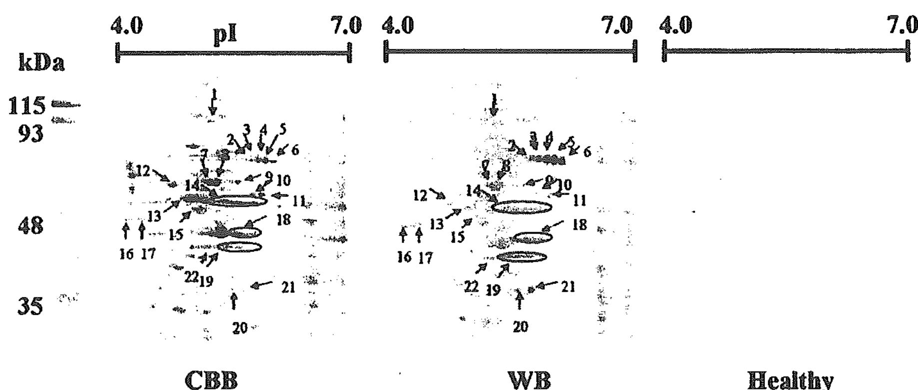

FIGURE 1 Structure of the novel protein "hepalaminin," which is encoded by AK094050. The number of the deduced amino acid (AA) residues of AK094050 was 381, which was much smaller than that of laminin β2 (1799 AA). The sequences of the first to 341st AA residues (618–1640 bp) and 342nd to 362nd AA residues (1641–1703 bp) are identical to those of the 1034th to 1374th AA residues (3998–5020 bp) and 1283rd to 1304th AA residues (5150–5212 bp) of laminin β-2, respectively. Only the short C-terminal stretch of 19 AA residues (designated as F3 portion) is of unique sequence, as shown.

Francisco, CA) for 1 hour. Finally, the bound antibodies were visualized by diaminobenzidine.

In-Gel Digestion, Mass Determination, and Mass Fingerprinting

Protein spots, which corresponded to the positive spots on the WB membranes, were recovered from the gels stained with Coomassie brilliant blue. Then, the recovered gel fragments were washed in double distilled water for 15 minutes, decolored in 50 μl decoloring solution (0.1 M ammonium hydrogen carbonate, 50% methanol) at 40°C for 15 minutes, and then cut into small pieces. The gel pieces were rehydrated in 20 μl trypsin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution (0.1 pmol/μl trypsin in 50 mM Tris-HCl) and incubated at 37°C. To extract the digested peptides, the gel fragments were immersed in 50 μl of 0.1% trifluoroacetic acid/50% acetonitrile, vortexed, and sonicated for 10 minutes. After centrifugation, the supernatant was recovered. After two more cycles of this extraction, a similar extraction was performed with 50 μl 0.1% trifluoroacetic acid/80% acetonitrile. The supernatants were collected and centrifuged again, filtered, and concentrated down to 50 μl in an evaporator, and then desalted using Zip-Tips (Millipore Corp., Bedford, MA). The sample peptide solution was stored at −20°C until mass spectrometric analysis.

The masses of the digested peptides in the samples

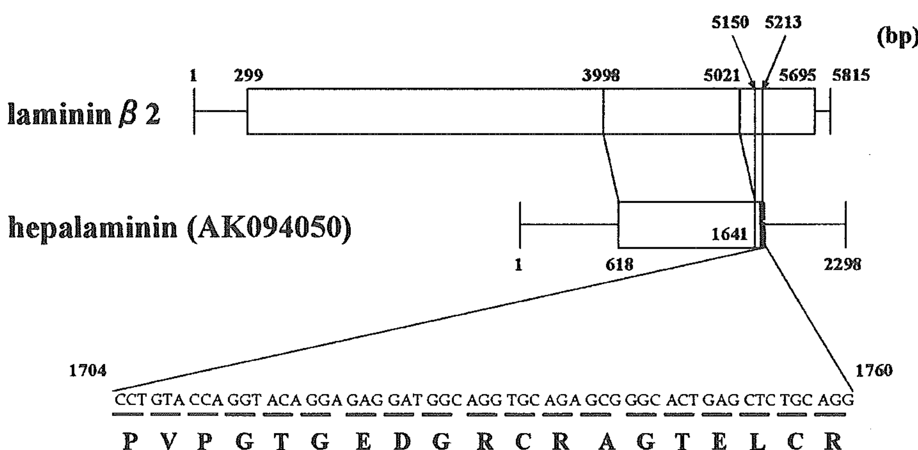

FIGURE 2 Detection of autoantigenic protein spots on gels after 2-dimensional electrophoresis (2DE). Total proteins extracted from HepG2 cells were separated by 2DE. Then the separated proteins were visualized by Coomassie's brilliant blue staining (CBB, left panel) or transferred onto a nitrocellulose membrane for Western Blotting (WB). Using WB pooled sera from 15 patients with chronic hepatitis C, more than 20 spots were found to be autoantigenic, as numbered in the left panel. The numbered spots in the right panel show overlapping points on the CBB-stained gel. Approximate molecular weights and pI values of the detected autoantigenic spots are shown in Table 1.

TABLE 1 Proteins identified by mass spectroscopy

Spot number	Protein name	MW	pI
22	AK094050 (hepalaminin)	40,479	5.17
13	Actin	40,194	5.55
7, 8	Heat shock protein 60	59,775	5.50
2–6	Heat shock protein 75	73,734	5.97

Molecular weights (MW) and isoelectric points (pI) were calculated based on the deduced amino acid sequences.

were determined using a mass spectrometer with matrix-assisted laser desorption/ionization-time of flight (Reflex 4, Bruker Daltonics Inc., Billerica, MA). α-Cyano-4-hydroxycinnamic acid was used as an assisting matrix. A list of the peptide masses so determined was compared with the mass fingerprinting using the Mascot software program (Matrix Science Ltd, London, UK), with which the National Center for Biotechnology Information (NCBI) protein databases were searched.

Preparation of Recombinant Proteins

According to the reported nucleotide sequence of AK094050 cDNA, we prepared two oligonucleotide primers to amplify by polymerase chain reaction a DNA fragment that was not homologous to laminin β-2 (designated as hepalaminin [HL] F3 in Figure 1). The nucleotide sequences of the two primers were as follows: 5′-TTTGAATTCGAGCAGACTCCTGTACCAGGTACAG GAGAGGATGGCAGGTGCAGAGCG (underlined: Eco RI recognition sequence for subcloning) and 5′-TTTGTCGACCCTGCAGAGCTCAGTGCCCGCTCTGCACCTG-CCATCCTCTCCTGT (underlined: Sal I recognition sequence for subcloning).

The resulting cDNA fragment was subcloned into the plasmid expression vector pMAL-eHis, a derivative of pMAL-c2 (New England Biolabs, Inc., Hitchin, Herts, UK). Using this construct, recombinant HL F3 protein was produced in *Escherichia coli* DH5α as a fusion with maltose binding protein (MBP). The recombinant protein was purified by the histidine-Ni^+ affinity purification method described previously [23].

ELISA

Ninety-six–well microtiter plates (Cook Dynatech, Alexandria, VA) were coated by placing into each well 50 μl of 10 μg/ml purified fusion proteins or MBP (as a control) in a carbonate buffer (50 mM sodium carbonate, pH 9.6) at 4°C for 8 hours. After washing five times with PBS containing 0.1% Tween 20, the plates were incubated overnight in 3% BSA–PBS-0.1% Tween 20 at 4°C. The plates were washed 10 times in PBS-0.1% Tween 20. Each serum sample was incubated in 3% BSA–PBS-0.1% Tween 20 with 100 μg/ml of bacterial lysate containing nonrecombinant pMAL products at room temperature for 2 hours to absorb the reactivity to

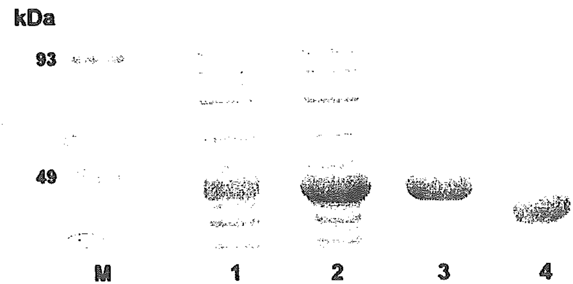

FIGURE 3 Preparation of a recombinant protein for the F3 region of hepalaminin. A DNA fragment encoding the hepalaminin-unique region (F3 portion) was synthesized chemically and then was inserted into pMAL-eHis. This construct produced the F3 portion as a maltose binding protein (MBP) fusion protein (HL-C19–MBP) in *Escherichia coli*. Lane 1, bacterial lysate before induction of hepalaminin-MBP; lane 2, bacterial lysate after induction; lane 3, purified HL-C19–MBP; lane 4, purified MBP alone as a negative control.

bacterial proteins and MBP. Fifty microliters of each pretreated serum sample was placed per well at room temperature for 2 hours. After washing 10 times with PBS-0.1% Tween 20, the plates were incubated with 2000-fold–diluted peroxidase-conjugated goat anti-human IgG antibodies at room temperature for 1 hour, and then washed 10 times with PBS-0.1% Tween 20. Color development was achieved by adding 100 μl of the peroxidase substrate, which consisted of 0.04% *o*-phenylene-diamine and 0.01% hydrogen peroxide in 0.1 M citrate/0.2 M N_2HPO_4 pH 5.0, to each well. After 15 minutes, the color reaction was stopped by adding 50 μl 6N H_2SO_4 to each well. The absorbance was measured

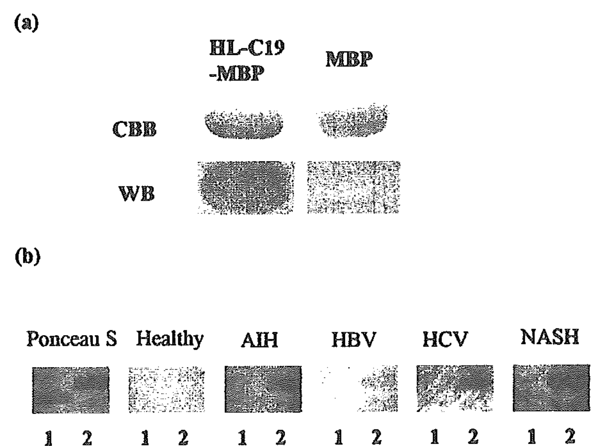

FIGURE 4 Western blotting of hepalaminin (HL)-C19–maltose binding protein (MBP). The serum mixture used for the original screening was used for WB of HL-C19–MBP and MBP as a negative control. The sera reacted to HL-C19–MBP, but not to MBP, demonstrating that HL was a genuine autoantigen and that the antigenicity differed from laminin β-2.

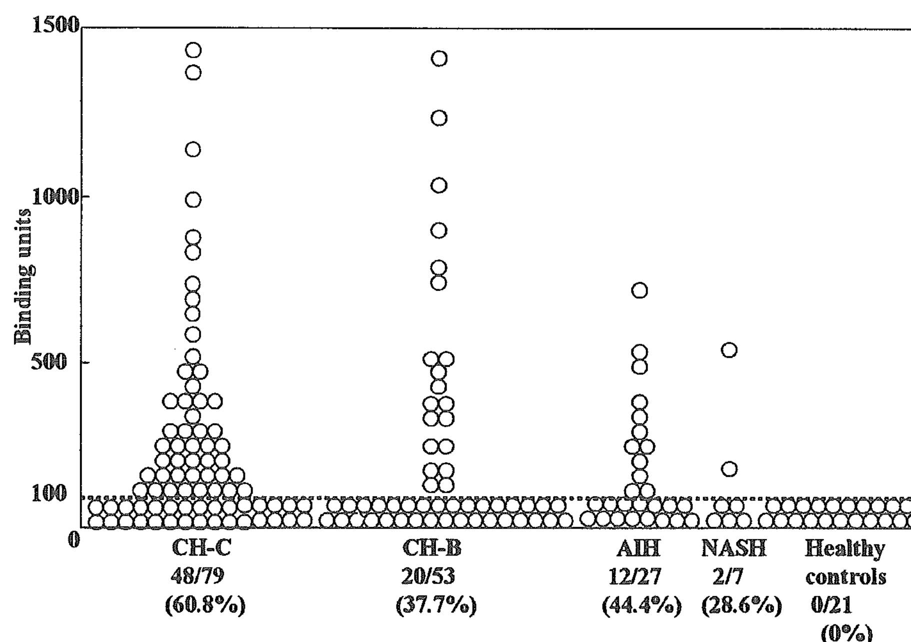

FIGURE 5 Prevalence of autoantibodies to hepalaminin (HL) determined by enzyme-linked immunosorbent assay using HL-C19–maltose binding protein in patients with chronic liver disease and healthy volunteers. One hundred binding units are defined as a cutoff value, which corresponded to the mean +3 standard deviations of the optical density obtained from the healthy volunteers.

with an ELISA microplate photometer at 492 nm and 630 nm. Each sample was measured in triplicate.

The reactivity to the fusion proteins was expressed in units according to the following formula:

binding unit = $\{OD_{sample}/(\text{mean } OD_{healthy} + 2 \text{ SD of normal sera})\} \times 100$

To obtain optical density $(OD)_{sample}$ and $OD_{healthy}$, the OD value for MBP was subtracted from that for the fusion protein in each assay. According to this formula, we defined the cutoff point as 100 binding units.

Immunohistochemistry

To demonstrate that the novel autoantigen detected here really was expressed in hepatocytes or liver tissue, a peptide consisting of the 20 amino acid residues of the HL F3 region was synthesized chemically. Then, rabbits were immunized with the peptide to raise polyclonal anti-HL F3 antibodies. Immunoglobulins were purified from the antisera using a peptide-immobilized column and used for staining liver biopsies with nonspecific rabbit IGs as a negative control.

Serum Viral Markers

Levels of HCV RNA were determined by the quantitative reverse transcriptase polymerase chain reaction assay (AMPLICOR HCV MONITOR Test Kit, Roche Diagnostic Systems, Branchburg, NJ). The HCV serotyping was performed using a commercial assay kit (Immuncheck F-HCV Gr Kokusai, International Co. Hyogo, Japan).

Statistical Analysis

Mann-Whitney's U test and Fisher's exact test were used to examine the significance of the difference of the laboratory parameters of the patients with or without autoantibodies. A p value < 0.05 was considered to be statistically significant.

RESULTS

Proteomic Analysis of Autoantigens in Patients With CH-C

We separated total proteins extracted from HepG2 cells by 2DE (Figure 2A), then transferred the separated proteins onto nitrocellulose membranes and reacted them with mixed serum samples from 15 patients with CH-C. Even though we concentrated on the area from pI 4.0 to 7.0 and of molecular weights greater than 20 kDa, 22 protein spots were found to be positive, as shown in Figure 1B. These proteins are considered as candidate autoantigens in CH-C.

Identification of the Autoantigens

Next, we tried to identify the proteins that were detected by WB. Four proteins were identified as actin, heat shock protein (HSP) 70, HSP60, and a laminin β-2–like protein (encoded by the cDNA, accession number AK094050) (Table 1).

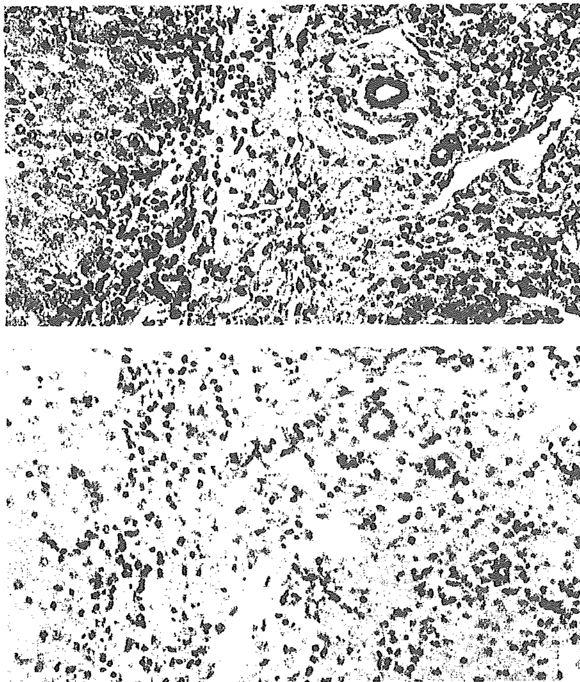

FIGURE 6 Immunohistochemical staining of hepalaminin (×200) of a biopsied liver obtained from a patient with chronic hepatitis C. The cytoplasm of hepatocytes and cholangiocytes were positively stained, but fibroblasts and vascular epithelium were negative.

Structure of the Novel Protein

We concentrated on the novel laminin β-2–like protein. The cDNA for the protein (AK094050) was registered by Kawakami *et al.* in the NEDO human cDNA sequencing project. Thus the functions of the protein, as well as its expression profile, have not been reported yet. The number of the deduced amino acid (AA) residues of AK094050 was 381, which was much smaller than that of laminin β-2 (1799 AA). The sequences of the first to 341st AA residues and 342nd to 362nd AA residues are identical to those of the 1034th to 1374th AA residues and 1283rd to 1304th AA residues of laminin β2, respectively. Only the short C-terminal stretch of 19 AA residues is of unique sequence (Figure 2).

Therefore, to distinguish autoantibodies to the product of AK094050, which we named "hepalaminin" for convenience, from those to laminin β-2 in further investigations, we prepared a recombinant protein which contained only the C-terminal 19 AA residues (HL-C19) as a fusion protein with MBP, designated as HL-C19–MBP (Figure 3). In WB using HL-C19–MBP, the serum mixture used in the original screening reacted to HL-C19–MBP, but not to MBP alone. This demonstrated that HL was a genuine autoantigen and that the antigenicity of HL was distinct from that of laminin β-2 (Figure 4).

Next, we investigated the prevalence of autoantibodies to HL by ELISA using HL-C19–MBP. Such autoantibodies were detected in 48 (60.8%) of 79 patients with CH-C, 20 (37.7%) of 53 patients with CH-B, 11 (42.3%) of 26 patients with AIH, two (28.6%) of seven patients with NASH, and two (10.0%) of 20 AsC-C, but in none of the 21 healthy volunteers (Figure 5).

Although the existence of HL was demonstrated by 2DE separation of HepG2 cells as shown in Figure 1, no other information on HL was available. To characterize HL, we prepared rabbit polyclonal Igs against the HL-C19 region and investigated the localization of HL in biopsied liver specimens obtained from seven patients with CH-C. Hepalaminin was positively stained in the cytoplasm of hepatocytes and cholangiocytes, but fibroblasts and vascular epithelium were negative (Figure 6). Thus we demonstrated that HL is expressed in liver-specific cells and that the localization of HL was quite different from that of laminin β-2, which is a known component of basal membranes.

Laboratory and Clinical Parameters of Patients With CH-C Who Do and Do Not Possess Anti-HL Autoantibodies

To investigate the pathophysiologic roles of the anti-HL autoantibodies in CH-C, we next compared clinical parameters between patients with the anti-HL autoantibodies and those without. We compared biochemical parameters and levels of HCV RNA and the ratio of serogroup 1 with serogroup 2 HCV (Table 2). However, no significant differences were noted.

Similarly, we compared the histologic activity of the liver biopsies between the two groups to elucidate the relationship of the anti-HL autoantibodies with inflammation and fibrosis. The anti-HL autoantibodies were detected more frequently in patients with more severe inflammation. Scores of category III (portal inflammation) and categories I + II + III (grading) were related to anti-HL autoantibody positivity. In contrast, no relationship was found between the positivity of anti-HL antibodies and the stage of liver fibrosis (category IV) (Table 3).

Finally, we compared the response to interferon-α therapy between the anti-HL autoantibody-positive and autoantibody-negative groups. Ten of 16 (62.5%) of the autoantibody-positive and six of 11 (54.5%) of the autoantibody-negative patients responded to interferon therapy, which showed no differences.

TABLE 2 Comparison of clinical parameters between antihepalaminin-positive and antihepalminin-negative groups in CH-C, CH-B, AIH, and NASH

Parameter	Antihepalaminin positive	Antihepalaminin negative	*p*
CH-C (n = 79)	n = 48	n = 31	
Age (years)	50.3 ± 10.9	45.7 ± 12.7	0.077
Sex (M:F)	34:14	20:11	0.556
AST (IU/I)	83.0 ± 48.3	75.4 ± 55.0	0.519
ALT (IU/I)	115 ± 78	115 ± 121	0.996
ANA (positive:negative)	6:42	4:26 (n = 30)	0.121
HCV serogroup (SG1:SG2)	18:28 (n = 46)	15:12 (n = 27)	0.173
Levels of HCV-RNA (KIU/ml)	245 ± 242	358 ± 401	0.121
CH-B (n = 53)	n = 20	n = 33	
Age (years)	39.3 ± 12.1	38.1 ± 13.1	0.758
Sex (M:F)	15:5	23:10	0.678
ALT (IU/I)	487 ± 755	326 ± 497	0.388
HBeAg (positive:negative)	13:2	13:13	0.046
HBV DNA (LGE/ml)[a]	6.44 ± 1.18	7.30 ± 1.03	0.086
AIH (n = 26)	n = 11	n = 15	
Age (years)	56.7 ± 6.2	49.6 ± 13.2	0.228
Sex (M:F)	5:6	3:12	0.165
ALT (IU/I)	419 ± 384	859 ± 905	0.154
IgG (mg/dl)	1767 ± 511	2319 ± 917	0.097
NASH (n = 7)	n = 2	n = 5	
Age (years)	52.5 ± 12.0	62.4 ± 7.1	0.214
Sex (M:F)	2:0	1:4	0.143
ALT (IU/I)	142 ± 15	82 ± 46	0.146

Abbreviations: AIH = autoimmune hepatitis; ALT = alanine aminotransferase; ANA = antinuclear antibody; AST = aspartate aminotransferase; CH-B = chronic hepatitis B; CH-C = chronic hepatitis C; HBeAg = hepatitis B e antigen; HBV = hepatitis B virus; HCV = hepatitis C virus; Ig = immunoglobulin; LGE = log genome equivalents; NASH = nonalcoholic steatohepatitis.

Laboratory and Clinical Parameters of Patients With CH-B, AIH, and NASH Who Do and Do Not Possess Anti-HL Autoantibodies

In addition to the analysis of CH-C, we compared laboratory and clinical parameters in patients with CH-B, chronic active AIH, and NASH who did and did not possess the anti-HL autoantibodies. As shown in Table 2, clinical parameters were similar between anti-HL positive and negative patients in CH-B, AIH, and NASH groups, except that hepatitis B e antigen positivity is high in anti–HL-positive patients in the CH-B group.

Histologic activities of the liver biopsies from the anti-HL positive and negative groups in CH-B and AIH are shown in Table 3. The anti-HL autoantibodies were detected more frequently in patients with more severe inflammation in CH-B. Scores of category II (intralobular degeneration and focal necrosis) and categories I + II + III (grading) were related to anti-HL autoantibody positivity. No relationship was found between positivity for anti-HL antibodies and the stage of liver fibrosis (category IV). In patients with AIH, there was no relationship between anti-HL positivity and the histologic findings (Table 3).

DISCUSSION

This study identified the targets of autoantibodies in the sera of patients with CH-C. Because the HepG2 cell line, which was established from a patient with hepatoblastoma and has been reported to retain many characteristics of native hepatocytes, was used for this study, the autoantibodies presumably were directed against hepatocyte components. Although the number of patients is limited, we may say that the components of hepatocytes targeted by autoantibodies in patients with CH-C were detected comprehensively in this study.

We could identify four protein spots from the 2D gel electrophoresis. Two spots were identified as HSP 60 and 70. Heat shock proteins are induced under stress and induce apoptosis. Autoantibodies against HSPs can modulate these steps and contribute to the pathogenesis of several diseases [24–27]. Indeed, antibodies against HSPs are found in the sera of patients with systemic lupus erythematosus [24], rheumatoid arthritis [25], and chronic active AIH [26]. However, the significance of the anti-HSP autoantibodies in CH-C has remains to be solved. Further studies are necessary to elucidate this aspect.

Another of the four spots was identified as actin.

TABLE 3 Comparison of histologic findings in the biopsied liver specimens between antihepalaminin-positive and antihepalaminin-negative groups in CH-C

Parameter	Antihepalaminin positive	Antihepalaminin negative	p
CH-C (n = 75)	n = 45	n = 30	
Category I	2.07 ± 1.56	1.53 ± 1.29	0.065
Category II	1.18 ± 0.33	1.13 ± 0.26	0.733
Category III	1.58 ± 0.84	1.13 ± 0.26	0.018
Category I + II + III	4.82 ± 5.06	3.80 ± 2.86	0.037
Category IV	1.73 ± 0.70	1.27 ± 1.10	0.106
CH-B (n = 32)	n = 16	n = 26	
Category I	3.38 ± 1.09	3.04 ± 1.18	0.362
Category II	3.13 ± 0.34	2.31 ± 1.09	0.006
Category III	2.88 ± 0.50	2.85 ± 0.54	0.864
Category I + II + III	9.38 ± 1.09	8.00 ± 2.06	0.019
Category IV	2.56 ± 0.81	2.35 ± 1.02	0.476
AIH (n = 19)	n = 4	n = 7	
Category I	3.25 ± 2.06	4.43 ± 2.23	0.409
Category II	2.50 ± 1.00	3.29 ± 0.49	0.108
Category III	2.50 ± 1.00	3.29 ± 1.25	0.314
Category I + II + III	8.25 ± 3.78	11.00 ± 3.56	0.258
Category IV	1.00 ± 0.00	2.14 ± 1.46	0.162

Abbreviations: AIH = autoimmune hepatitis; CH-B = chronic hepatis B; CH-C = chronic hepatis C.

F-actin, which is known to be an autoantigen in autoimmune liver disease, possesses a filamentous structure that is unlikely to be detected by WB. Accordingly, this autoantibody may differ from anti-smooth muscle antibody, which is often detected in AIH and sometimes in CH-C [13, 14, 28–30].

The last of the four spots was a novel protein consisting of domains derived from laminin β-2 fused to an unknown polypeptide. Laminin β-2 is an isoform of the laminin β-chains. In normal liver, this isoform exists as laminin-4 (α-2, β-2, γ-1) on the walls of sinusoids and vessels [31, 32]. Laminin is found in the basement membrane and modulates the adhesion, differentiation, and movement of cells. The results of immunohistochemical study showed that this new protein exists exclusively in hepatocytes and cholangiocytes. Therefore, this new protein should have different functions from laminin. Because the amino acid sequences of the F3 domain of this new protein are quite different from proteins reported previously, the function of this protein is difficult to predict.

It is of interest that positivity for the antibody to this novel protein, which we have named "hepalaminin," is related to the histologic grading. This coincides with the finding that positivity for this autoantibody among asymptomatic HCV carriers was low. The results suggest that infiltration of inflammatory cells within the portal tract might be related to anti-HL. Indeed, the presence of anti-HL autoantibodies may simply reflect the release of HL from destroyed hepatocytes or cholangiocytes. The roles of this novel protein and corresponding antibody are worthy of further evaluation.

Serum anti-HL was detected in about 60% of patients with CH-C. It was also detected in many patients with CH-B or AIH. The results suggest that an autoantibody response to HL reflects common mechanisms that modify hepatic inflammation in both chronic viral hepatitis and autoimmune chronic hepatitis.

In conclusion, we identified four autoantibodies present in the sera of patients with CH-C. Autoimmunity to this protein may exacerbate inflammation in CH. Additionally, 2DE-WB and mass fingerprinting were found to be powerful tools to understand the profiles of autoantibodies in CH.

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Autoantibodies to CD69 in Patients with Chronic Hepatitis Type C: A Candidate Marker for Predicting the Response to Interferon Therapy

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Key Words
Chronic hepatitis C · Autoimmunity · Autoantibody · CD69 · Hepatitis C virus · Interferon

Abstract
Objective: To understand the autoimmunity associated with chronic hepatitis C (CHC), we investigated autoantibodies (autoAbs) to CD69. ***Methods:*** With this aim, we tested the reactivity of serum samples from patients with CHC and asymptomatic carriers of hepatitis C virus (HCV), as well as from patients with chronic hepatitis B (CHB) and autoimmune hepatitis (AIH), to recombinant CD69 molecules. ***Results:*** Frequencies of anti-CD69 autoAbs were 38.7% in CHC, 15.8% in AIH and 12.3% in CHB. None of the tested asymptomatic HCV carriers had autoAbs to CD69. It is important clinically that the presence of anti-CD69 autoAbs was found to be associated with a poor response to interferon-α (IFN-α) therapy. In the epitope analysis, multiple epitopes were mapped on CD69, indicating antigen-driven production of the autoAbs. ***Conclusion:*** We evidenced existence of anti-CD69 autoAbs in patients with CHC, and found that the anti-CD69 autoAb may have potential for predicting responses to IFN-α therapy.

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Introduction

Infection with hepatitis C virus (HCV) gives rise to serious liver disorders. Specifically, about 80% of patients with acute hepatitis due to HCV become chronic HCV carriers [1]. A substantial portion of chronic HCV carriers develop chronic hepatitis which progresses steadily and finally leads to liver cirrhosis and hepatocellular carcinoma [2–11]. An inadequate and/or insufficient immune response to HCV allows persistence of the virus and thus also of chronic liver injury, although the precise mechanisms remain unclear [12–17]. In this regard, autoimmune phenomena, like autoantibodies (autoAbs) to nuclear proteins, smooth muscle and thyroid, are often observed in patients with chronic hepatitis C (CHC) [18–21]. Such autoimmune phenomena may reflect inade-

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quate immune responses to HCV and thus could be a clue to understanding immune responses to HCV. Further, the autoimmunity found in HCV infection may play a role in chronic inflammation of the liver. Therefore, the autoimmunity in HCV infection should be investigated in more detail. In this context, the anti-lymphocyte antibody (ALA) is one of the autoAbs which should be investigated in HCV infection, since ALA is detected frequently in systemic inflammatory diseases [22, 23]. However, to our knowledge, nor report is currently available that describes ALA in patients with CHC or HCV infection. Therefore, in the present study, we investigated the existence of ALA, in particular autoAbs to CD69, one of the activation surface marker molecules of T cells. We chose this approach because the existence of autoAbs to CD69, detected in 38% of patients with rheumatoid arthritis (RA) in our previous study [24], was related with the severity of RA, and profiles of the autoimmunity in CHC and RA have similar aspects, such as frequent detection of rheumatoid factors. Further, in both conditions, the inflamed tissue, liver in CHC and synovium in RA, is infiltrated with CD69-positive T cells [25–28].

Specifically, we prepared recombinant CD69 molecules and tested their reactivity to serum samples from patients with CHC as well as from asymptomatic carriers, patients with chronic hepatitis B (CHB) and autoimmune hepatitis (AIH) and healthy donors. We found that 38.7% of the tested CHC patients possessed anti-CD69 autoAbs, whereas only 12.3, 15.8 and 0% of the CHB patients, AIH patients and asymptomatic HCV carriers, respectively, possessed anti-CD69 autoAbs. Further, the CHC patients with anti-CD69 autoAbs were found to be resistant to interferon-α (IFN-α) therapy. Our data suggest that mechanisms for the production of anti-CD69 autoAbs may be involved in the pathological process of CHC and that anti-CD69 autoAbs would be useful for predicting the effectiveness of IFN-α therapy.

Patients and Methods

Patients, Liver Specimens and Serum Samples

Serum samples were obtained at the time of liver biopsy with informed consent from 127 patients who were admitted to the St. Marianna University hospital during the period from August 1994 to December 1999. They consisted of 61 patients with CHC (43 males and 18 females; mean age 47.5 years, ranging between 23 and 77 years), 47 patients with CHB (32 males and 15 females; mean age 37.7 years, ranging between 20 and 65 years), 19 patients with AIH (7 males and 12 females; mean age 50.1 years, ranging between 26 and 78 years) and 19 asymptomatic HCV carriers as defined by normal serum aspartate or alanine aminotransferase levels on periodical blood examination (7 males and 12 females; mean age 50.1 years, ranging between 26 and 78 years). The mean HCV RNA level was 637.2 KIU/ml. All of the 61 patients with CHC underwent liver biopsy and were subsequently treated with IFN-α (504 MU in total). Histological activity of hepatitis C was evaluated using the biopsied liver samples by the scoring system reported by Knodell et al. [29] or the METAVIR algorithm [30]. Serum samples from age- and sex-matched healthy donors were used as controls for each disease category. The research was carried out in accordance with the human experimentation guidelines of our institution and the Helsinki Declaration.

Viral Markers

Levels of HCV RNA were determined by quantitative reverse transcriptase polymerase chain reaction assay (Ampricor HCV Monitor Test Kit, Roche Diagnostic Systems, Branchburg, N.J., USA). The HCV serotyping was performed using a commercial assay kit (Immuncheck F-HCV Gr, Kokusai International Co., Hyogo, Japan).

Recombinant CD69 Fusion Proteins

The plasmids for production of the CD69 fusion proteins used in this study were described in our previous study [24]. Briefly, pMAL-CD69^{full}, pMAL-CD69^{F1}, pMAL-CD69^{F2}, pMAL-CD69^{F3} and pMAL-CD69^{F3d} were used for transformation of *Escherichia coli*. The CD69 proteins produced as fusion proteins with maltose-binding protein (MBP) cover the entire CD69-full (amino acid residues 1–199), CD69-F1 (amino acid residues 1–98), CD69-F2 (amino acid residues 91–146), CD69-F3 (amino acid residues 138–199) and CD69-F3d (amino acid residues 184–199), respectively (fig. 1). Each of the fusion proteins was purified using amylose resin columns (New England Biolabs, Beverly, Mass., USA) according to the manufacturer's instructions. pMAL-C, an expression vector producing MBP alone, was also used as a control.

Enzyme-Linked Immunosorbent Assay

Ninety-six-well microtiter plates (Cook Dynatech, Alexandria, Va., USA) were coated by placing in each well 50 μl of 10 μg/ml purified CD69 fusion proteins or MBP (as a control) in a carbonate buffer (50 m*M* sodium carbonate, pH 9.6) at 4° for 8 h. After washing with phosphate-buffered saline (PBS) containing 0.1% Tween 20 5 times, the plates were incubated in 3% bovine serum albumin (BSA)-PBS-0.1% Tween 20 at 4° overnight. The plates were washed in PBS-0.1% Tween 20 10 times. To absorb the reactivity of the serum sample to bacterial proteins and MBP, each serum sample was incubated in 3% BSA-PBS-0.1% Tween 20 with 100 μg/ml bacterial lysate containing nonrecombinant pMAL products at room temperature for 2 h in advance. Fifty microliters of each serum sample was placed in each well at room temperature for 2 h. After washing 10 times with PBS-0.1% Tween 20, the plates were incubated in 4,000-fold diluted peroxidase-conjugated goat anti-human IgG antibodies at room temperature for 1 h, and then washed 10 times with PBS-0.1% Tween 20. Color development was achieved by adding 100 μl of peroxidase substrate, which consisted of 0.04% *o*-phenylene-diamine and 0.01% hydrogen peroxide in 0.1 *M* citrate-0.2 *M* N_2HPO_4, pH 5.0, to each well. After 15 min, the color reaction was stopped by adding 50 μl of 6 *N* H_2SO_4 to each well. The absorbance was measured with an enzyme-linked immunosorbent assay (ELISA) microplate photometer at 492 nm and 630 nm. Each sample was measured in triplicate.

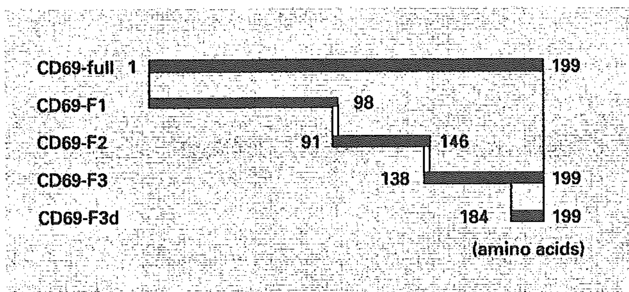

Fig. 1. Map of the recombinant CD69 proteins used in this study. Each of CD69-full, F1, F2, F3 and F3d was produced as a fusion protein with MBP using the plasmid vector pMAL.

The reactivity to the fusion proteins was expressed in units according to the following formula: binding units = [OD_{sample}/(mean $OD_{healthy}$ + 3 SD of normal sera)] × 100, where OD is the optical density. To obtain OD_{sample} and $OD_{healthy}$, the OD value of MBP was subtracted from that of the fusion protein in each assay. According to this formula, we defined the cutoff point as 100 binding units.

Western Blot Analysis

Western blotting was performed as described previously [31]. Briefly, 5 μg of each purified fusion protein or MBP (as a control) was separated by 10% SDS-PAGE and then transferred onto a nitrocellulose membrane. After blocking with PBS containing 1% BSA and 0.1% Tween 20 for 2 h and washing in PBS with 0.1% Tween 20 for 5 min 3 times, each membrane was then incubated with goat anti-human CD69 antibody (Santa Cruz Biotechnology, Santa Cruz, Cal-

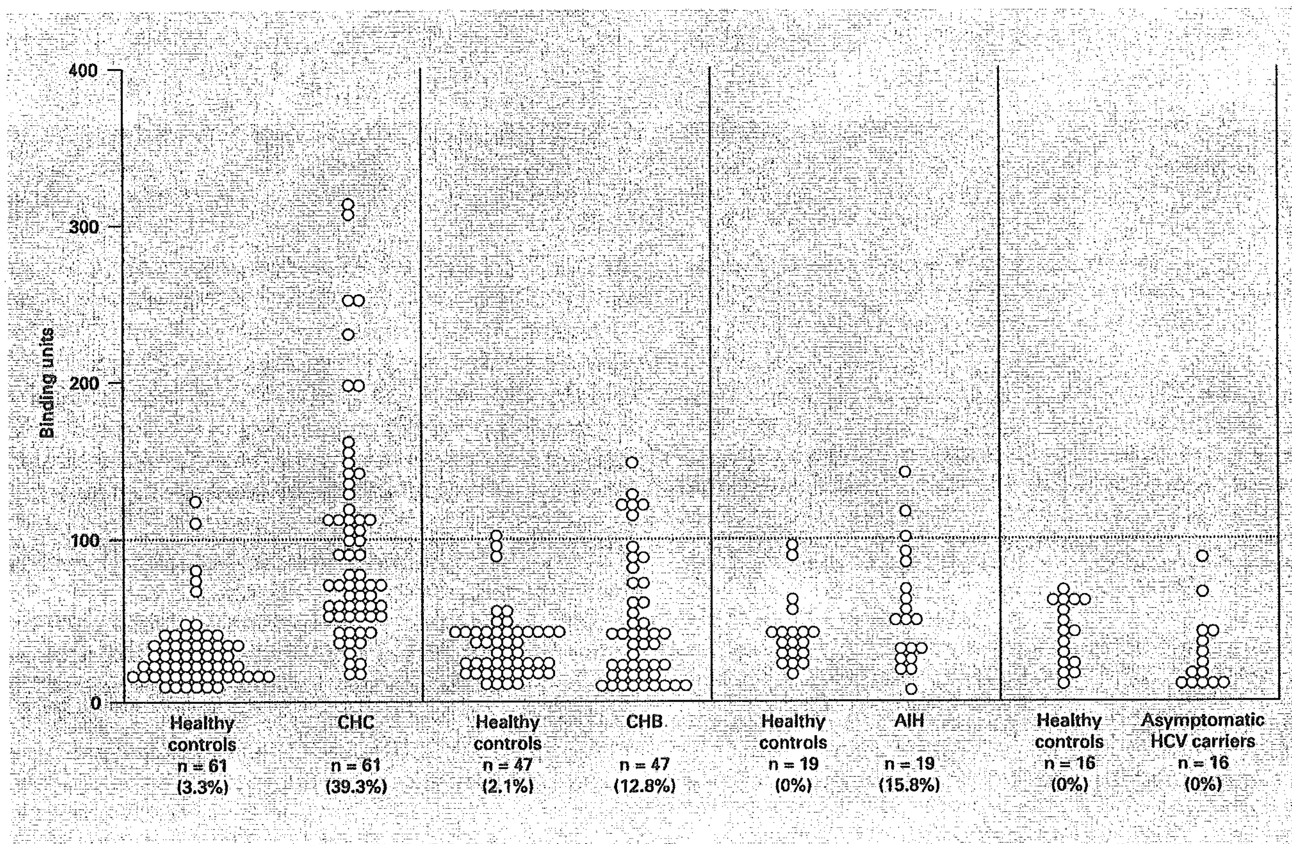

Fig. 2. Anti-CD69 autoAbs detected by ELISA. AutoAbs to the recombinant CD69-full protein (fig. 1) were titered by ELISA. Serum samples were diluted at 1:500. Identical numbers of serum samples from sex- and age-matched healthy controls were also titered for each of the disease categories. The OD value of each sample is indicated by binding units, calculated according to the formula given in Patients and Methods. The dotted line indicates the positive cutoff value of 100 binding units. The positivity of anti-CD69 autoAbs is indicated by percentages. n = Numbers of samples tested.

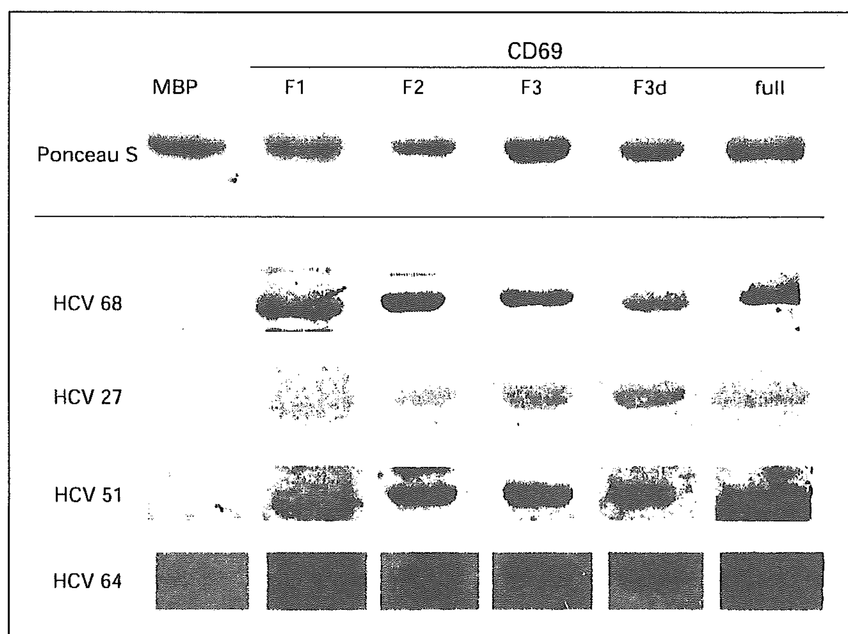

Fig. 3. Representative results of Western blotting. The recombinant CD69-full protein and MBP as a control were separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were reacted with serum samples diluted at 1:100 and then with horseradish peroxidase-labeled anti-human IgG antibodies. The bound autoAbs were visualized with diaminobenzidine.

if., USA), goat anti-MBP antibody (Santa Cruz Biotechnology) and each serum sample for 2 h. Before the membrane was incubated with the serum sample, the serum sample was diluted at 1:100 with PBS containing 1% BSA and 0.1% Tween 20 and preincubated with 2 mg/ml bacterial lysate containing nonrecombinant pMAL-c product for 2 h at room temperature. Following membrane incubation, the membrane was washed 5 times in PBS with 0.1% Tween 20, and the bound antibodies were reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (Medical & Biological Laboratories, Nagoya, Japan) or goat anti-human IgG (Zymed Laboratories, San Francisco, Calif., USA) diluted at 1:3,000–10,000 with PBS containing 1% BSA and 0.1% Tween 20 for 1 h. The bound antibodies were visualized with diaminobenzidine.

Statistical Analysis

Laboratory parameters are expressed as the mean ± SEM. The Mann-Whitney U test and Fisher's exact test were used to examine the significance of the difference in the laboratory parameters of the CHC patients with or without anti-CD69 autoAbs. p values of less than 0.05 were considered to be statistically significant. A multiple logistic regression analysis was used to elucidate independent predictive factors for the sustained response to IFN treatment.

Results

Reactivity of the Sera of Patients with Various Liver Diseases to Recombinant CD69 Protein

We investigated whether autoAbs to CD69 exist in sera of patients with various liver diseases by ELISA using the full-length recombinant CD69 molecule (fig. 1). As a result, IgG-type anti-CD69 autoAbs were detected in 24 out of the 61 patients with CHC (38.7%), in 6 out of the 47 patients with CHB (12.8%) and in 3 out of the 19 patients with AIH (15.8%) (fig. 2). On the other hand, autoAbs were detected in only 0–3.2% of the age- and sex-matched healthy donor groups for each category. Interestingly, none of the 19 asymptomatic HCV carriers tested showed positive reactivity to the CD69 molecule (fig. 2). Each positive serum sample was further confirmed to possess anti-CD69 autoAbs by Western blot analysis. Representative results are shown in figure 3.

Considering these data together, we found that autoAbs to CD69 existed in sera of patients with various types of chronic hepatitis. It is of interest that autoAbs to CD69 were much more common in patients with CHC than in those with CHB or AIH and were not detected in asymptomatic HCV carriers.

Epitope Mapping of CD69 Using Serum Samples from Patients with CHC

As described above, autoAbs to CD69 were much more common in patients with CHC than in those with CHB or AIH or in the asymptomatic HCV carriers. Thus, we recommend further studies concentrating on CHC. In our previous study, a predominant autoepitope was mapped at the C terminus of the CD69 molecule, using serum samples from patients with RA [24]. To under-

Table 1. Reactivity of the serum samples from patients with CHC to the four truncated CD69 proteins F1, F2, F3 and F3d by Western blotting

Sample No.	CD69			
	F1	F2	F3	F3d
6	+	+	+	+
8	+	+	+	+
16	±	+	+	+
19	+	+	+	+
21	–	+	+	±
25	–	+	+	+
27	±	±	±	+
31	+	+	+	±
34	+	+	+	+
41	±	+	+	±
43	+	+	+	+
44	+	+	+	+
46	+	+	+	+
47	+	+	+	+
49	+	+	+	+
51	+	+	+	±
52	+	+	+	±
59	+	+	+	+
60	–	+	+	±
61	±	+	+	–
64	+	+	+	+
68	+	+	+	+
70	±	+	+	–
71	+	+	±	+
Reactivity	21/24 (87.5)	24/24 (100)	24/24 (100)	22/24 (91.7)

The serum samples used were positive for the anti-CD69 autoantibody by ELISA.

Figures in parentheses represent percentages.

stand whether the mechanism for the production of anti-CD69 autoAbs in the patients with CHC was the same as that in RA patients or not, we investigated autoepitopes on the CD69 molecule, using the anti-CD69-positive serum samples from the patients with CHC. Specifically, we prepared four truncated fusion proteins of F1, F2, F3 and F3d [24] (fig. 1) and then investigated their antigenicity for the serum samples by Western blotting. F1, F2 and F3 covered the entire region of the CD69 molecules with overlaps. F3d is a small peptide region that carried the predominant autoepitope detected in our RA patients [24]. As a result, 19 out of the 24 serum samples (79%) reacted to all four fusion proteins F1, F2, F3 and F3d, and the remaining 5 serum samples reacted to three of the four regions (table 1). Representative results are shown in figure 4. This result was different from that obtained from the study on RA [24], in which most of the serum samples reacted solely to F3d.

Laboratory and Clinical Parameters of Patients with CHC with or without Anti-CD69 AutoAbs

To investigate pathophysiological roles of anti-CD69 autoAbs in CHC, we next compared clinical parameters between patients with anti-CD69 autoAbs and those without them. First, we compared biochemical parameters by blood examinations, including aspartate aminotransferase, alanine aminotransferase, albumin and prothrombin time, as shown in table 2. Also, we examined amounts of HCV RNA and the ratio of serogroup 1 to serogroup 2 of HCV (table 3). However, no significant difference was detected. Similarly, we compared the histological activity of the biopsied liver samples by the criteria of the histological activity index of Knodell et al. [29] between the two groups to elucidate the relation of anti-CD69 autoAbs with inflammation and fibrosis. However, no significant difference was observed (table 4).

Lastly, we compared the response to IFN-α therapy between the anti-CD69 autoAb-positive and -negative groups. Interestingly, 26 out of the 34 anti-CD69 autoAb-negative patients (76.5%) showed a sustained response (defined by negative HCV RNA 24 weeks after treatment) to IFN-α treatment (table 5). In contrast, only 5 out of the 20 anti-CD69 autoAb-positive patients (25%) met the criteria for a sustained response. Thus, the anti-CD69 autoAb could be a marker to predict response to IFN-α treatment. To evaluate whether the anti-CD69 autoAb was an independent factor for prediction, we applied multiple logistic regression analysis to the presence of anti-CD69 autoAbs as well as the amounts of HCV RNA and HCV serotypes tested here. As shown in table 6, although a high amount of HCV RNA (≥ 100 kcopies/ml) was the strongest predictive factor for the sustained response to IFN-α treatment ($p = 0.003$), the presence of the anti-CD69 autoAb was found to be another strong independent predictive factor ($p = 0.010$). HCV serotypes did not reach significance ($p = 0.086$). These data indicate that the anti-CD69 autoAb is an independent predictive factor for a sustained response to IFN-α treatment.

Laboratory and Clinical Parameters of Patients with CHB or AIH with or without Anti-CD69 AutoAbs

In addition to the analysis of CHC, we compared laboratory and clinical parameters in patients with CHB or AIH who did and did not demonstrate anti-CD69 au-

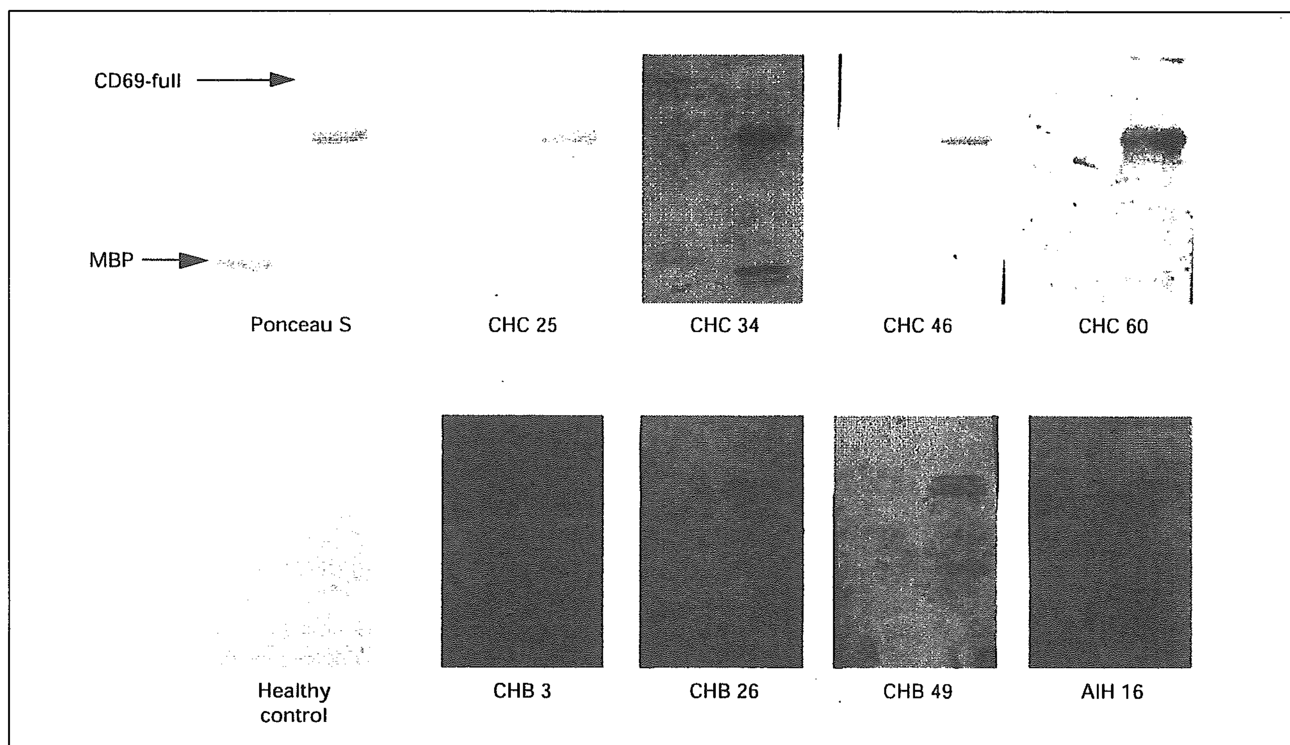

Fig. 4. Representative results of Western blotting of the truncated CD69 fusion proteins. The recombinant proteins CD69-full, F1, F2, F3 and F3d and MBP as a control were separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were reacted with serum samples diluted at 1:100 and then with horseradish peroxidase-labeled anti-human IgG antibodies. The bound autoAbs were visualized with diaminobenzidine.

toAbs. As a result, no significant difference was detected, as shown in tables 7 and 8, respectively.

Discussion

This study investigated the existence, production mechanisms and clinical significance of anti-CD69 autoAbs in patients with chronic hepatitis. Our results are as follows: 37.8% of the patients with CHC had anti-CD69 autoAbs, whereas only 12.8% of the patients with CHB and 15.8% of the patients with AIH had the autoAbs. None of the asymptomatic HCV carriers tested were positive for anti-CD69 autoAbs. Multiple epitopes on the CD69 molecule were recognized by most of the serum samples. The presence of anti-CD69 autoAbs had a reverse correlation with a sustained response to IFN-α therapy.

With regard to the first point, hepatitis C has been reported to be accompanied by various autoAbs like anti-nuclear antibodies, anti-smooth-muscle antibodies, anti-thyroid antibodies and anti-cardiolipin antibodies [18–21]. However, there have been no reports so far on anti-lymphocyte autoAbs. Therefore, we here investigated anti-CD69 autoAbs, one of the ALAs, which we first reported in patients with RA. As a result, we found that anti-CD69 autoAbs were detected most frequently in patients with CHC. Since more than 10% of the patients with CHB or AIH had anti-CD69 autoAbs, anti-CD69 autoAbs are not a specific marker for HCV infection. However, anti-CD69 autoAbs were 2–3 times as frequent as anti-nuclear antibodies and even more frequent than cryoglobulin, as shown in table 2. Thus, the anti-CD69 autoAb could be one of the representative autoimmune phenomena in CHC.

Second, the asymptomatic HCV carriers tested did not have anti-CD69 autoAbs, indicating that the presence of

Table 2. Comparison of blood biochemical parameters between anti-CD69 autoAb-positive and -negative patients with CHC

Parameter	AutoAbs to CD69		p
	positive (n = 24)	negative (n = 37)	
Aspartate aminotransferase, IU/l	80.6 ± 47.2	78.7 ± 52.0	NS
Alanine aminotransferase, IU/l	122.5 ± 71.7	120.0 ± 118.1	NS
Prothrombin time, %	88.1 ± 12.2	93.1 ± 11.0	NS
Albumin, g/dl	4.4 ± 0.5	4.4 ± 0.4	NS
Platelets, $\times 10^4/\mu l$	15.8 ± 4.9	16.9 ± 5.1	NS
ICG, R15%	12.5 ± 10.2	12.4 ± 8.7	NS
Rheumatoid factor, U/ml	11.7 ± 6.9	425 ± 2,035	NS
Erythrocyte sedimentation rate, mm/h	15.4 ± 27	12.5 ± 21	NS
C-reactive protein, mg/dl	<0.3	<0.3	NS
IgG, mg/dl	1,801 ± 391	1,662 ± 463	NS
IgA, mg/dl	222 ± 124	240 ± 122	NS
IgM, mg/dl	120 ± 42	129 ± 177	NS
TTT, U	9.7 ± 9.8	7.6 ± 4.8	NS
ZTT, U	17.7 ± 4.8	15.5 ± 6.2	NS
Positive ANA[1]	3/22 (13.6)	6/34 (17.6)	NS
Positive cryoglobulin[2]	6/15 (40	11/23 (48)	NS

p values were calculated using the Mann-Whitney U test. Figures in parentheses represent percentages. ICG = Indocianin green test; R15% = remnant rate after 15 min (%); TTT = thymol turbidity test; ZTT = zinc turbidity test; ANA = antinuclear antibody; NS = not significant.

1 Data from 56 patients with CHC were available.

2 Data from 38 patients with CHC were available.

Table 3. Comparison of the subtypes and concentrations of HCV between anti-CD69 autoAb-positive and -negative patients with CHC

HCV	Anti-CD69 autoAbs		p
	positive (n = 22)	negative (n = 33)	
Subtype[1]			
Serogroup 1	11 (50.0)	11 (33.3)	NS
Serogroup 2	11 (50.0)	22 (66.7)	NS
Serum HCV RNA, kcopies/ml[2]	399 ± 411 (n = 14)	257 ± 308 (n = 30)	NS

p values were calculated using the Mann-Whitney U test. Figures in parentheses represent percentages. NS = Not significant.

1 Data from 55 patients with CHC were available.

2 Data from 34 patients with CHC were available.

HCV itself is not linked to production of anti-CD69 autoAbs. Thereby, the anti-CD69 autoAb could be produced in the inflammatory process in the liver caused by HCV. Alternatively, the anti-CD69 autoAb could be one of the triggers to start the inflammatory process in the liver under CHV-infected conditions. Further studies will be needed to elucidate these mechanisms.

With regard to the third point, we previously reported that the anti-CD69 autoAb was detected in 38% of patients with RA. CHC and RA have several immunological disorders in common, such as frequent detection of rheumatoid factors and cryoglobulins [32]. Our study indicates that the anti-CD69 autoAb is another immunological disorder common to CHC and RA. In our previous

Table 4. Comparison of histological activity between anti-CD69 autoAb-positive and -negative patients with CHC

	HAI score	Anti-CD69 autoAbs		p
		positive (n = 24)	negative (n = 37)	
I.	Periportal ± bridging necrosis	1.75±1.11	2.08±1.38	NS
II.	Intralobular degeneration and focal necrosis	2.00±0.00	1.11±0.46	NS
III.	Portal inflammation	1.42±0.83	1.49±0.87	NS
IV.	Fibrosis	1.54±0.98	1.68±1.00	NS

p values were calculated using the Mann-Whitney U test. NS = Not significant; HAI = histological activity index.

Table 5. Difference in the responses to IFN-α therapy between anti-CD69 autoAb-positive and -negative patients with CHC

Response to IFN-α	Anti-CD69 autoAbs	
	positive (n = 20)	negative (n = 34)
Sustained response	5 (25.0)	26 (76.5)
No response	15 (75.0)	8 (23.5)

p = 0.0002 by Fisher's exact test. Figures in parentheses represent percentages.

Table 6. Variables related with resistance to IFN-α treatment by multiple logistic regression analysis

Variables	Odds ratio	95% CI	p
HCV RNA			
<100 kcopies/ml	1		
≥100 kcopies/ml	8.771	0.028–0.468	0.003
HCV serotype			
2	1		
1	3.831	0.052–1.308	0.086
Anti-CD69 autoAbs			
Negative	1		
Positive	8.850	0.028–0.457	0.010

Table 7. Comparison of blood biochemical parameters between anti-CD69 autoAb-positive and -negative patients with CHB

Parameters	Anti-CD69 autoAbs		p
	positive (n = 6)	negative (n = 41)	
Age, years	38.2±15.9	42.3±16.2	NS
Sex (M:F)	5:1	24:17	NS
Alanine aminotransferase, IU/l	312±283	193±172	NS
HBsAg positive	6	41	NS
HBeAg positive	4	34	NS
Anti-HBe positive	2	7	NS
HBV DNA, pg	615±197	833±199	NS
Histology of the liver			
Fibrosis[1]	2	1.5	NS
Overall histological activity[1]	1	2.3	NS

p values were calculated using the Mann-Whitney U test or Fisher's exact test. HBsAg = Hepatitis B s antigen; HBeAg = hepatitis B e antigen; HBV = hepatitis B virus; NS = not significant.

[1] Based on the METAVIR system.

Table 8. Comparison of blood biochemical parameters between anti-CD69 autoAb-positive and -negative patients with AIH

Parameters	Anti-CD69 autoAbs		p
	positive (n = 3)	negative (n = 16)	
Age, years	45.7±16.7	42.3±16.2	NS
Sex (M:F)	2:1	10:6	NS
Alanine aminotransferase, IU/l	279±283	810±899	NS
IgG, mg/dl	2,409±890	2,315±693	NS
Type of AIH			
Type I	3	14	NS
Type IV	0	2	NS
Histology of the liver			
Fibrosis[1]	1	1.4	NS
Overall histological activity[1]	1.7	2.3	NS

p values were calculated using the Mann-Whitney U test or Fisher's exact test. NS = Not significant.

[1] Based on the METAVIR system.

study [24], we found that most of the anti-CD69-positive RA serum samples recognized only one epitope nearly exclusively. The amino acid sequence of the predominant epitope is homologous to a part of low density lipoprotein receptor-related protein 2 (LRP-2), and, in fact, autoAbs to the predominant epitope of CD69 cross-reacted with the homologous region of LRP-2 [24]. Thus, we speculated that the anti-CD69 autoAb could belong to the anti-LRP-2 autoAbs. To investigate whether this scenario can be applied to anti-CD69 autoAbs in CHC, we mapped autoepitopes on CD69 using the serum samples of the tested CHC patients. As shown in table 3 and figure 4, the CHC serum samples mostly recognized all four fragments of CD69. This recognition of multiple epitopes indicates an antigen-driven immune response, not cross-reactive recognition. Thus, the mechanisms for the production of anti-CD69 autoAbs would be different between CHC and RA.

The last finding that the presence of anti-CD69 autoAbs had a significant correlation with resistance to IFN-α treatment in CHC is of clinical importance. Further, multiple logistic regression analysis revealed that the anti-CD69 autoAb was an independent predictive factor for a sustained response to treatment. Diagnostically, previous studies elucidated that the response to IFN-α treatment was influenced by several factors like viral genotypes, amounts of the viral RNA, the amino acid sequence of the IFN sensitivity-determining region and the histological grading [33–37]. However, there remain some patients whose response to IFN-α therapy cannot be predicted by the proposed predictive factors. Thus, the presence or absence of anti-CD69 autoAbs, as reported here, may be a novel useful marker for the prediction of response to treatment.

The question of how the presence of anti-CD69 autoAbs is linked to resistance to IFN-α therapy remains to be answered. Here, we could not detect substantial binding of anti-CD69 autoAbs to native CD69 on lymphocytes from CHC patients (data not shown). On this point, we previously reported the binding of anti-CD69 autoAbs to native CD69 on lymphocytes from RA patients; however, the binding was very weak [24]. Combining these data, autoAbs to recombinant CD69 may not bind substantially to natural CD69 on live lymphocytes. Therefore, although the anti-CD69 autoAb is a good marker, it would not have a direct effect on the resistance to IFN-α therapy, as is the case with the anti-Sm autoAb, which is a specific marker for systemic lupus erythematosus, but not has been shown to play a pathological role in the disease. Alternatively, we here detected autoAbs to recombinant CD69 molecules, which would not form conformational epitopes. AutoAbs to conformational epitopes, expressed only on native CD69, may have some pathological role. Further studies will be needed.

In conclusion, we have shown that the anti-CD69 autoAb is detected frequently in patients with CHC and that the presence of these autoAbs is linked to a negative response to IFN-α treatment. Further studies on CD69 autoAbs would provide us with clues to elucidate the inflammatory process of CHC and with diagnostic benefit in being able to predict responses to IFN-α therapy.

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Distinct Geographic Distributions of Hepatitis B Virus Genotypes in Patients With Acute Infection in Japan

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Genotypes of hepatitis B virus (HBV) were determined in 145 patients with acute hepatitis B from various districts in Japan to establish their geographic distribution and evaluating the influence on the clinical illness and outcome. Genotypes were A in 27 (19%) patients, B in 8 (5%), C in 109 (75%) and mixed with B and C in the remaining one (1%). Genotype A was more frequent in metropolitan than the other areas (21/69 (30%) vs. 6/76 (8%), $P < 0.001$). On phylogenetic analysis, seven of the nine (78%) HBV/A isolates selected at random clustered with those from Europe and the United States, while the remaining two with those of subgroup A′ prevalent in Asia and Africa. Maximum ALT levels were lower (2069 ± 1075 vs. 2889 ± 1867 IU/L, $P = 0.03$) and baseline HBV DNA titers were higher (5.90 ± 1.45 vs. 5.13 ± 1.36 log genome equivalents (LGE)/ml, $P = 0.002$) in patients infected with genotype A than C. Hepatitis B surface antigen persisted longer in patients infected with genotype A than C (1.95 ± 1.09 vs. 1.28 ± 1.42 months, $P = 0.02$). HBV infection became chronic in one (4%) patient with genotype A and one (1%) with genotype C infection. Fulminant hepatic failure developed in none of the patients with genotype A, one (13%) with genotype B and five (5%) with genotype C. The point mutation in the precore region (A1896) or the double mutations in the basic core promoter (BCP) region (T1762/A1764) were detected in none of the patients with genotype A, two (25%) with genotype B and 27 (26%) with genotype C. In conclusion, genotype A is frequent in patients with acute hepatitis B in metropolitan areas of Japan, probably reflecting particular transmission routes, and associated with longer and milder clinical course than genotype C. ***J. Med. Virol. 77:39–46, 2005.*** © 2005 Wiley-Liss, Inc.

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INTRODUCTION

The clinical outcome in patients with acute hepatitis B varies widely. Although hepatitis is self-limited in most patients, the clinical features range from asymptomatic to fulminant hepatic failure, while some patients become carriers of hepatitis B virus (HBV) [Chan HL and Lok, 1999; Chan HLY, 1999]. Factors that determine the clinical outcome remain unknown.

Viral nucleotide (nt) mutations have been shown to influence the clinical outcome of acute hepatitis B. Mutations in the precore region (A1896) and the basic core promoter (BCP) region (T1762/A1764) are common in patients with fulminant hepatic failure [Carman et al., 1991; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Hawkins et al., 1994; Sato et al., 1995; Baumert et al., 1996; Chu et al., 1996]. Viral factors other than these mutations may influence the clinical outcome of acute hepatitis B.

Eight genotypes of HBV have been identified by sequence divergence greater than 8% in the entire genome, and they are designated by capital alphabet letters from A to H [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. Furthermore, recombinant HBV strains consisting of two different genotypes have been reported [Bollyky et al., 1996; Morozov et al., 2000]. Genotype distribution is different in different countries and even in distinct areas of the same country [Orito et al., 2001a; Kao, 2002; Kato et al., 2002; Miyakawa and Mizokami, 2003]. Therefore, surveys on genotype distribution may be helpful in identifying transmission routes and evaluating clinical relevance.

It has been shown that the clinical outcome of chronic hepatitis B is influenced by HBV genotypes. In Asian patients with chronic hepatitis B, genotype C is associated with later seroconversion of hepatitis B e antigen (HBeAg) and more severe liver damage than genotype B [Kao et al., 2000; Orito et al., 2001b; Chu et al., 2002; Ding et al., 2002; Sugauchi et al., 2002a]. Likewise, a study from India has shown that genotype D is associated with more severe liver disease than genotype A [Thakur et al., 2002]. Genotype A is peculiar in that A1896 in the precore region occurs infrequently, because it causes instability of the stem-loop structures of the pregenome encapsidation signal [Li et al., 1993; Lok et al., 1994]. These reports suggest that HBV genotypes also influence the clinical characteristics of acute hepatitis. Recent studies on small numbers of patients with acute hepatitis B suggest that the clinical course may differ among infections with distinct HBV genotypes [Mayerat et al., 1999; Kobayashi et al., 2002; Ogawa et al., 2002]. However, the association between viral genotype and severity of liver disease remains uncertain in acute HBV infection.

To evaluate the effect of HBV genotypes on the clinical characteristics of acute hepatitis B, a multi-center study on 145 patients was conducted in Japan.

MATERIALS AND METHODS

Patients

During 1992 through 2001, serum samples were collected from 147 patients diagnosed with acute hepatitis B in our institutions. Only patients from whom sera at the onset of hepatitis were stored were included in this study. Sixty-nine (47%) patients lived in metropolitan areas (Kawasaki, Tokyo and Tokorozawa), while the others in Kurume, Ube, Osaka, Gifu, Nagoya and Sapporo. Criteria for the diagnosis of acute hepatitis B were: (1) Acute onset of liver injury without a history of liver dysfunction and detection of hepatitis B surface antigen (HBsAg) in serum; and (2) IgM antibody to HBV core (anti-HBc) in high titer. Co-infection with hepatitis A virus or hepatitis C virus was excluded by serological tests.

Among the 147 patients, acute hepatitis B in six (4%) was complicated by hepatic encephalopathy and prolonged prothrombin time for the diagnosis of fulminant hepatic failure. Other two (1%) patients remained positive for HBsAg for longer than 6 months, and they were considered to have acquired chronic infection.

Sera from the 147 patients with acute hepatitis B were examined virologically, and the results were correlated with clinical and demographic characteristics. Informed consent was obtained from each patient for the purpose of this study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Ethics Committees of our institutions.

Determination of HBV DNA

Levels of HBV DNA were determined using transcription-mediated amplification (TMA) and hybridization-protection assay (Chugai Diagnostics Science Co., Ltd., Tokyo, Japan) after the protocol as reported [Kamisango et al., 1999]. The range of detection by TMA was from 3.7 log genome equivalents (LGE)/ml ($10^{3.7}$ copies/ml corresponding to 5,000 copies/ml) to 8.7 LGE/ml ($10^{8.7}$ copies/ml). In 16 of 86 studied sera, levels of HBV DNA were under 3.7 LGE/ml and categorized in 3.7 LGE/ml.

Genotyping HBV

HBV genotypes in most samples were determined with commercial enzyme immunoassay kits (HBV Genotype EIA, Institute of Immunology Co. Ltd., Tokyo, Japan) involving monoclonal antibodies to genotype-specific epitopes in the preS2-region, as reported previously [Usuda et al., 1999, 2000; Kato et al., 2001]. Genotypes in 18 (12%) samples were determined by genotype-specific probe assay (Smitest HBV Genotyping Kit, Genome Science, Fukushima, Japan). In brief, DNA extracted from serum was amplified by the polymerase chain reaction (PCR) with three sense primers (s1: 5′-ACC AAC CCT CTG GGA TTC TTT CC-3′, s2: 5′-ACC AAT CCT CTG GGA TTC TTC CC-3′ and s3: 5′-AGC AAT CCT CTA GGA TTC CTT CC-3′ [nt 2902–2924]) and an antisense primer (as1: 5′-GAG CCT GAG GGC TCC ACC C-3′ [nt 3091–3073]) biotinated at the 5′-end;

they were deduced from conserved sequences in the preS1 region of HBV. The biotin-labeled and amplified HBV DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one or other of the seven genotypes (A–G) immobilized on wells of a 96-well microplate. Thereafter, hybiridization was detected by staining with the streptavidine-horseradish peroxidase (HRP) conjugate [Kato et al., 2003].

Subtypes of genotype B, in terms of Ba with the recombination with genotype C and Bj without it were determined by direct sequencing of precore and core regions by the method reported previously [Sugauchi et al., 2002b].

Amplifying and Sequencing HBV DNA of Genotype A Isolates

A subgroup of genotype A is reported with the designation of A' from South Africa, Philippines, Malawi, and Belgium [Bowyer et al., 1997; Kramvis et al., 2002; Sugauchi et al., 2004]. Randomly selected HBV/A samples were classified into genotype A and subtype A' by sequencing the S region. For amplification and sequencing, the entire S region was divided into two fragments, spanning nt 3130–478 and nt 378–878, respectively, and they were amplified by two-stage PCR. The outer primers for amplification of the 1st fragment were: 5′-ACC AAT CGG CAG TCA GGA AG-3′ (sense: nt 3121–3140) and 5′-CTG GAA TTA GAG GAC AAA CG-3′ (antisense: nt 488–469) and the inner primers were: 5′-CAG TCA GGA AGG CAG CCT ACT-3′ (sense: nt 3130–3150) and 5-AGG ACA AAC GGG CAA CAT AC-3′ (antisense: nt 478–459). The outer primers for amplification of the 2nd fragment were: 5′-TGT CCT GGT TAT CGC TGG AT-3′ (sense: nt 359–378) and 5′-CAA CGT ACC CCA ACT TCC AA-3′ (antisense: nt 909–890) and the inner primers were: 5′-TGT GTC TGC GGC GTT TTA TC-3′ (sense: nt 378–397) and 5′-ATG AAG TTT AGG GAA TAA CC-3′ (antisense: nt 878–859).

The first stage of amplification was carried out in a thermal cycler for 40 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in 100 μl of the reaction mixture containing 200 μM dNTPs, 1.0 μM each of primers and 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.001% (wt/vol) gelatin) and 2 U of Ampli-Taq polymerase (Perkin Elmer Cetus Corp., Connecticut). PCR products (2 μl) were subjected to the second stage of amplification under the same conditions as the first stage. Standard precautions to avoid contamination were exercised during PCR, with a negative control serum included in each run.

Amplification products were purified on Wizard PCR preps DNA purification resin (Promega, Wisconsin), and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, California) using the PCR primers. Sequencing was performed in an automated DNA sequencer (ABI 377: PE Applied Biosystems).

The nucleotide sequences of HBV/A isolates from patients were compared with those of 25 reference HBV/A strains including subtype A' retrieved from the DDBJ/EMBL/GenBank database, as well as representatives of the other six major genotypes (B–G). Phylogenetic trees were constructed with the mega program version 2.1 using the Kimura two-parameter matrix and the neighbor-joining method [Sugita et al., 1991]. To confirm the reliability of phylogenetic tree analysis, bootstrap resampling, and reconstruction were carried out 500 times.

Detection of Point Mutations in the Precore and BCP Regions of HBV

Mutation in the precore region for A1896 was detected by enzyme-linked minisequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostics, Tokyo, Japan) and mutations in the BCP region for T1762/A1764 were detected by enzyme-linked specific probe assay (Smitest HBV Core Promoter Mutation Detection Kit; Genome Science Laboratory, Tokyo, Japan) according to the manufacturer's instructions, after the principles described previously [Orito et al., 2001b]. The results were recorded as "the wild-type" and "the mutant-type" expressed dominantly by HBV isolates.

Statistical Analysis

Data were analyzed by chi-square test or Fisher's exact test for categorical data and Student's *t*-test or Mann–Whitney *U*-test for continuous variables. *P*-values less than 0.05 were regarded as statistically significant. Logistic regression (backward logistic regression) was used in the multivariate analysis to evaluate the factors associated with differences between genotypes A and C.

RESULTS

Distribution of HBV Genotypes

HBV genotypes were determined in 145 of the 147 (99%) patients with acute hepatitis B; they were untypeable in the remaining two patients (Table I). Genotype A was detected in 27 (19%) patients, B in 8 (5%), C in 109 (75%), and mixed genotypes with B and C in the remaining one (1%). In the 69 patients with acute hepatitis B from metropolitan areas (Tokyo, Kawasaki, and Tokorozawa), genotype A was found in 21 (30%), B in 5 (7%), and C in 43 (63%). In the 76 patients from the other areas in the mainland, by contrast, genotype A occurred in 6 (8%), B in 3 (4%), C in 66 (87%), and mixed genotypes with B and C in one (1%). Thus, genotype A was significantly more frequent in patients with acute hepatitis B from the metropolitan than the other areas (30% vs. 8%, $P < 0.001$).

Demographic and Clinical Differences Among Patients Infected With HBV of Distinct Genotypes

Clinical and demographic backgrounds in patients with acute hepatitis B who were infected with HBV of