viral loads and predicting the response to antiviral treatments [7–9], and for determining the natural history of chronic hepatitis B [10, 11]. Therefore, HBsAg and HBV DNA would be instrumental in foretelling the outcome of acute hepatitis B. However, the clinical utility of these markers in patients with acute hepatitis B is largely unknown.

Therefore, the aim of the present study was to examine differences in viral kinetics among patients with acute hepatitis B, who were infected with HBV of different genotypes, and evaluate the usefulness of quantifying HBsAg and HBV DNA for predicting the clinical outcome.

PATIENTS AND METHODS

Patients

This was a retrospective study of patients who were diagnosed with acute hepatitis B in our institutions during 1994 through 2010. Criteria for the diagnosis of acute hepatitis B were (1) acute onset of liver injury without a previous history of liver dysfunction; (2) detection of HBsAg in the serum; (3) immunoglobulin M (IgM) antibody to HBV core (anti-HBc) in high titers (detectable in serum samples diluted 10-fold) [3]; (4) absence of a past or family history of chronic HBV infection; and (5) exclusion of coinfection with hepatitis A virus, hepatitis C virus, or other hepatotropic viruses by serologic testing. Among the 232 patients who met these criteria, 215 patients (159 men and 56 women with a mean age of 31.8 ± 10.0 years) whose serum samples were available for virologic analyses were included in the study. No patient developed liver failure.

No patient received antiviral treatment. Of the 215 patients, 159 (74.0%) patients could be regularly followed up until the confirmation of clinical outcomes. Based on the duration of HBsAg (defined as the interval between the onset [defined by the first visit] and the last visit with detectable HBsAg), we classified the 159 patients into the following 4 groups (the duration of HBsAg is indicated in parentheses): group 1 (<3 months); group 2 (3–6 months); group 3 (>6–12 months); and group 4 (>12 months). Changes in virologic parameters were analyzed in relation with clinical characteristics. The study was approved by the ethics committees of our institutions, and written informed consent was obtained from each patient.

Quantification of Serologic Markers for HBV Infection and HBV DNA

HBsAg had been measured quantitatively by chemiluminescent enzyme-linked immunosorbent assay (ELISA; Sysmex JAPAN Co, Ltd, Kobe, Japan) every 2–4 weeks, until the clinical outcome was known. It has a dynamic range of 0.03–2, 500 IU/mL. Serum samples scaling out from this range were diluted so as to contain them within it. Antibody to hepatitis B s antigen (anti-HBs), hepatitis B e antigen (HBeAg), and IgM anti- HBc were determined by ELISA (Abbott JAPAN Co, Ltd, Tokyo, Japan). Levels of HBV DNA were determined using the COBAS TaqMan HBV v.2.0 kit (Roche Diagnostics, Basel, Switzerland), which has a dynamic range over 2.1–9.0 log copies/mL.

HBV Genotyping

The HBV genotype was determined by a genotype-specific probe assay (Smitest HBV genotyping Kit, Genome Science, Fukushima, Japan) as previously reported [12].

Molecular Evolutionary Analyses

HBV genotype A started to prevail in Japan merely several years ago, suggesting that it was imported to Japan only recently [3, 13]. Therefore, genomic sequences of HBV genotype A (HBV/A), recovered from sera of patients with acute HBV infection, would be closely related to one another and with those reported from abroad. To evaluate this possibility, 20 HBV/A samples were selected randomly and sequenced by the method reported previously [14].

The number of nucleotide substitutions per site was estimated by the 6-parameter method [15], and a phylogenetic tree was constructed by the neighbor-joining method [16] based on the numbers of substitutions. To confirm the credibility of phylogenetic analyses, bootstrap resampling tests were carried out 1000 times [17].

Statistical Analyses

Categorical variables were compared by χ^2 test or Fisher exact test, and continuous variables by the Mann-Whitney U test. $P < .05$ was considered statistically significant. Receiver operating characteristic (ROC) analysis was performed to compute the area under the ROC curves for viral markers to determine cutoff points for predicting the outcome.

RESULTS

Distribution of HBV Genotypes in Patients With Acute Hepatitis B

HBV genotypes were determined in 215 of the 232 (93%) patients with acute hepatitis B. Of the 215 patients, genotype A was detected in 113 (52%), B in 26 (12%), C in 73 (33%), D in 1 (1%), E in 1 (1%), and F in 1 (1%). The distribution of genotypes was compared among 4 periods during 1994 through 2010 (Table 1). The proportion of patients with genotype A gradually increased to 65.9% in 2007–2010; it was higher than those in the earlier periods (34.4% in 1994–1998 [$P = .002$], 36.8% in 1999–2002 [$P = .002$], and 51.9% in 2003–2006 [$P = .093$]).

Phylogenetic Relationship Among HBV Strains of Genotype A

We randomly selected 11 HBV/A strains sampled in 2007–2010 and 9 of those in 2001–2006, and constructed a molecular evolutionary tree (Figure 1). All 20 samples had similar nucleotide sequences with a concordance of 99%. They were close to previously

Table 1. Prevalence of Hepatitis B Virus Genotypes in Patients With Acute Hepatitis B During 4 Chronologic Periods

Period	Genotype A	Genotype B	Genotype C	Others
1994–1998 (n = 32)	11[a] (34.4%)	3 (9.3%)	18 (56.3%)	0
1994–1998 (n = 38)	14[b] (36.8%)	4 (10.5%)	20 (52.7%)	0
1994–1998 (n = 54)	28[c] (51.9%)	6 (11.1%)	19 (35.1%)	1 (1.9%)
1994–1998 (n = 91)	60[a,b,c] (65.9%)	13 (14.3%)	16 (17.6%)	2 (2.2%)
Total (N = 215)	113 (52.5%)	26 (12.0%)	73 (34.0%)	3 (1.5%)

[a] $P = .0032$.

[b] $P = .0014$.

[c] $P = .02$.

reported genotype A2 sequences from Western countries. The results support the possibility that HBV/A was imported to Japan only recently and has been spreading throughout the country.

Clinical Features Among Patients Infected With HBV of Different Genotypes

Clinical features of patients with acute hepatitis B of different genotypes are compared in Table 2. The mean age was no different among patients infected with HBV of different genotypes. The proportion of men was higher in genotype A or B than C infection (93.8% or 80.7% vs 39.7% [A vs C, $P < .001$; B vs C, $P < .001$]).

The maximum alanine aminotransferase (ALT) level was lower in patients with genotype A than in those with genotype C (2126 ± 938 vs 2857 ± 1668 IU/L, $P = .002$). The maximum bilirubin level was higher in patients with genotype A (7.1 ± 6.4 mg/dL) or C (9.0 ± 7.5 mg/dL) than in those with genotype B (4.8 ± 3.3 mg/dL) (A vs B, $P = .003$; B vs C, $P < .001$). Regarding viral markers, the peak HBV DNA level was higher in patients with genotype A than in those with genotype C (6.3 ± 1.7 vs 4.9 ± 1.5 log copies/mL, $P < .001$). HBeAg was detected in 95 of the 121 (77.3%) patients with genotype A, 24 of the 28 (88.5%) with genotype B, and 37 of the 58 (65.5%) with genotype C (A vs C, $P = .036$). Men who have sex with men were more frequently represented among patients with genotype A than B or C (31.4% vs 4.8% or 11.3% [A vs B, $P = .017$; A vs C, $P = .002$]). Antibody to human immunodeficiency virus (anti-HIV) was examined in 72 of the 113 (63.7%) patients with genotype A, 7 of the 26 (26.9%) with genotype B, 58 of the 73 (79.5%) with genotype C, and 1 with genotype E. Anti-HIV was detected in 7 of the 72 (9.7%) patients with genotype A, and the other 96 patients tested for anti-HIV showed negative results. All of the 7 patients with anti-HIV cleared HBsAg from the serum within 6 months without antiviral treatment.

Among the 215 patients whose HBV genotypes were determined, 159 could be followed until the confirmation of clinical outcomes. The distribution of HBsAg-positive period is compared among patients with different genotypes. Group 1 (HBsAg persisting for <3 months) comprised 84 patients; group 2 (3–6 months) comprised 54 patients; group 3 (>6–12 months) comprised 15 patients; and group 4 (>12 months) comprised 6 patients. HBsAg remained >6 months in 21 of the 215 (9.8%) patients, including 14 of the 113 (12.4%) with genotype A, 1 of the 26 (3.8%) with genotype B, and 6 of the 73 (8.2%) with genotype C. Among the 21 patients, 15 (71.4%) cleared HBsAg within 12 months from the onset, and were classified into group 3. The remaining 6 (5 with genotype A and 1 with genotype B) who failed to clear HBsAg within 12 months were classified into group 4. All of the 6 were negative for anti-HIV. The proportion of group 4 was 6.0% in the patients with genotype A, 4.0% in those with genotype B, and 0% in those with genotype C.

The mean duration of HBsAg was 13.9 ± 8.7 weeks in patients with genotype A, 7.1 ± 5.3 weeks in those with genotype B, and 9.6 ± 7.6 weeks in those with genotype C, presuming the duration of HBsAg in group 4 at 12 months. The duration was longer in patients with genotype A than in those with B or C (A vs B, $P < .001$; A vs C, $P = .04$).

Prediction of the Outcome by the Duration of HBsAg

Table 2 shows that the duration of HBsAg among patients with genotype A varied to a higher extent than that among those with other genotypes. Therefore, we determined HBsAg and HBV DNA levels serially, and evaluated them for the ability to predict the outcome of acute hepatitis B in patients with genotype A.

Serial changes in HBsAg levels are shown in Supplementary Figure 1*A*. HBsAg levels declined more slowly in group 2 than group 1, as well as in group 3 than group 2. In group 4, HBsAg reelevated at 12 weeks after the onset. Figure 2 compares HBsAg levels among groups 1–4 at different intervals from the onset. HBsAg at 8 weeks from the onset was useful for distinguishing group 3 or 4 from group 1 or 2. Likewise, HBsAg at 12 weeks from the onset was helpful for discriminating among groups 2, 3, and 4.

Prediction of the Outcome by HBV DNA

We also studied serial changes of HBV DNA in patients with genotype A, and examined if they also were useful for predicting the clinical outcome of acute hepatitis B. Supplementary Figure 1*B* shows serial changes in HBV DNA levels in patients in 4 groups. Although the reelevation of HBV DNA was not observed, the decline of HBV DNA was quite slow in group 4. Figure 3 compares HBV DNA levels among groups 1–4 at different intervals from the onset. HBV DNA at 4 weeks from

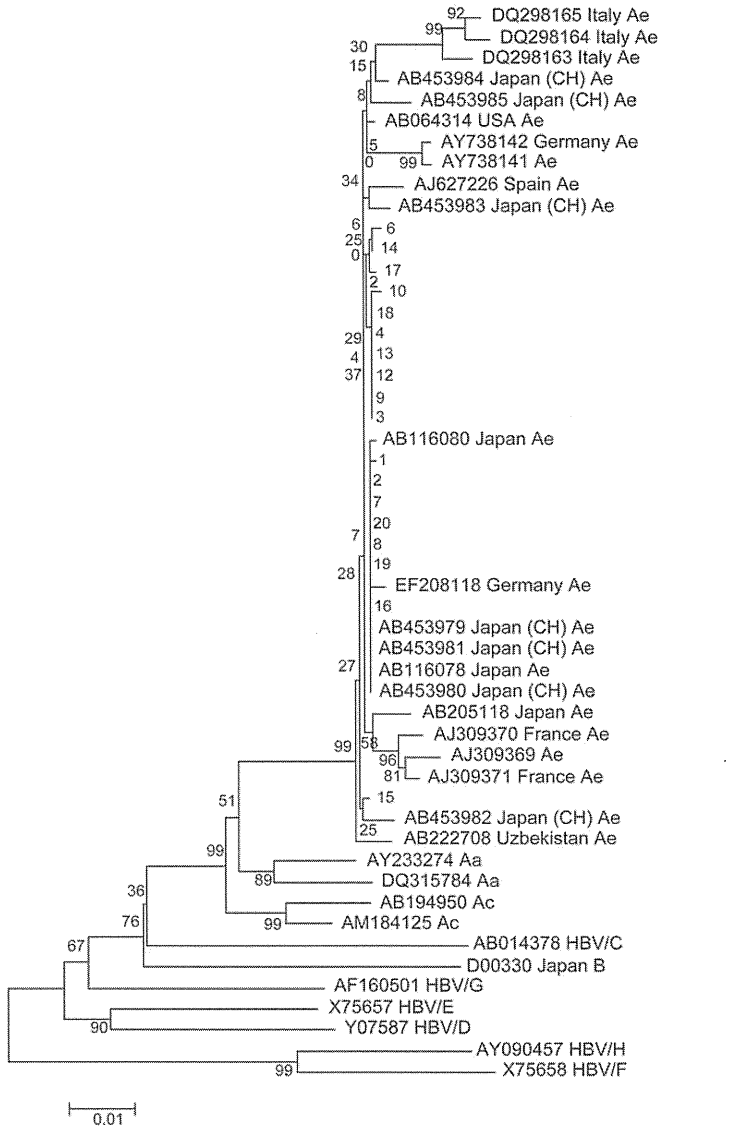

Figure 1. Evolutionary relationships of 86 hepatitis B virus genotype A taxa, including 20 from the present cases. The evolutionary history, inferred using the neighbor-joining method, shows that all 20 samples had similar nucleotide sequences close to previously reported genotype A2 sequences from Western countries.

the onset was useful for distinguishing group 3 or 4 from group 1 or 2. Likewise, HBV DNA levels at 8 weeks from the onset were useful for discriminating between group 4 and group 3, as well as for distinguishing group 3 or 4 from group 1 or 2.

Levels of HBsAg and HBV DNA for Predicting Persistent Infection

As the levels of HBsAg at 12 weeks and HBV DNA at 8 weeks from the onset were useful for distinguishing group 4 from the other groups, we evaluated the appropriate levels for predicting persistent infection in patients with genotype A. When we set the cutoff value of HBsAg at 1000 IU/mL based on the ROC analysis, both the positive predictive value and the negative predictive value were 100% with high sensitivity (100%) and specificity (98.1%). Likewise, when we set the cutoff value of HBV DNA at 10^6 log IU/mL based on the ROC analysis, both the positive predictive value and the negative predictive value were 100% with high sensitivity (100%) and specificity (96.4%). Therefore, HBsAg at 12 weeks >1000 IU/mL or HBV DNA at 8 weeks >10^6 log copies/mL is useful for predicting persistent infection.

DISCUSSION

In Japan, as shown in Table 1, the dominant HBV in acute hepatitis has been shifting from genotype C to A [3, 5, 14, 18]. The fact that nucleotide sequences of HBV/A isolates from patients

Table 2. Baseline Characteristics and the Duration of Hepatitis B Surface Antigen in Patients With Acute Hepatitis B Virus With Different Hepatitis B Virus Genotypes

	HBV Genotypes					
Features	A (n = 113)	B (n = 26)	C (n = 73)	D (n = 1)	E (n = 1)	F (n = 1)
Age, y	30.8 ± 9.5	32.3 ± 9.5	33.3 ± 10.9	27	26	58
Male	106 (93.8%)[a]	21 (80.7%)[b]	29 (39.7%)[a,b]	0	0	1 (100%)
Transmission routes Identified	102 (90.2%)	21 (80.8%)	53 (72.6%)	1 (100%)	1 (100%)	1 (100%)
Heterosexual	70 (68.6%)	19 (90.4%)	47 (88.7%)	1 (100%)	1 (100%)	1 (100%)
MSM	32 (31.4%)[c,d]	1 (4.8%)[c]	6 (11.3%)[d]	0	0	0
ALT, IU/L	2126 ± 938[e,*]	2394 ± 820	2857 ± 1668[e]	4180	1175	1533
Bilirubin, mg/dL	7.1 ± 6.4[f*]	4.8 ± 3.3[f,g]	9.0 ± 7.5[g]	6.8	3.9	3.5
HBV DNA, log copies/mL	6.3 ± 1.7[h,*]	5.5 ± 2.3	4.9 ± 1.5[h]	5.2	7.4	4.8
HBeAg	95/121 (77.3%)[i,*]	24/28 (88.5%)	37/58 (65.5%)[i]	1/1 (100%)	1/1 (100%)	1/1 (100%)
Anti-HIV	7/72 (9.7%)	0/7 (0%)	0/23 (0%)	Not tested	0/1 (0%)	Not tested
Duration of HBsAg*						
Group (mo)						
1 (<3)	35 (42.2%)	16 (64.0%)	31 (64.6%)	0	1	1
2 (3–6)	34 (41.0%)	8 (32.0%)	11 (22.9%)	1	0	0
3 (>6–12)	9 (10.8%)	0	6 (12.5%)	0	0	0
4 (>12)	5 (6.0%)	1 (4.0%)	0	0	0	0

Abbreviations: ALT, alanine aminotransferase; anti-HIV, antibody to human immunodeficiency virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MSM, men who have sex with men.

[a] $P < .001$.

[b] $P < .001$.

[c] $P = .017$.

[d] $P = .002$.

[e] $P = .002$.

[f] $P = .003$.

[g] $P < .001$.

[h] $P < .001$.

[i] $P = .036$.

* Data from anti-HIV-positive patients are excluded.

with acute hepatitis B in this study were very close to one another suggests that most HBV/A strains were imported recently and have spread rapidly, which may be attributed to the features of HBV/A in transmission routes and viral kinetics. We have reported that patients with genotype A tend to have multiple sexual partners [5]. Consequently, chances of secondary transmission of HBV/A would be higher than those of other genotypes, which may increase the number of patients who contract HBV/A infections. On the other hand, HBsAg persisted longer in patients with genotype A than B or C, which is consistent with the in vivo experiment using chimera mice carrying human hepatocytes showing that proliferation of HBV starts later and lasts longer in genotype A than in B or C infection [19].

Our results have shown that 6% of the patients with genotype A develop persistent infection. Because liver cirrhosis or hepatocellular carcinoma can develop in a substantial population of HBV carriers [20, 21], it is important to distinguish the patients in whom HBV infection becomes chronic, and follow them carefully. Although polymorphisms in host genes may be useful for identifying patients who are prone to develop chronic HBV infection [22], simple surrogate markers for the outcome have not been reported. Our data indicate that it would be difficult to predict the clinical outcome based on serum levels of viral markers at the first visit alone. This is understandable, because the dose of infecting virus, as well as the interval between infection and the first visit, can vary widely. Hence, we set out to analyze changes in serum levels of viral markers.

As seen in Figure 2, HBsAg levels at 12 weeks from the onset were most useful for discriminating among groups 2, 3, and 4 in the genotype A infection. Therefore, the outcome of acute hepatitis B may be predictable at this time point. Of note is the reelevation of HBsAg observed in group IV (Supplementary Figure 1*A*). Reelevation of viral markers suggests prolonged viral proliferation in the liver, and may be useful to identify the patients who may develop chronic infection.

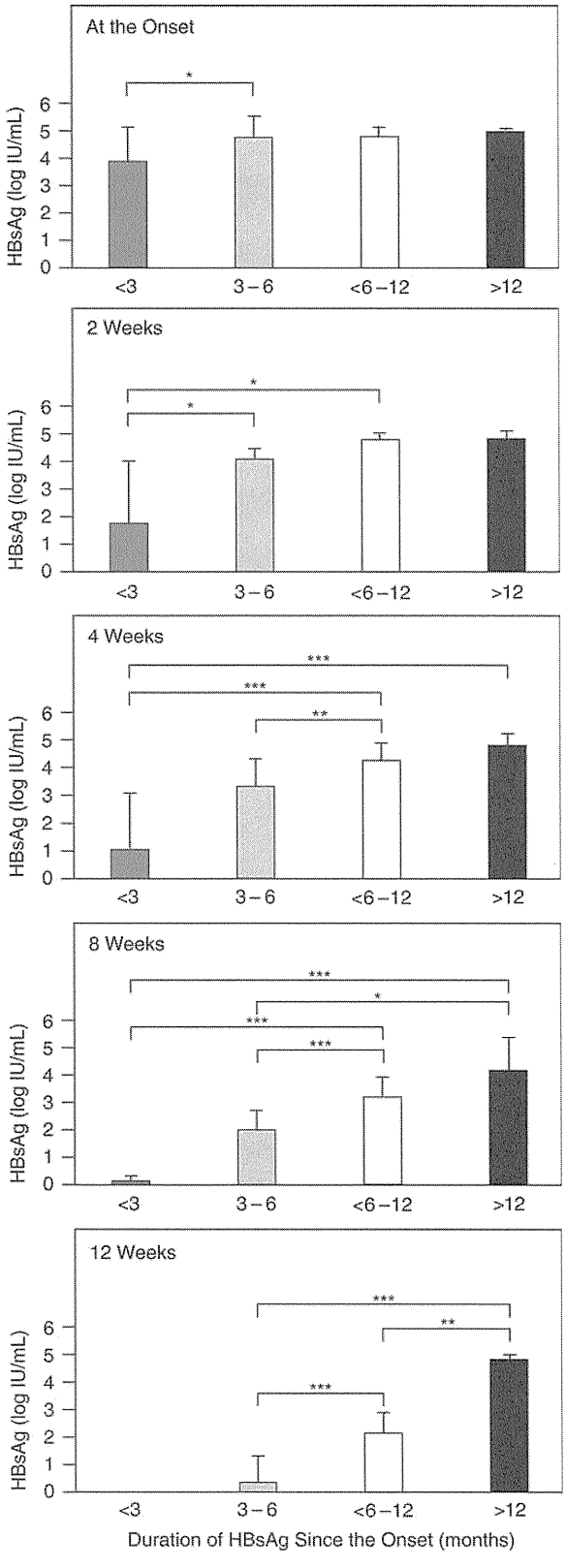

Figure 2. Levels of hepatitis B surface antigen in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B genotype A *P < .05; **P < .01; ***P < .001. Abbreviation: HBsAg, hepatitis B surface antigen.

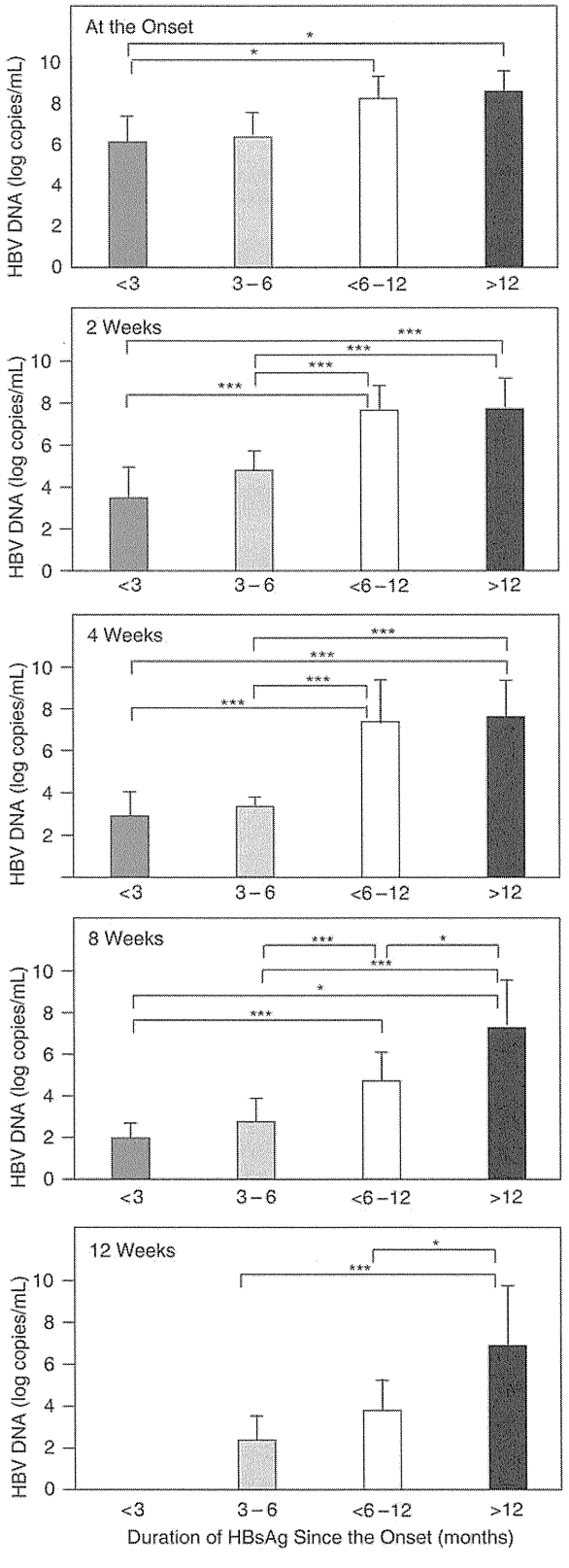

Figure 3. Levels of hepatitis B virus DNA in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B genotype A. *P < .05; **P < .01; ***P < .001. Abbreviations: HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

As shown in Figure 3, HBV DNA levels at 4 weeks from the onset can discriminate groups 1/2 from groups 3/4. Furthermore, HBV DNA levels at 8 weeks from the onset can distinguish group 4 from group 1, 2, or 3. Therefore, the combination of HBV DNA levels at weeks 4 and 8 would be useful for predicting the outcome. For the prediction of a chronic outcome, HBV DNA level at 8 weeks from the onset is a useful surrogate marker of the outcome as well as HBsAg level at 12 weeks. There were differences in viral kinetics among groups 1, 2, 3, and 4.

Our present study showed that 15 of the 215 patients (7.0%) cleared HBsAg from >6 to 12 months after the onset. Sixty percent of the 15 patients had HBV/A. Although these patients met the criteria of chronic infection, they finally cleared HBsAg from the sera. Therefore, we would like to propose that transition to chronic infection in acute hepatitis B be judged at 12 months from onset in patients with genotype A; further studies in larger cohorts are necessary. One reason for our proposal is the indication of antiviral treatment. Antiviral treatment in patients with acute hepatitis B is not indicated because previous studies failed to show the efficacy of antiviral treatments in the patients with acute hepatitis B [23, 24]. However, if patients who actually develop chronic infection can be identified and treated by antiviral treatment, the number of those who develop secondary infection may be markedly reduced. Evaluation of the efficacy of antiviral treatments by prospective studies, based on surrogate markers for the outcome, should be conducted as the next step. HBeAg, which was reported to be useful as a surrogate marker for chronicity, should also be assessed as a surrogate marker [25, 26].

Our study has some limitations. First, the lack of data in early stages made it difficult to study viral kinetics precisely. Second, viral kinetics in the infection with each HBV genotype were obtained from a restricted number of patients, not large enough to establish the usefulness of changes in viral markers in earlier stages of HBV infection. Third, anti-HIV was not checked in all patients due to the lack of informed consent. Fourth, HBsAg and HBV DNA were not determined 24 weeks after onset when discrimination between groups 3 and 4 may be possible more easily. Fifth, the maximum levels of ALT and bilirubin may be affected by the time of blood test. Validation studies in larger cohorts are necessary to evaluate the feasibility of our hypotheses.

In conclusion, we have shown that viral kinetics and the clinical outcome are different among patients with acute hepatitis B who are infected with HBV of distinct genotypes. HBsAg levels at 12 weeks and HBV DNA at 8 weeks after the onset would be useful to predict the clinical outcome of patients with acute hepatitis B.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Original Article

Fibrosis score consisting of four serum markers successfully predicts pathological fibrotic stages of chronic hepatitis B

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Aim: In order to evaluate and judge a fibrotic stage of patients with chronic hepatitis B, multivariate regression analysis was performed using multiple fibrosis markers.

Method: A total of 227 patients from seven hepatology units and institutes were diagnosed by needle biopsy as having chronic liver disease caused by hepatitis B virus. Twenty-three variables and their natural logarithmic transformation were employed in the multivariate analysis. Multiple regression function was generated from data of 158 patients in one hospital, and validation was performed using the other data of 69 patients from six other hospitals.

Results: After stepwise variable selection, multivariate regression analysis finally obtained the following function: $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) $+ 1.24 \times ln$ (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) $+ 1.19 \times ln$ (α-2-macroglobulin) (mg/dL) $- 9.15$. Median values of fibrosis scores of F1 ($n = 73$), F2 ($n = 42$), F3 ($n = 31$) and F4 stages ($n = 12$) were calculated as 0.95, 2.07, 2.98 and 3.63, respectively. Multiple regression coefficient and coefficient of determination were 0.646 and 0.418, respectively. Validation with patient data from other institutions demonstrated good reproducibility of fibrosis score for hepatitis B (FSB), showing 1.33 in F1 ($n = 27$), 2.20 in F2 ($n = 20$), 3.11 in F3 ($n = 20$) and 5.30 in F4 ($n = 2$), respectively.

Conclusion: A concise multiple regression function using four laboratory parameters successfully predicted pathological fibrosis stage of patients with hepatitis B virus infection.

Key words: chronic hepatitis, hepatitis B virus, liver cirrhosis, liver fibrosis, multiple regression analysis, stage

INTRODUCTION

WHEN HEPATITIS B virus (HBV)-related chronic liver disease is found by biochemical and virological examination, liver biopsy can establish the definitive diagnosis of chronic hepatitis and its fibrotic staging. Although these pathological procedures are reliable and informative both in diagnosis and treatment, they sometimes require medical invasion and financial costs, including the risk of bleeding from needle puncture, some pain experienced during the procedure and hospital stays of a few days. The pathological examination is, therefore, rarely performed repeatedly in a short period of time, unless disease activity is severe or progression of liver disease is highly suspected. Recently, many authors described the usefulness of ultrasonographic elastography and multiple resonance imaging technology in the estimation of staging of chronic hepatitis and cirrhosis.[1–5] These ways of estimation using the imaging apparatuses seem truly useful for current patients, but they cannot evaluate and compare with past fibrotic states of patients retrospectively. Moreover,

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the same apparatus for elastometry will not be available for repeated measurement for a follow-up examination, for example, several years later.

In spite of the accuracy of biopsy and convenience of elastography in chronic liver disease, clinical diagnosis based on biochemistry and hematology is still indispensable for the daily practice of many patients with HBV-related liver disease. Recently, several studies were published about estimation of hepatitis stages, using one or more serum biomarkers. Discriminant functions or multivariate analyses demonstrated that approximately 60–90% of patients with chronic hepatitis B were correctly classified as having mild hepatitis and severe hepatitis with advanced fibrosis.[2,6–13] Up to the present time, however, the usefulness of the discriminant functions are less valuable for a few reasons. First, these functions were made for the purpose of discrimination of severe hepatic fibrosis from mild fibrosis, and four histological classifications (F1–F4) were neglected in almost of the studies. Second, some studies analyzed both hepatitis B and hepatitis C virus infection, although the significance and actual values of each liver function test in the evaluation of the severity of liver disease were not similar among each viral hepatitis and alcoholic liver disease. Third, biochemical markers for liver fibrosis (e.g. hyaluronic acid, type IV collagen, procollagen III peptide)[14–16] were not always included in those previous studies.

We tried to generate a function estimating fibrotic stages of HBV-related chronic hepatitis, which were objectively diagnosed by liver biopsy. The purpose of this study is, therefore, to make a reliable multiple regression function and to obtain practical coefficients for significant variables also using fibrosis markers.

METHODS

Patients

A TOTAL OF 273 Japanese patients with chronic hepatitis B were recruited for the study from seven hospitals in Japan: Toranomon Hospital, Hiroshima University Hospital (K. Chayama, M.D.), Ehime University Hospital (M. Onji, M.D.), Musashino Red Cross Hospital (N. Izumi, MD), Shishu University Hospital (E. Tanaka, M.D.), Showa University Hospital (M. Imawari, M.D.) and Osaka University Hospital (T. Takehara, M.D.). Inclusion criteria for this study were: (i) positive hepatitis B surface antigen for more than 6 months; (ii) persistent or intermittent elevation in aspartate aminotransferase (AST)/alanine aminotransferase (ALT) levels; and (iii) liver biopsy showing chronic hepatitis (F1–F4). We excluded those patients with overt alcoholic liver disease or fatty liver, association of other types of liver disease (e.g. hepatitis C, primary biliary cirrhosis, autoimmune hepatitis), or those associated with hepatocellular carcinoma or other malignancy. Among the patients, 244 patients fulfilled the conditions for the study: complete demographic data, basic laboratory data of hematology and biochemistry, required liver biopsy specimens, and sufficient amount of frozen sera. Also, we excluded additional 17 patients with eventual histological diagnosis as F0 stage.

Finally, a total of 227 patients who were diagnosed as having chronic hepatitis or cirrhosis (F1–F4) were analyzed for the following hematological, biochemical and histopathological examination. There were 172 males and 55 females aged 16–70 years (median, 39 years).

All the patients presented written informed consent in individual hospitals and medical centers, and the study was approved in each ethical committee.

Hematological and biochemical examination

Hematological and standard biochemical evaluation had been performed in each medical institution: white blood cells, red blood cells, hemoglobin, platelets, total bilirubin, AST, ALT, AST/ALT ratio (AAR), γ-glutamyl transpeptidase (γ-GTP), total protein, albumin and γ-globulin.

Special biochemical examinations including "fibrosis markers" were carried out using stored frozen sera at −20°C or lower: α-2-macroglobulin, haptoglobin concentration, haptoglobin typing, apolipoprotein A1, hyaluronic acid, tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2, procollagen III peptide and type IV collagen 7S.

Histological diagnosis of chronic hepatitis and cirrhosis

All the 227 cases fulfilled required standards of histological evaluation: sufficient length of specimen, hematoxylin–eosin staining, and at least one specimen with fiber staining. Four independent pathologists (Y. T., J. F., F. K. and T. F.), who were not informed of patients' background and laboratory features except for age and sex, evaluated the 227 specimens regarding the stages of fibrosis and activity. Pathological classification of chronic hepatitis staging was based on Desmet *et al.*[17]

Before judgment of histological staging of individual specimens, the pathologists discussed the objective and reproducible judgment of pathological diagnosis of

hepatitis. They made a panel about obvious criteria using typical microscopic pictures for each stage, and it was always referred to during the procedure of pathological judgment. When inconsistent results were found in the diagnosis of hepatitis stage among the pathologists, the final judgment accepted majority rule among them.

Statistical analysis

Non-parametric procedures were employed for the analysis of background characteristics and laboratory data among patients in each stage, including Mann–Whitney *U*-test, Kruskal–Wallis test and χ^2-test.

The normality of the distribution of the data was evaluated by a Kolmogorov–Smirnov one-sample test. Because certain variables partly did not conform to a normal distribution, natural logarithmic transformation of bilirubin, AST, ALT, γ-GTP, α-2-macroglobulin, hyaluronic acid, type IV collagen 7S and TIMP-2 were also analyzed in the following calculation. The natural logarithmic transformation of the results yielded a normal distribution or symmetrical distribution for all the analyzed factors. After the procedures, the following multiple regression analysis became rationally robust against deviations from normal distribution. In order to avoid introducing into the model any variables that were mutually correlated, we checked the interaction between all pairs of the variables by calculating variance inflation factors. Of the highly correlated variables, less significant factors were removed from the viewpoint of multicollinearity.

Multivariate regression analysis was performed using 158 patient data from Toranomon Hospital (training dataset) to generate a training data of predicting function. We used a stepwise method for selection of informative subsets of explanatory variables in the model. Multiple regression coefficient and coefficient of determination were also taken into account in the selection of variables. Next, we validated the obtained predictive function using the remaining 69 patient data from the other six liver institutions (validation dataset).

A *P*-value of less than 0.05 with two-tailed test was considered to be significant. Data analysis was performed using the computer program SPSS ver. 19.[18]

For evaluation of the efficiency and usefulness of obtained function for fibrosis estimation, we compared various fibrosis scores for hepatitis B and C, including AAR,[19] AST-to-platelet ratio index (APRI),[20] FIB-4,[21] FibroTest[22] and discrimination function of cirrhosis from hepatitis in Japanese patients.[23]

RESULTS

Pathological diagnosis

FOUR PATHOLOGISTS INDEPENDENTLY judged the fibrotic stages and inflammatory activity for 227 specimens of chronic hepatitis/cirrhosis caused by HBV. One hundred patients (44.1%) had a fibrosis stage of F1, 62 (27.3%) F2, 51 (22.5%) F3 and 14 (6.2%) F4. In the subgroup of the 158 patients in the training group, judgment as F1 was made in 73 cases, F2 in 42, F3 in 31 and F4 in 12. Of the 69 patients in the validation group, judgment as F1 was made in 27, F2 in 20, F3 in 20 and F4 in two.

According to hepatitis activity classification, A0 was found in five (2.2%), A1 in 100 (44.1%), A2 in 107 (47.1%) and A3 in 15 (6.6%).

Laboratory data of each hepatitis stage in the training group

There were 124 men and 34 women with a median age of 39 years ranged 16–70 years. Laboratory data of 158 patients in the training group are shown in Table 1. Although several individual items were well correlated with the severity of hepatic fibrosis, significant overlap values were noted among F1–F4 stages: platelet count, γ-globulin, α-2-macroglobulin, haptoglobin, hyaluronic acid, TIMP-2 and type IV collagen 7S.

Significant variables serving staging of hepatitis

Univariate analyses using trend analysis with the Cochran–Armitage method showed that the fibrotic stage of chronic hepatitis B (FSB) was significantly correlated with platelet count (Spearman: $r = -0.45$, $P < 0.001$), γ-GTP ($r = 0.19$, $P = 0.017$), γ-globulin ($r = 0.29$, $P < 0.001$), α-2-macroglobulin ($r = 0.32$, $P < 0.001$), hyaluronic acid ($r = 0.36$, $P < 0.001$), TIMP-2 ($r = 0.16$, $P = 0.043$), procollagen III peptide ($r = 0.30$, $P < 0.001$) and type IV collagen 7S ($r = 0.55$, $P < 0.001$).

Regression function generated from training patient group

After stepwise variable selection, multivariate regression analysis finally obtained the following function: $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) $+ 1.24 \times ln$ (TIMP-2) (ng/mL) $+ 1.19 \times ln$ (α-2-macroglobulin) (mg/dL) $-$ 9.15. Median values of the fibrosis score of F1 ($n = 73$), F2 ($n = 42$), F3 ($n = 31$) and F4 stages ($n = 12$) were calculated as 0.95, 2.07, 2.98 and 3.63, respectively

Table 1 Demography and laboratory data of 158 patients in training group

	F1 ($n = 73$)	F2 ($n = 42$)	F3 ($n = 31$)	F4 ($n = 12$)
Demographics				
Men : women	58:15	33:9	23:8	10:2
Age (median, range)	36 (16–70)	39.5 (18–66)	39 (25–64)	43 (32–59)
Laboratory data (median, range)				
WBC (×1000/mm^3)	5.4 (2.5–10.6)	5.1 (2.4–8.7)	4.9 (3.0–8.7)	4.1 (3.7–6.6)
Hemoglobin (g/dL)	15.3 (10.3–18.8)	15.4 (12.5–17.9)	15.2 (11.5–17.2)	14.45 (12.1–18.2)
Platelet (×1000/mm^3)	204 (124–341)	173 (82–308)	155 (96–220)	130 (86–230)
Albumin (g/dL)	4.1 (3.2–4.9)	4.0 (3.2–5.1)	4.0 (3.3–4.9)	3.95 (3.4–4.6)
Bilirubin (mg/dL)	0.8 (0.2–1.7)	0.8 (0.3–2.3)	0.9 (0.4–5.4)	0.85 (0.6–2.3)
AST (IU/L)	48 (16–450)	55 (17–588)	54 (17–1446)	76.5 (27–396)
ALT (IU/L)	102 (10–839)	90 (12–886)	85 (19–2148)	89 (18–809)
γ-GTP (IU/L)	37 (7–247)	55 (8–687)	44 (14–564)	69 (33–262)
γ-Globulin (g/dL)	1.29 (0.78–2.11)	1.495 (0.62–3.20)	1.43 (0.90–2.30)	1.735 (0.92–2.47)
γ-Globulin (%)	17.3 (10.8–26.1)	19.3 (8.5–35.6)	19.9 (12.9–28.6)	22.55 (13.9–30.2)
α-2-Macroglobulin (mg/dL)	226 (116–446)	276 (148–495)	261 (202–565)	286.5 (166–425)
Haptoglobin (mg/dL)	77 (<5–318)	59 (<5–238)	61 (<5–151)	48.5 (<5–145)
Apolipoprotein A-I (mg/dL)	134 (89–212)	143 (78–250)	133 (87–189)	125 (73–169)
Hyaluronic acid (μg/L)	16 (<5–130)	32.5 (<5–204)	38 (<5–418)	49 (24–335)
TIMP-1 (ng/mL)	168 (93–271)	172 (116–314)	157 (119–365)	192 (145–365)
TIMP-2 (ng/mL)	80 (41–135)	80.5 (35–121)	92 (38–251)	85.5 (70–123)
Procollagen III peptide (U/mL)	0.75 (0.53–1.90)	0.835 (0.45–1.20)	0.89 (0.58–2.50)	1.05 (0.71–2.20)
Type IV collagen 7S (ng/ml)	4.0 (2.7–7.7)	4.6 (2.6–9.6)	5.6 (2.3–15.0)	7.2 (4.2–14.0)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; TIMP, tissue inhibitor of matrix metalloproteinase; WBC, white blood cells.

(Fig. 1). The multiple regression coefficient and coefficient of determination were 0.646 ($P < 0.001$) and 0.418 ($P < 0.001$), respectively.

Because the generated regression function was obtained by multivariate analysis with stepwise variable selection, several variables were removed from the function due to multicollinearity among them. Mutual correlation among the fibrosis predictors are shown in Table 2.

A 28-year-old man of F1 fibrotic stage (Fig. 2a) had a serum type IV collagen concentration of 4.4 ng/mL, platelet 221×10^3 count/mm^3, TIMP-2 75 ng/mL and α-2-macroglobulin 226 mg/dL. The regression function provided a fibrosis score of 0.99. Another man aged 46 years had F3 fibrosis on histological examination (Fig. 2b). His type IV collagen was 5.3 ng/mL, platelet 137×10^3 count/mm^3, TIMP-2 92 ng/mL and α-2-macroglobulin 255, and the regression function calculated his fibrosis score as 3.10.

Validation of discriminant function

Validation data of 69 patients (Table 3) were collected from the other six institutions in Japan. When applying

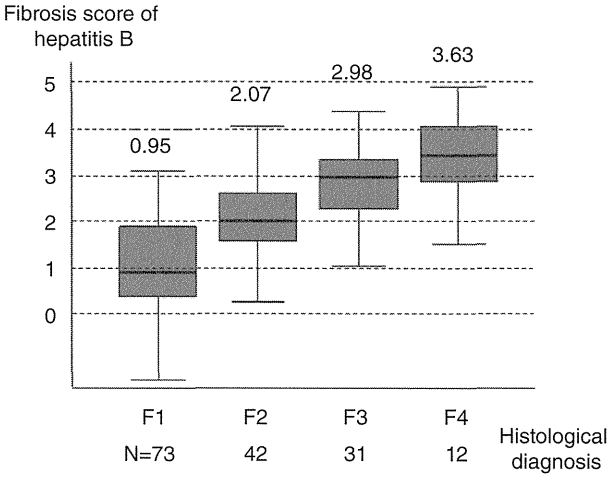

Figure 1 Box and whisker plots of fibrotic score of each histological fibrosis group in the training dataset. The fibrosis score of hepatitis B was generated by the function, $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) − $0.017 \times$ (platelet count) (×1000^3/mm^3) + $1.24 \times ln$ (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) + $1.19 \times ln$ (α-2-macroglobulin) (mg/dL) − 9.15.

Table 2 Correlation coefficients (Spearman's ρ) among fibrosis predictors used in multivariate analysis

	Platelet	gamma-globulin	*ln* (α-2-macroglobulin)	*ln* (hyaluronate)	*ln* (P-III-P)	*ln* (IV collagen)	*ln* (TIMP-2)
Platelet ($\times 10^3/mm^3$)	1.000	−0.214 ($P = 0.008$)	−0.260 ($P = 0.001$)	−0.384 ($P < 0.001$)	−0.045 ($P = 0.58$)	−0.297 ($P < 0.001$)	0.094 ($P = 0.24$)
γ-Globulin (g/dL)		1.000	0.276 ($P = 0.001$)	0.349 ($P < 0.001$)	0.342 ($P < 0.001$)	0.414 ($P < 0.001$)	0.268 ($P = 0.001$)
ln (α-2-macroglobulin) (mg/dL)			1.000	0.281 ($P < 0.001$)	0.141 ($P = 0.078$)	0.171 ($P = 0.032$)	−0.079 ($P = 0.32$)
ln (hyaluronic acid) (mg/L)				1.000	0.373 ($P < 0.001$)	0.493 ($P < 0.001$)	0.089 ($P = 0.27$)
ln (procollagen III peptide) (U/mL)					1.000	0.600 ($P < 0.001$)	0.145 ($P = 0.071$)
ln (type IV collagen) (mg/L)						1.000	0.358 ($P < 0.001$)
ln (TIMP-2) (mg/L)							1.000

TIMP, tissue inhibitor of matrix metalloproteinase.

the regression function for the validation set, the fibrosis score demonstrated good reproducibility, showing 1.33 in patients with chronic hepatitis of F1 ($n = 27$), 2.20 of F2 ($n = 20$), 3.11 of F3 ($n = 20$) and 5.30 of F4 ($n = 2$), respectively (Fig. 3). Although F4 fibrosis stage consisted of only two patients and the score 5.30 was regarded as of rather higher value, the scores of other stages of fibrosis were concordant with histological fibrosis.

Comparisons of efficacy with various fibrosis scores (Fig. 4)

In order to evaluate the efficacy and usefulness of the obtained FSB, we compared it with previously reported fibrosis scores using training data. AAR, APRI and FibroTest showed only slight correlation with actual histological stage. FIB-4 demonstrated an increasing trend of the score associated with histological fibrosis, but significant overlapping scores were found in F1–F4. Spearman's correlation coefficients of AAR, APRI, FIB-4 and FibroTest were 0.199 ($P = 0.012$), 0.265 ($P = 0.001$), 0.412 ($P < 0.001$) and 0.330 ($P < 0.001$), respectively. Our FSB showed a Spearman's correlation coefficient of 0.625 ($P < 0.001$), and was a much higher value than the others. The dichotomous discrimination function for cirrhosis and hepatitis C in Japanese patients[23] showed good differentiation also in patients with hepatitis B virus.

DISCUSSION

RECOGNITION OF SEVERITY of chronic hepatitis is essential in managing patients with chronic HBV infection: estimation of length of infection, existence of any previous hepatitis activity, presumption of current fibrotic stage, and prediction of future fibrosis progression and hepatocarcinogenesis. Differential diagnosis of cirrhosis from chronic hepatitis is especially important in the evaluation of chronic HBV infection. Identification of liver cirrhosis often leads to an important change in management of the patient: need for fiberscopic examination for esophageal varices, ultrasonographic exploration for the association of liver cancer, and prediction of hepatic decompensation. Guidelines published by the American Association of Study of Liver Disease[24] recommend liver biopsy for HBV carriers with aminotransferase elevation or for any candidates of antiviral therapy, because hepatic fibrosis sometimes shows unexpectedly far advancement to cirrhosis, and because it is very difficult to evaluate and translate the liver function tests or ultrasonographic findings compared to chronic hepatitis type C.

Table 3 Demography and laboratory data of 69 patients in training group

	F1 ($n = 27$)	F2 ($n = 20$)	F3 ($n = 20$)	F4 ($n = 2$)
Demographics				
Men : women	18:9	15:5	13:7	2:0
Age (median, range)	36 (13–64)	45 (14–64)	36.5 (24–59)	32 (25–39)
Laboratory data (median, range)				
WBC (×1000/mm^3)	5.0 (2.8–8.7)	5.8 (2.8–11.6)	5.3 (3.2–8.1)	3.85 (2.7–5.0)
Hemoglobin (g/dL)	14.8 (12.4–17.4)	15.0 (12.4–16.9)	14.4 (11.1–16.4)	14.4 (12.5–16.3)
Platelet (×1000/mm^3)	204 (86–322)	180 (90–275)	147 (90–276)	130 (67–183)
Albumin (g/dL)	4.4 (2.8–5.2)	4.2 (3.5–5.1)	4.3 (3.4–4.9)	4.45 (4.0–4.9)
Bilirubin (mg/dL)	0.9 (0.4–6.4)	0.8 (0.2–1.6)	0.75 (0.4–1.7)	1.15 (1.1–1.2)
AST (IU/L)	52 (17–575)	50.5 (21–272)	65 (22–284)	248.5 (51–446)
ALT (IU/L)	84 (16–1101)	101.5 (19–554)	86.5 (16–1113)	453.5 (74–833)
γ-GTP (IU/L)	42 (14–332)	54 (16–205)	52.5 (13–191)	193 (57–329)
γ-Globulin (g/dL)	1.30 (1.04–1.59)	1.35 (1.18–2.53)	1.62 (1.16–1.97)	1.545 (1.51–1.58)
γ-Globulin (%)	17.9 (14.3–22.1)	19.6 (15.5–30.8)	22.0 (16.5–24.6)	20.15 (19.3–21.0)
α-2-Macroglobulin (mg/dL)	287 (160–687)	270 (89–452)	272.5 (211–463)	389 (313–465)
Haptoglobin (mg/dL)	58 (<5–229)	74 (<5–154)	56.5 (<5–198)	<5 (<5–<5)
Apolipoprotein A-I (mg/dL)	146 (95–216)	137 (87–162)	120 (88–170)	100.5 (74–127)
Hyaluronic acid (μg/L)	27 (<5–113)	36 (10–1050)	59 (14–439)	331 (225–437)
TIMP-1 (ng/mL)	168.5 (83–302)	176 (127–408)	182 (104–303)	390.5 (283–498)
TIMP-2 (ng/mL)	76 (25–143)	86.5 (28–154)	77.5 (32–141)	100.5 (91–110)
Procollagen III peptide (U/mL)	0.71 (0.27–2.20)	0.88 (0.63–2.80)	0.995 (0.60–2.10)	1.75 (1.50–2.00)
Type IV collagen 7S (ng/ml)	3.6 (2.7–17.0)	5.25 (3.3–13.0)	5.7 (3.0–16.0)	15.5 (15.0–16.0)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; TIMP, tissue inhibitor of matrix metalloproteinase; WBC, white blood cells.

Recently, non-invasive estimation of severity of liver fibrosis has been reported in patients with HBV-related chronic hepatitis.[2,6–13] However, these studies were principally aimed at differentiation of advanced fibrotic stages of F3 or F4 from mild fibrotic stages of F1 or F2. Those discrimination functions were insufficient to recognize the stepwise progression of viral hepatitis from F1–F4. This dichotomy (mild or severe) of chronic hepatitis B seemed less valuable in the study of disease progression, disease control abilities of antiviral drugs and estimation of histological improvement after anti-inflammatory drugs. A histology-oriented, practical and reliable formula is therefore required for the diagnosis and investigation of chronic hepatitis B.

This study aimed to establish non-invasive evaluation and calculation of liver fibrosis for patients with chronic hepatitis B virus infection. Although it was retrospectively performed as a multicenter study of eight institutions, judgment of histological diagnosis was independently performed by four pathologists in another hospital, who were informed only of the patient's age, sex and positive HBV infection. Objective judgment of the histological staging and grading in sufficient biopsy specimens could be obtained.

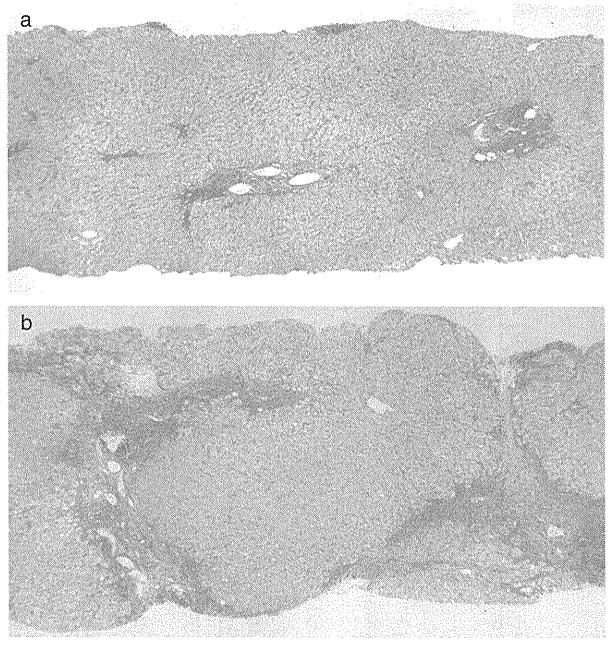

Figure 2 Case presentations of the training set. (a) A 28-year-old man with F1 fibrosis. Final regression function provided his fibrosis score as 0.99. (b) A 45-year-old man with F3 fibrosis. His regression coefficient was calculated as 3.10. Silver stain, ×40.

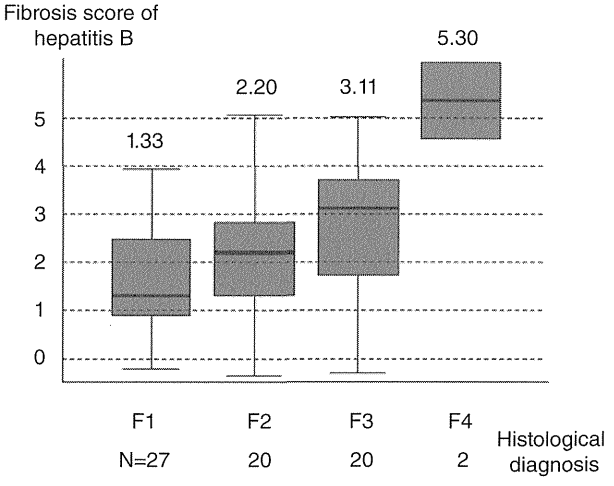

Figure 3 Box and whisker plots of fibrotic score of each group of histological fibrosis in the validation dataset. The fibrosis score of hepatitis B was generated by the function, $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) − $0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) + $1.24 \times ln$ (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) + $1.19 \times ln$ (α-2-macroglobulin) (mg/dL) − 9.15.

As many as 227 patients with chronic hepatitis B were analyzed in this study, who had been diagnosed as having chronic hepatitis or cirrhosis by liver biopsy performed in experienced liver units in Japan. To obtain the most suitable equation approximating histological fibrotic stage, multivariate analysis was performed using two demographic parameters (age and sex) and 21 hematological and biochemical markers with or without logarithmic transformation. They included many kinds of fibrosis markers: α-2-macroglobulin, haptoglobin concentration, haptoglobin typing, apolipoprotein A1, hyaluronic acid, TIMP-1, TIMP-2, procollagen III peptide and type IV collagen 7S Multiple regression analysis finally generated a first-degree polynomial function consisting of four variables: type IV collagen 7S, platelet count, TIMP-2 and α2-macroglobulin. A constant numeral (−9.15) was finally adjusted in the regression equation in order to obtain fitted figures for a fibrotic stage of F1–F4. From the magnitude of the standardized partial regression coefficient of individual variable in the function, platelet count demonstrated the most potent contribution toward the prediction of liver fibrosis. Type IV collagen 7S and *ln* (TIMP-2) proved to be the second and third distinctive power in the model, respectively.

The FSB was sufficiently fitted to actual fibrotic stages with certain overlapping as is usually found in histological ambiguity judged by pathologists. Because judgment of fibrosis in chronic hepatitis often shows a transitional histological staging, pathological examination cannot always make a clear-cut diagnosis discriminating F1–F4. Considering the limitation of the pathological difficulty in differentiating the four continuous disease entities, the obtained regression function showed satisfactory high accuracy rates in the prediction of liver disease severity. The FSB can provide one or two decimal places (e.g. 3.2 or 3.24) and the utility of the score is possibly higher than the mere histological stage of F1–F4. The reproducibility was confirmed by the remaining 67 patients' data obtained from the other six hospitals. Although the validation data were collected from a different geographic area and different chronological situation, the FSB showed similar results in prediction of histological staging.

The FSB seemed a very useful quantitative marker in evaluating fibrotic severity of hepatitis B patients without invasive procedures and without any specialized ultrasonography or magnetic resonance imaging. The FSB also has an advantage of measurement, in which old blood samples are available for retrospective assessment of varied clinical settings: for example, old sera from 20 years prior to the time of initial liver biopsy, or paired sera before and after long-term antiviral therapy. These kinds of retrospective assessments of fibrotic staging will be valuable in estimating a long-term progression of liver disease, in evaluating efficacy of long-term medication or other medical intervention, or in making a political judgment from the viewpoints of socioeconomic efficacy.

The score can be calculated for any patients with chronic HBV infection. Although this multiple regression model dealt with appropriate logarithmic transformation for non-normal distribution parameters, the regression analysis was based on a linear regression model. Very slight fibrosis can be calculated as less than 1.00, which is commonly found to a slight degree in chronic hepatitis with tiny fibrotic change as F0. Very severe fibrosis might be calculated as more than 4.00, which is an imaginary and nonsense number in the scoring system of fibrosis. The FSB is, however, very useful and valuable in a real clinical setting: estimation of severity of liver fibrosis in an outpatient clinic, evaluation of the natural progression of a patient's fibrosis over 10 years and assessment of a long-term administration of interferon in patients with chronic hepatitis B from the viewpoint of fibrotic change. Recent development of new nucleoside/nucleotide analogs requires evaluation for long-term histological advantage, for aggravation of hepatitis stage during viral and biochemical breakthrough caused by HBV mutation, and even for

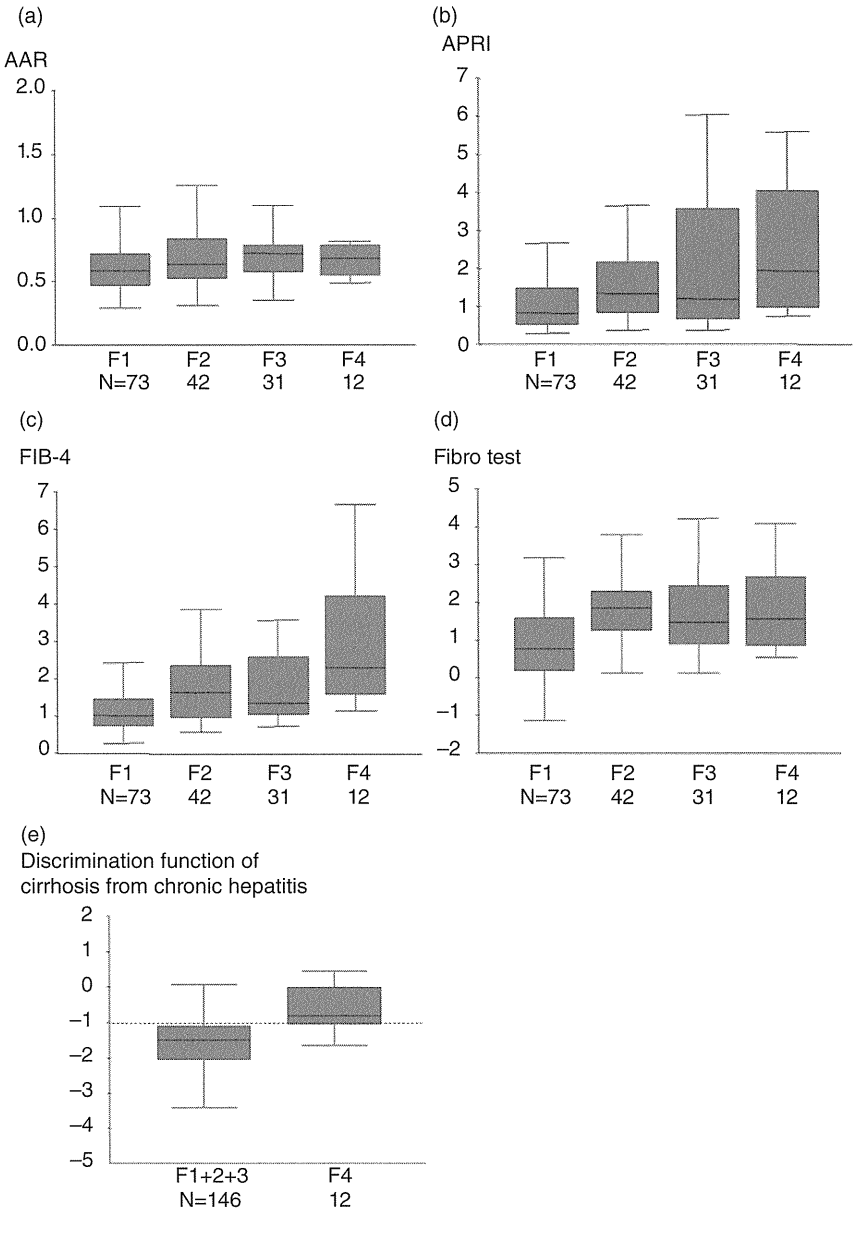

Figure 4 Previously published fibrosis scores. (a) Aspartate aminotransferase/alanine aminotransferase ratio (AAR),[19] (b) aspartate aminotransferase-to-platelet ratio index (APRI),[20] (c) FIB-4,[21] (d) FibroTest[22] and (e) discrimination function of cirrhosis from hepatitis in Japanese patients.[23]

the best management of patients with chronic hepatitis B. The FSB seems one of the ideal methods of approximating the fibrotic stage of chronic hepatitis B. Repeated measurement is quite suitable for patients with an unestablished treatment or trial, every 1 or 2 years, for example. Because the current regression function was generated from the data of HBV-related chronic liver disease, this equation would not be suitable for the recognition of hepatitis C virus-related chronic liver disease, alcoholic liver disease, and other congenital or autoimmune liver diseases. To recognize the latter diseases, other studies of individual diseases must be performed.

We compared the usefulness of the FSB with that of other fibrosis scores.[19–23] The more simple and less expensive AAR or APRI could not estimate fibrotic stages with poor correlation coefficients of 0.199 and 0.265, which are much lower than the coefficient of the FSB of 0.625. FibroTest, which contained three costly fibrosis markers (α-2-macroglobulin, haptoglobin and apolipo-

protein A1), also showed a low correlation coefficient of 0.330, suggesting that its usefulness was limited in HBV positive oriental patients. Although FIB-4 demonstrated the best coefficient of 0.412 among the fibrosis scores, significant overlaps were found between neighboring stages and obtained scores were not coordinated for real histological classification.

In conclusion, the FSB was a useful and reliable biomarker for prediction of liver fibrosis in patients with chronic HBV infection. The FSB is expected to be introduced and utilized in varied kinds of studies and trials. Its accuracy and reproducibility require further validation using higher numbers of patients in several countries other than Japan.

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Original Article

Effect of the infectious dose and the presence of hepatitis C virus core gene on mouse intrahepatic CD8 T cells

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Aim: Chronic hepatitis C viral (HCV) infections often result in ineffective CD8 T-cell responses due to functional exhaustion of HCV-specific T cells. However, how persisting HCV impacts CD8 T-cell effector functions remains largely unknown. The aim of this study is to examine the effect of the infectious dose and the presence of HCV core gene.

Methods: We compared responses of intrahepatic CD8 T cells during infection of wild-type or HCV core transgenic (Tg) mice with various infectious doses of HCV-NS3-expressing recombinant adenovirus (Ad-HCV-NS3).

Results: Using major histocompatibility complex class I tetramer and intracellular interferon (IFN)-γ staining method to track HCV-NS3-specific CD8 T cells, we found that a significant expansion of HCV-NS3-specific CD8 T cells was restricted to a very narrow dosage range. IFN-γ production by intrahepatic CD8 T cells in HCV core Tg mice was suppressed as compared with wild-type mice. Higher levels of expression of regulatory molecules, Tim-3 and PD-1, by intrahepatic CD8 T cells and PD-L1 by intrahepatic antigen-presenting cells were observed in HCV core Tg mice following Ad-HCV-NS3 infection, and the expression increased dependent on infectious dose. Furthermore, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules in antigen-specific intrahepatic CD8 T cells.

Conclusion: High infectious dose and the presence of HCV core gene were strongly involved in ineffective CD8 T-cell responses. We consider that HCV core Tg mouse infected with high infectious dose of Ad-HCV-NS3 is useful as a chronic infection model in the development of immunotherapy for chronic hepatitis C.

Key words: core, functional exhaustion, hepatitis C, infectious dose, T cell

INTRODUCTION

HEPATITIS C VIRUS (HCV) is a positive-sense single-stranded RNA virus of the genus *Hepacivirus* in the family *Flaviviridae*, and it infects 170 million people worldwide.[1] Approximately 10–60% of the patients clear HCV spontaneously during the acute phase of infection,[2] while the others develop chronic persistent HCV infection that eventually leads to liver cirrhosis and hepatocellular carcinoma.[3] HCV-specific cytotoxic T lymphocytes (CTL) play a major role in viral control during acute infection.[4] Nevertheless, during persistent infection, HCV-specific CTL effector functions are significantly impaired.

T-cell exhaustion is one of the remarkable features of chronic HCV infection. In chronically HCV-infected individuals, the frequencies of CTL are relatively low; similarly, the proliferative capacity as well as effector functions of HCV-specific T cells are impaired, and the production of type I cytokines (i.e. interleukin-2 and interferon [IFN]-γ) is dramatically suppressed.[5–8]

It appears that the major factors which determine duration and magnitude of an antiviral immune response are antigen (Ag) localization, dose and kinetics.[9] For example, high doses of widely disseminating strains of lymphocytic choriomeningitis virus (LCMV) exhaust antiviral CTL leading to establishment of a persistent infection.[10] Physical deletion of anti-LCMV CTL is most likely preceded by their functional impairment with the inability to produce effector cytokines.[11,12]

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Moreover, Wherry *et al.* showed that not only the persistence of a viral Ag, but also the initial Ag level is an important factor determining the quality of the antiviral memory response.[13]

Hepatitis C virus core protein has been reported to suppress T-cell response. HCV core-mediated inhibition of T-cell response can occur via either modulation of pro-inflammatory cytokine production by antigen-presenting cells (APC; i.e. monocyte and dendritic cells)[14] or direct effect on T cells.[15–17] Because the liver is the major site of HCV infection, it is crucial to understand the regulation of host immunity by HCV core in the liver compartment and the impact of HCV core-induced immune dysregulation in facilitating HCV persistence.

Hepatitis C virus does not infect small laboratory animals. The lack of a small animal model has hampered studies attempting to elucidate the mechanism of HCV-mediated suppression of antiviral CD8 T-cell activity and caused difficulty in the development of a therapeutic and/or prophylactic HCV vaccine.

Adenoviral vectors efficiently and reproducibly transfer foreign DNA into the livers of immunocompetent experimental animals. i.v. administration of adenoviral vectors of more than 10^9 infectious units/mouse results in infection and Ag expression in more than 90% of hepatocytes and acute self-limiting viral hepatitis.[18,19]

In this study, to develop a useful animal model in the development of immunotherapy for chronic hepatitis C, we examined the responses of intrahepatic CD8 T cells of HCV core transgenic (Tg) mice with various infectious doses of HCV-NS3-recombinant adenovirus (Ad-HCV-NS3).

METHODS

Mice

C57BL/6 MICE WERE purchased from Clea Japan (Tokyo, Japan), and Tokyo Laboratory Animal Science (Tokyo, Japan). Production of HCV core Tg mice has been described.[20] The core gene of HCV placed downstream of a transcriptional regulatory region from hepatitis B virus, which has been shown to allow an expression of genes in Tg mice without interfering with mouse development,[21] was introduced into C57BL/6 mouse embryos (Clea Japan). Eight- to 10-week-old mice were used for all experiments. The mice were housed in appropriate animal care facilities at Saitama Medical University (Saitama, Japan) and were handled according to international guidelines. The experimental protocols were approved by the Animal Research Committee of Saitama Medical University (#855).

HCV-NS3 recombinant adenovirus

Adenovirus HCV-NS3 expressing the fusion protein, comprising the entire HCV-NS3 and 3X flag, was constructed by using the AdEasy XL adenoviral vector system (Agilent Technologies, Santa Clara, CA, USA). The HCV-NS3 gene corresponding to amino acid residues 1027–1657 was amplified from the plasmid pBRTM/HCV1-3011con which contains the entire DNA sequence derived from the HCV H77 clone (kindly provided by Charls M. Rice, The Rockefeller University, New York, NY, USA)[22] by polymerase chain reaction. The recombinant Ad-HCV-NS3 vector was linearized by PacI digestion, and then transfected into 293 cells using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) to generate adenovirus. Ad-HCV-NS3 expressing transgene NS3 was amplified in 293 cells, purified by a series of cesium chloride ultracentrifugation gradients and stored at −80 °C until use. Mice were injected i.v. with 2×10^7, 1×10^9 and 1×10^{10} plaque-forming units (PFU) of Ad-HCV-NS3 or Adψ5 control vector. The experimental protocol regarding construction of recombinant adenovirus and infection of mice was approved by the Recombinant DNA Advisory Committee of Saitama Medical University (#1073).

Isolation of intrahepatic leukocytes

The liver was perfused with phosphate-buffered saline (PBS) plus 0.05% collagenase via the portal vein. Perfused livers were smashed through a 100-μm cell strainer (BD Biosciences, San Jose, CA, USA). The cell suspension was centrifuged with 35% Percoll at 320 *g* for 10 min, and the cell pellet was cultured in a plastic Petri dish in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; R-10) for 1.5 h to remove adherent cells. Then, non-adherent cells were harvested, washed twice with R-10 and used as intrahepatic lymphocytes (IHL). Adherent cells were used as intrahepatic APC).

Intracellular IFN-γ staining

The IHL were resuspended in R-10. In each well of a 96-well round-bottomed plate, 2×10^6 IHL were incubated for 5 h at 37 °C in R-10 containing 50 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MI, USA), 1 μM ionophore A23187 (Sigma-Aldrich) and 1 μg/mL brefeldin-A (BD Biosciences). The cells were then washed twice with ice-cold PBS (−) and incubated for 10 min at 4 °C with a rat antimouse

CD16/CD32 monoclonal Ab (mAb; Fc Block; BD Biosciences) at a concentration of 1 μg/well. Following incubation, the cells were washed twice with ice-cold PBS (−) and stained with a PE-conjugated HCV-NS3 H-2Db tetramer (Tet-603; GAVQNEVTL; Medical and Biological Laboratories, Nagoya, Japan)[23] and peridinin chlorophyll protein (PerCP)-conjugated rat antimouse CD8 MAb (clone 53-6.7; BD Biosciences) for 30 min at 4 °C in staining buffer (PBS with 1% FCS and 0.1% NaN_3). After the cells were washed twice, they were fixed and permeabilized by using a Cytofix/Cytoperm kit (BD Biosciences) and stained with a fluorescein isothiocyanate (FITC)-conjugated rat antimouse IFN-γ mAb (clone XMG1.2; BD Biosciences). After the cells were washed, flow cytometric analyses were performed with a FACScanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and the data were analyzed with FACSdiva software (Becton Dickinson).

PD-1 and Tim-3 staining

Intrahepatic lymphocytes were prepared and treated with an antimouse CD16/CD32 mAb as described above for intracellular IFN-γ staining and then stained with a PE-conjugated HCV-NS3 H-2Db tetramer, PerCP-conjugated anti-CD8a (BD Biosciences), FITC-conjugated anti-PD-1 (eBioscience, San Diego, CA, USA) and Alexa647-conjugated anti-Tim-3 (Biolegend, San Diego, CA, USA) for 30 min at 4 °C. After the cells were washed twice, they were fixed with PBS containing 1% formaldehyde and 2% FCS and analyzed by flow cytometry.

PD-L1 staining

Intrahepatic APC were prepared and treated with an antimouse CD16/CD32 mAb as described above for intracellular IFN-γ staining and then stained with a FITC-conjugated anti-CD11c (BD Biosciences) and PE-conjugated anti-PD-L1 (eBioscience) for 30 min at 4 °C. After the cells were washed twice, they were fixed with PBS containing 1% formaldehyde and 2% FCS and analyzed by flow cytometry.

HCV core Ag detection

For the detection of HCV core Ag in the liver, liver tissue samples isolated 7 and 14 days post-infection were homogenized in RIPA B buffer (50 mM Tris pH 7.5, 1% NP40, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride) to make 10% (w/v) extract. Liver tissue extracts were assessed using Lumispot Eiken HCV Ag assay kit (Lumispot-Ag; Eiken Chemical, Tokyo, Japan).

Histology and immunohistology staining

Liver tissue samples isolated 7 and 14 days post-infection were used for histological studies. Paraffin sections (4-μm thick) were stained with hematoxylin–eosin safranin O. For immunohistology, 5-μm thick acetone-fixed frozen sections were incubated with rat anti-CD8 (BD Biosciences), followed by biotin-conjugated antirat immunoglobulin G and ABC staining system (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Persisting HCV-NS3 Ag detection

For the detection of persisting HCV-NS3 Ag in the liver, liver tissue samples isolated 21 days post-infection were homogenized in RIPA C buffer (50 mM Tris pH 7.5, 1% Triton X-100, 300 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.02% NaN_3) to make 2% (w/v) extract and used for immune precipitation/western blot assay. Liver tissue extracts were incubated with protein-G sepharose beads for 30 min at 4 °C to remove non-specifically bound proteins. After centrifugation, supernatants were incubated with anti-Flag-M2 antibody (Sigma-Aldrich) coupled protein-G sepharose beads for 2 h at 4 °C. After centrifugation, HCV-NS3-3xFlag fusion protein bound to the beads were dissolved in sample buffer and separated on 10% sodium dodecylsulfate polyacrylamide gel electrophoresis gels (Mini PROTEAN TGX gel; Bio-Rad, Hercules, CA, USA) for immunoblot analysis using anti-Flag-M2 antibody and goat anti-mouse Ig horseradish peroxidase (KPL, Gaithersburg, MD, USA). Electrochemiluminescence Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) was used for chemiluminescent detection.

Statistical analysis

Mann–Whitney *U*-tests were used to evaluate the significance of the differences. Correlations between parameters were tested for statistical significance by Pearson correlation.

RESULTS

Functional exhaustion of Ag-specific CD8 IHL with high infectious dose and the impaired Ag-specific CD8 IHL responses in core Tg mice

TO DETERMINE THE effect of the amount of virus dose, we evaluated hepatic inflammation and compared the magnitude of HCV-NS3-specific CD8

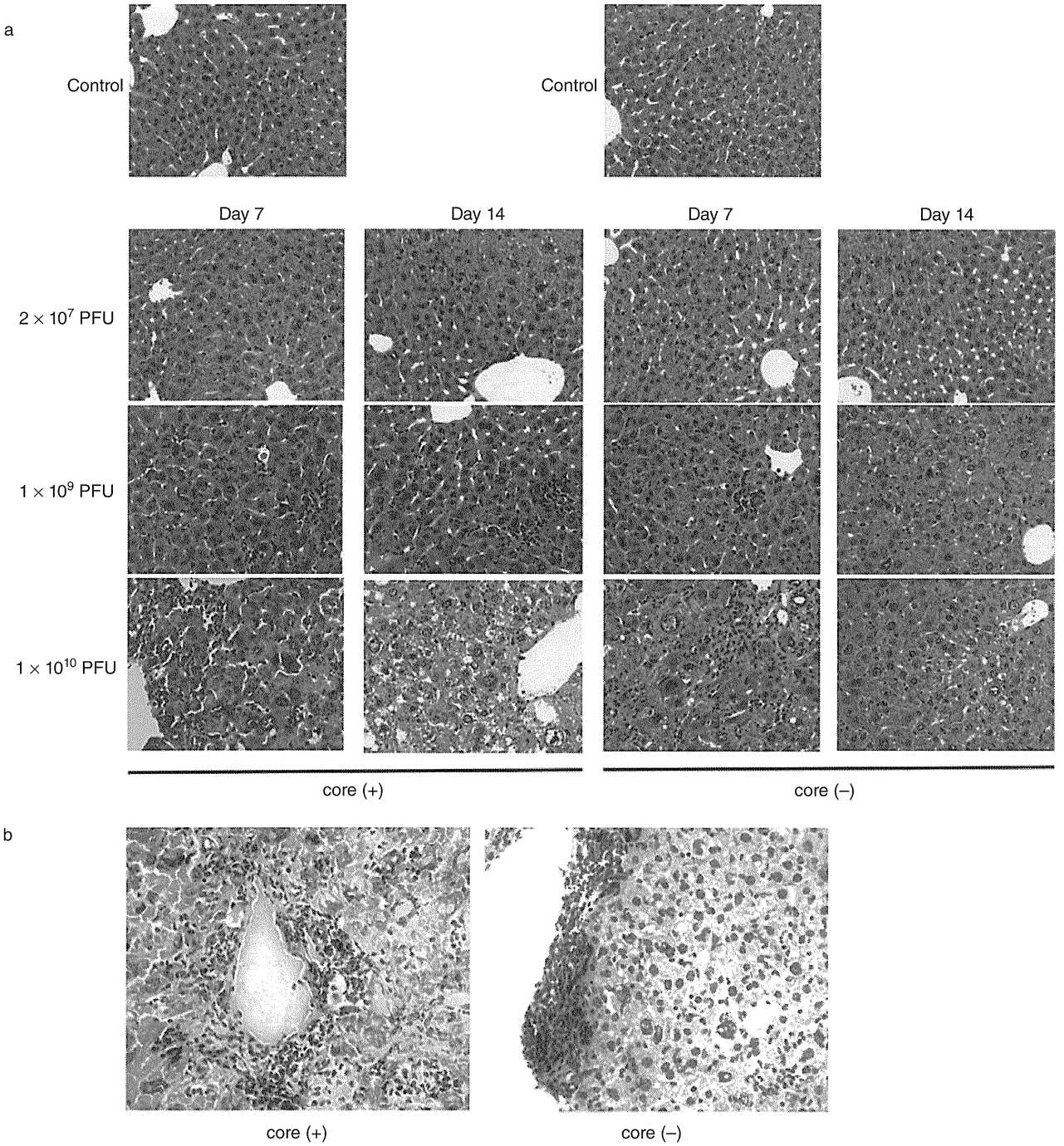

Figure 1 Adenovirus (Ad) infection-mediated hepatic inflammation in mouse liver. Hepatitis C virus (HCV) core (+) and core (–) mice were infected with 2×10^7, 1×10^9 and 1×10^{10} plaque-forming units (PFU) of Ad-HCV-NS3 i.v. and analyzed at 7 and 14 days post-infection. (a) Harvested liver tissues were analyzed by hematoxylin–eosin staining for assessment of hepatic inflammation. (b) Frozen liver sections were analyzed by CD8 staining. Liver infected with 1×10^{10} PFU and harvested at 7 days post-infection was used (original magnifications: [a] ×100; [b] ×200). (c,d) The frequency and number of hepatic CD8 lymphocytes were assessed by flow cytometric analysis. There were no differences in the frequency and number of hepatic CD8 lymphocytes between core (+) mice and core (–) mice. (e) Detection of HCV core antigen in the liver. Liver tissue extracts were assessed using Lumispot Eiken HCV Ag assay kit. There were no differences in core protein expression between Ad-infected and non-infected livers. ■, day 7; ▩, day 14; □, day 21.