sequences around the boundary of the deletions are depicted. The deleted regions were represented as nucleotide positions as those corresponding to HCV-J1 by numbers between the dotted tagged boxes. Boundaries of the deletions are shown as amino acid positions corresponding to those of HCV RNA (HCV-J1) on the bottom line of the graphs. **C**: Images of agarose gel electrophoresis of LD-RT-PCR products amplified from in vitro non-truncated (**lane 1**) G14 and truncated (**lane 2**) RNA transcripts from Donor G14 and Patient 207 cDNAs, respectively.

Sequencer (Gene Code Corporation, Ann Arbor, MI), MacVector (Accelrys K.K., Tokyo, Japan), and EMBOSS [Rice et al., 2000] software packages.

Construction of HCV cDNA Expression Vectors

HCV RNA reverse transcribed with HC1b9405R from Patient 207 liver biopsy was subjected to PCR using the primers T7-HCLongH1 (5′-TCTAGTCGACGGCCAG-TGAATTGTAATACGACTCACTATAGGGCGGCCAG-CCCCCTGATGGGGGCGACACTCCACC-3′) and core-cla-as (5′-GCCGCATGTAAGGGTATCGATGACC-3′) in order to amplify the entire 5′-UTR, and cloned (LV207-H1-Cla). To obtain the 3′-UTR cDNA (LV373-3′UTR), cDNA was synthesized with HC8913F primer (5′-CTTGAAAAAGCCCTGGATTGTCAGAT-3′) from the minus strand RNA of Patient 373 liver sample. cDNA was amplified by PCR with HC8913F and RP2 (5′-ACATGATCTGCAGAGAGGCC-3′), and followed by PCR with HC8939F and R1 (5′-ACATGATCTGCAGA-GAGGCCAGTATCAGCACTCTC-3′).

Ligation of the fragments from LV207-0193-1, -15, and -6, the 5′-UTR-core fragment and the 3′-UTR fragments gave the chimeric HCV cDNA (LV207cont). The cDNA encoding the HCV subgenome was inserted into pcDNA3.1 (Invitrogen) to construct pcD/LV207cont. A Cla I site in the core region and an Asc I site in the NS2 region of pcD/LV207cont were introduced using Quick-Change II site-directed mutagenesis kits with primers. Insertion of the corresponding fragment from E1 to NS2, which was obtained from HCV-J1 cDNA [Aizaki et al., 1998] by PCR with core-cla-s (5′-GGTCATCGA-TACCCTTACATGCGGC-3′) and Asc-M-as (5′-CCTTC-CTCGGCGCGCCGAGACRGGTAGACCCRAGATGAT-GTCCCCACA-3′) generated pcD/J1NLV.

In Vitro Synthesis of Truncated and Non-Truncated RNA Transcripts and Subsequent RT-PCR

Truncated cDNA, LV207cont, having the T7 promoter sequence was inserted into pBluescript II (Stratagene) to give pLV207cont. A plasmid carrying non-truncated HCV cDNA was constructed by inserting full-length cDNA derived from G14 plasma, in which only full-length HCV RNA was detected, into pBluescript II. Truncated and non-truncated RNA was synthesized using MEGAscript T7 kit (Ambion, Inc.) according to the manufacturer's instructions.

RNA transcripts (10^4 copies) were mixed with RNA extracted from uninfected liver tissue, and were reverse-transcribed and amplified by same protocol used to obtain truncated cDNAs. In addition, RNA transcripts and extracted RNA from plasma or serum were amplified by RT-PCR using primers targeting the junction site of LV207 cDNA. RNA was reverse-transcribed and amplified using QuantiTect SYBR GREEN RT-PCR Kit (QIAGEN) with sense, LV207-1S (5′-GCGTCCCC-ACTAAGGCAATA-3′), and antisense primers, LV207-3AS (5′-AGCAGGAGTTTGGTGATGATCCG-3′), for the

TABLE II. List of Primers Used for Detecting Truncated and Non-Truncated HCV Genomes

		Primer sets									
		a	b	c	d	e	f	g	h	i	j
Reverse transcription	Sense	HC3945R	HC3481R	HC3945R	HC3945R	HC3297R	HC3945R	HC3945R	HC3945R	HC3945R	HC3481R
	Antisense	HClongA1	HClongA1	HC813S	HC813S	HClongA1	HClongA1	HClongA1	HClongA1	HC813S	HClongA1
1st PCR	Sense	HC3945R	HC3481R	HC3945R	HC3174AS	HC3297R	HC3945R	HC3945R	HC3945R	HC3297R	HC3481R
	Antisense	HC85F	HC85F	HC841S	HC841S	HC85F	HC85F	HC85F	HC841S	HC841S	HC85F
2nd PCR		HC3297R	HC3297R	HC3759R	HC3111AS	HC3174AS	HC3297R	HC3759R	HC3759R	HC3174S	HC3297R