Liver International 2006: 26: 90–96

Liver International
DOI: 10.1111/j.1478-3231.2005.01200.x

Clinical Studies

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.
Liver International 2006: 26: 90–96. © Blackwell Munksgaard 2005

Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

Eiji Tanaka,[1] Akihiro Matsumoto,[1] Fumitaka Suzuki,[2] Mariko Kobayashi,[2] Masashi Mizokami,[3] Yasuhito Tanaka,[3] Takeshi Okanoue,[4] Masahito Minami,[4] Kazuaki Chayama[5], Michio Imamura[5], Hiroshi Yatsuhashi[6], Shinya Nagaoka[6], Hiroshi Yotsuyanagi[7], Sumio Kawata[8], Tatsuji Kimura[9], Noboru Maki[9], Shiro Iino[10], Kendo Kiyosawa[1], and HBV Core-Related Antigen Study Group

[1]Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan, [2]Department of Research Institute for Hepatology, Toranomon Hospital, Minato-ku, Tokyo, Japan, [3]Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Science, Nagoya, Japan, [4]Department of Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan, [5]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan, [6]Clinical Research Center, National Nagasaki Medical Center, Omura, Japan, [7]Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University, Kawasaki, Japan, [8]Department of Gastroenterology, School of Medicine, Yamagata University, Yamagata, Japan, [9]Advanced Life Science Institute, Inc., Wako, Japan, [10]Kiyokawa Hospital, Tokyo, Japan

Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

Eiji Tanaka, MD, Department of Medicine, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto 390-8621, Japan.
Tel: +81-263-37-2634
Fax: +81-263-32-9412
e-mail: etanaka@hsp.md.shinshu-u.ac.jp

Received 19 June 2005,
accepted 8 August 2005

Kiyomi Yasuda (Kiyokawa Hospital, Tokyo, Japan); Hitoshi Togashi and Takatumi Saito (Department of Gastroenterology, School of Medicine, Yamagata University); Masataka Tsuge (Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan); Rumiko Nakao (Clinical Research Center, National Nagasaki Medical Center, Omura, Japan); Chiaki Okuse and Hideaki Takahashi (Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University, Kawasaki, Japan).

Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 μL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance Negative (*n* = 54)	 Positive (*n* = 27)	*P*
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	>0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	>0.2†
HBV genotype (A/B/C)	2/2/50	0/1/26	>0.2‡
HBe antigen (positive %)	59%	70%	>0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	>0.2†
At 6 months	27 (11–115)	30 (15–92)	>0.2†
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	>0.2†
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073†
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡χ^2-test.

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it (*P*>0.2).

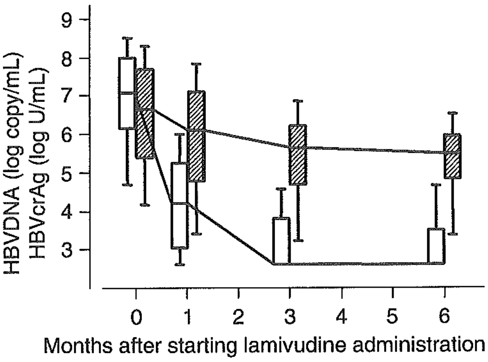

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, *P*<0.001), 3 (3.60 log copy/ml vs. 0.83 log U/ml, *P*<0.001) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, *P*< 0.001) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P < 0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

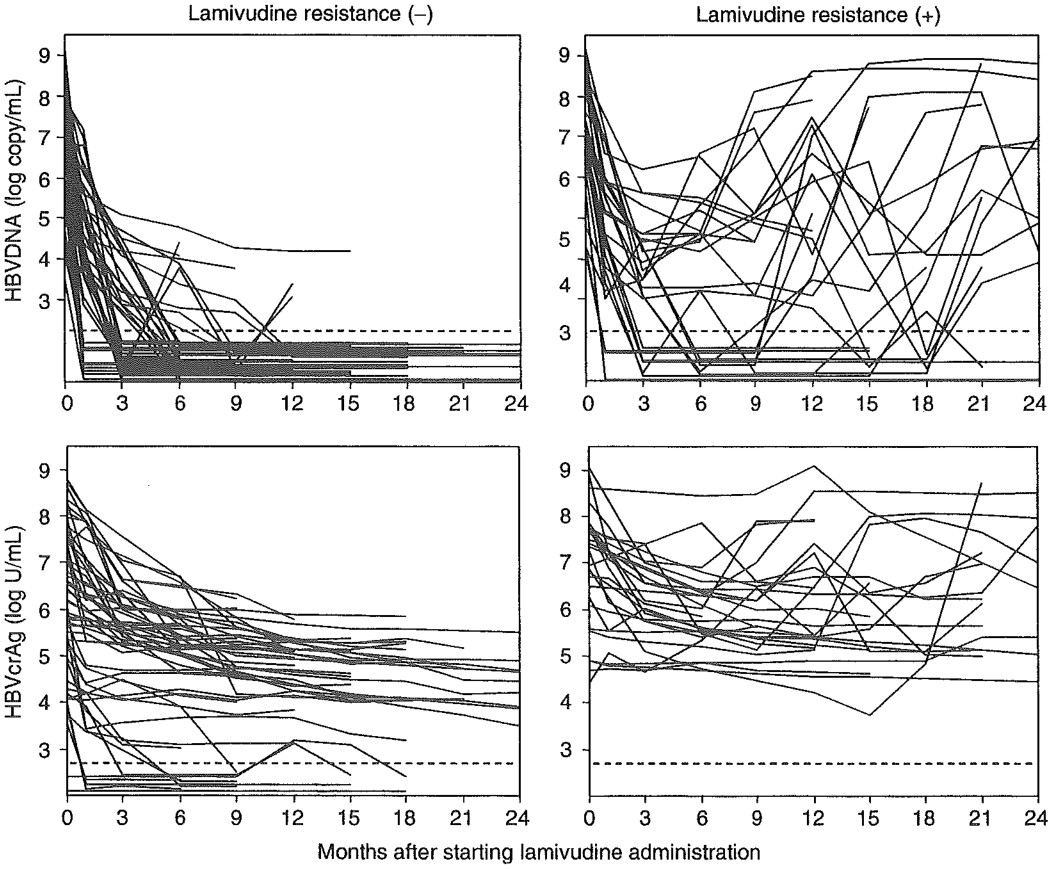

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

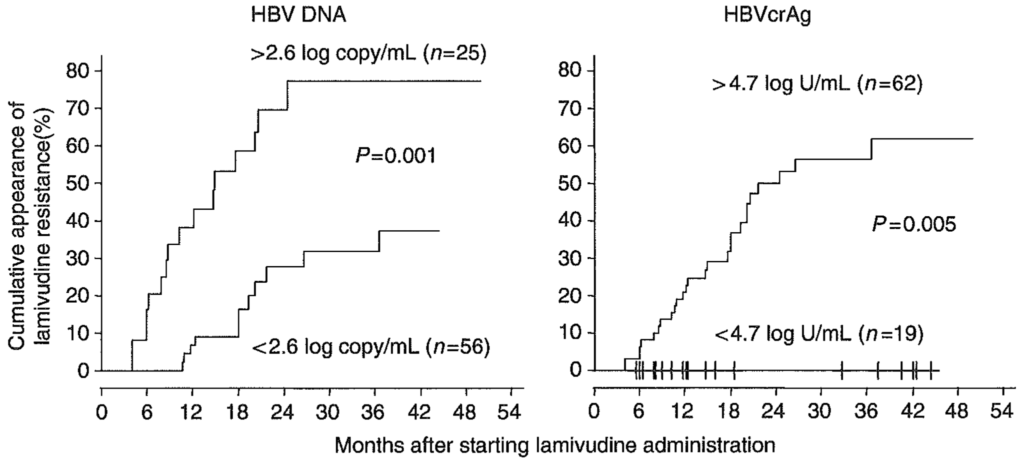

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

Acknowledgements

This research was supported in part by a research grant on hepatitis from the Ministry of Health, Labour and Welfare of Japan.

References

1. Doong S L, Tsai C H, Schinazi R F, Liotta D C, Cheng Y C. Inhibition of the replication of hepatitis B virus in vitro by 2′, 3′-dideoxy-3′-thiacytidine and related analogues. Proc Natl Acad Sci USA 1991; 88: 8495–9.
2. Benhamou Y, Dohin E, Lunel-Fabiani F, Poynard T, Huraox J M, Katlama C, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. Lancet 1995; 345: 396–7.
3. Dienstag J L, Goldin R D, Heathcote E J, Hann H W, Woessner M, Stephenson S L, et al. Histological outcome during long-term lamivudine therapy. Gastroenterology 2003; 124: 105–17.
4. Dienstag J L, Perrillo R P, Schiff E R, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995; 333: 1657–61.
5. Lai C L, Chien R N, Leung N W, Chang T T, Guan R, Tai D I, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998; 339: 61–8.
6. Liaw Y F, Sung J J, Chow W C, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004; 351: 1521–31.
7. Matsumoto A, Tanaka E, Rokuhara A, et al. Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2,795 patients. Hepatol Res 2005; 32: 173–84.
8. Ling R, Mutimer D, Ahmed M, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 1996; 24: 711–3.
9. Lok A S, Lai C L, Leung N, Yao G B, Cui Z Y, Schiff E R, et al. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. Gastroenterology 2003; 125: 1714–22.
10. Tipples G A, Ma M M, Fischer K P, Bain V G, Kneteman N M, Tyrrell D L. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 1996; 24: 714–7.
11. Liaw Y F, Chien R N, Yeh C T, Tsai S L, Chu C M. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. Hepatology 1999; 30: 567–72.
12. Suzuki F, Tsubota A, Arase Y, Suzuki Y S, Akuta N, Hosaka T, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology 2003; 46: 182–9.
13. Zollner B, Schafer P, Feucht H H, Schroter M, Petersen J, Laufs R. Correlation of hepatitis B virus load with loss of e antigen and emerging drug-resistant variants during lamivudine therapy. J Med Virol 2001; 65: 659–63.
14. Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Klyosawa K, et al. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J Clin Microbiol 2002; 40: 439–45.
15. Rokuhara A, Tanaka E, Matsumoto A, Kimura T, Yamaura T, Orii K, et al. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. J Viral Hepatol 2003; 10: 324–30.
16. Bruss V, Gerlich W H. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. Virology 1988; 163: 268–75.
17. Garcia P D, Ou J H, Rutter W J, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. J Cell Biol 1988; 106: 1093–104.
18. Lee W M. Hepatitis B virus infection. N Engl J Med 1997; 337: 1733–45.
19. Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 1999; 450: 66–71.
20. Kobayashi S, Shimada K, Suzuki H, Tanikawa K, Sata M. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). Hepatol Res 2000; 17: 31–42.
21. Mason W S, Halpern M S, England J M, Seal G, Egan J, Coates L, et al. Experimental transmission of duck hepatitis B virus. Virology 1983; 131: 375–84.
22. Summers J, Smith P M, Horwich A L. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. J Virol 1990; 64: 2819–24.
23. Tuttleman J S, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell 1986; 47: 451–60.

24. Moraleda G, Saputelli J, Aldrich C E, Averett D, Condreay L, Mason W S. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. J Virol 1997; 71: 9392–9.
25. Werle-Lapostolle B, Bowden S, Locarnini S, Worsthorn K, Petersen J, Lau G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology 2004; 126: 1750–8.
26. Zhu Y, Yamamoto T, Cullen J, et al. Kinetics of hepadnavirus loss from the liver during inhibition of viral DNA synthesis. J Virol 2001; 75: 311–22.
27. Lau D T, Khokhar M F, Doo E, Ghany M G, Herion D, Park Y, et al. Long-term therapy of chronic hepatitis B with lamivudine. Hepatology 2000; 32: 828–34.
28. Yuen M F, Sablon E, Hui C K, Yuan H J, Decraemer H, Lai C L. Factors associated with hepatitis B virus DNA breakthrough in patients receiving pro longed lamivudine therapy. Hepatology 2001; 34: 785–91.
29. Mihm U, Sarrazin C, Herrmann E, Teuber G, von Wagner M, Kronea berger B, et al.Response predictors and results of a long-term treatment with lamivudine in patients with chronic hepatitis B. Z Gastroenterol 2003; 41: 249–54.

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

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

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

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

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

---

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

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

Received March 16, 2005; accepted August 29, 2005.

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

DOI 10.1002/hep.20917

Potential conflict of interest: Nothing to report.

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

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

Patients and Methods

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

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

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

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

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

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

HCV Proteins. The recombinant full-length HCV core protein (amino acid residues 1-191), E2 protein (amino acid residues 384-746), NS3 protein (amino acid residues 1027-1657), and NS5B protein (amino acid residues 2421-3011) were expressed and purified from *Escherichia coli* using the expression vector as previously described.[22,23] Control proteins were expressed as carboxy-terminal fusion proteins with human superoxide dismutase in *E. coli*.

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

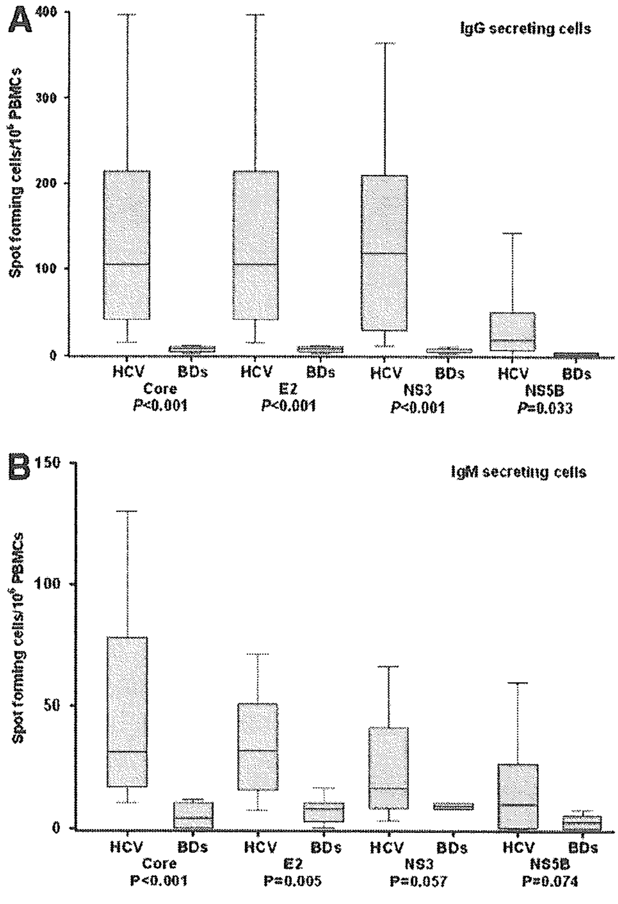

Fig. 1. Detection of anti-HCV antibody-secreting B cells in patients with HCV infection and volunteer blood donors. Boxes represent the IQR of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group,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

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

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

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

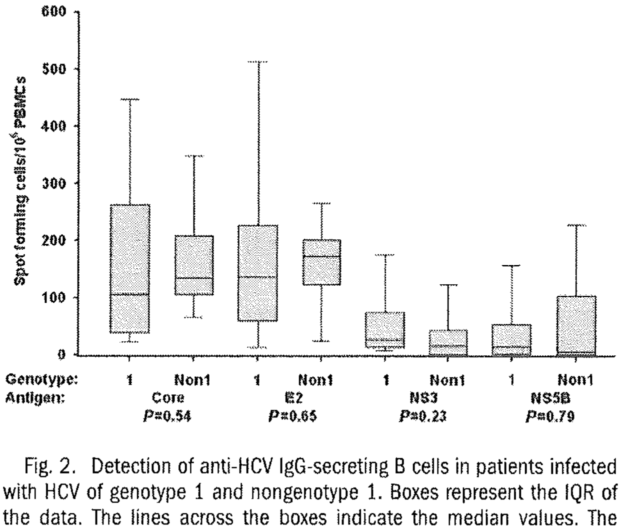

Fig. 2. Detection of anti-HCV IgG-secreting B cells in patients infected with HCV of genotype 1 and nongenotype 1. Boxes represent the IQR of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group,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

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

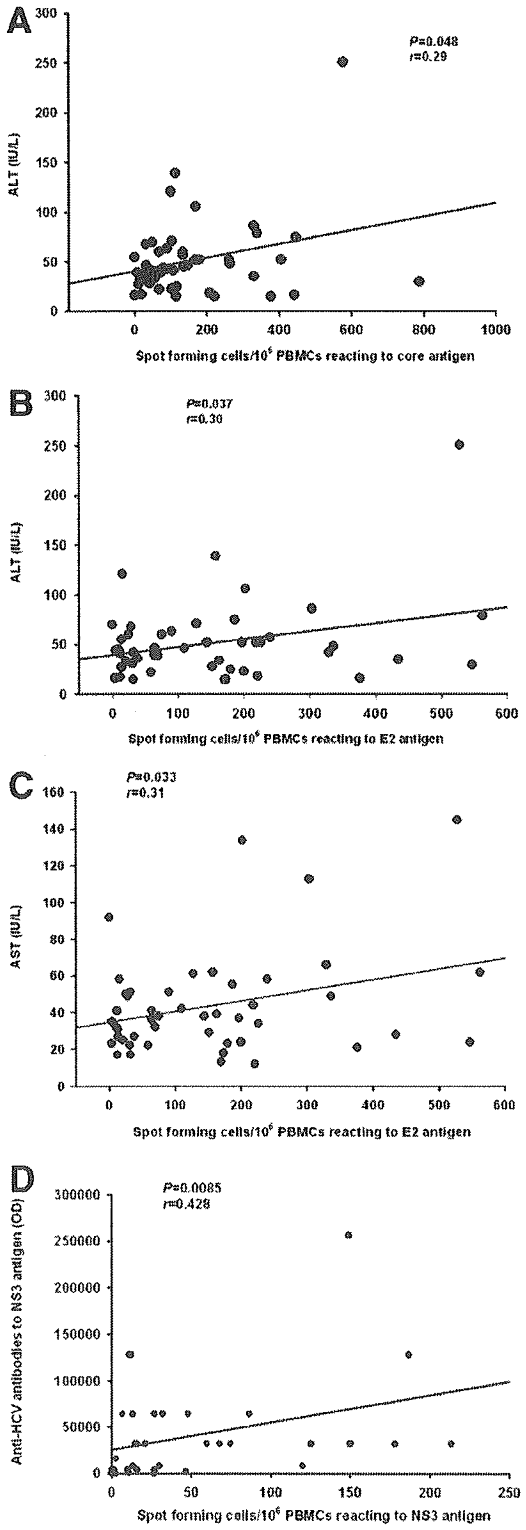

Fig. 3. Correlation of the number of anti-HCV IgG-secreting B cells and clinical characteristics in 48 patients with HCV infection. (A) Frequency of circulating anti-HCV IgG-secreting B cells to core antigen was significantly correlated with the value of ALT ($r = 0.29$, $P = .048$). (B-C) Frequency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

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

Discussion

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

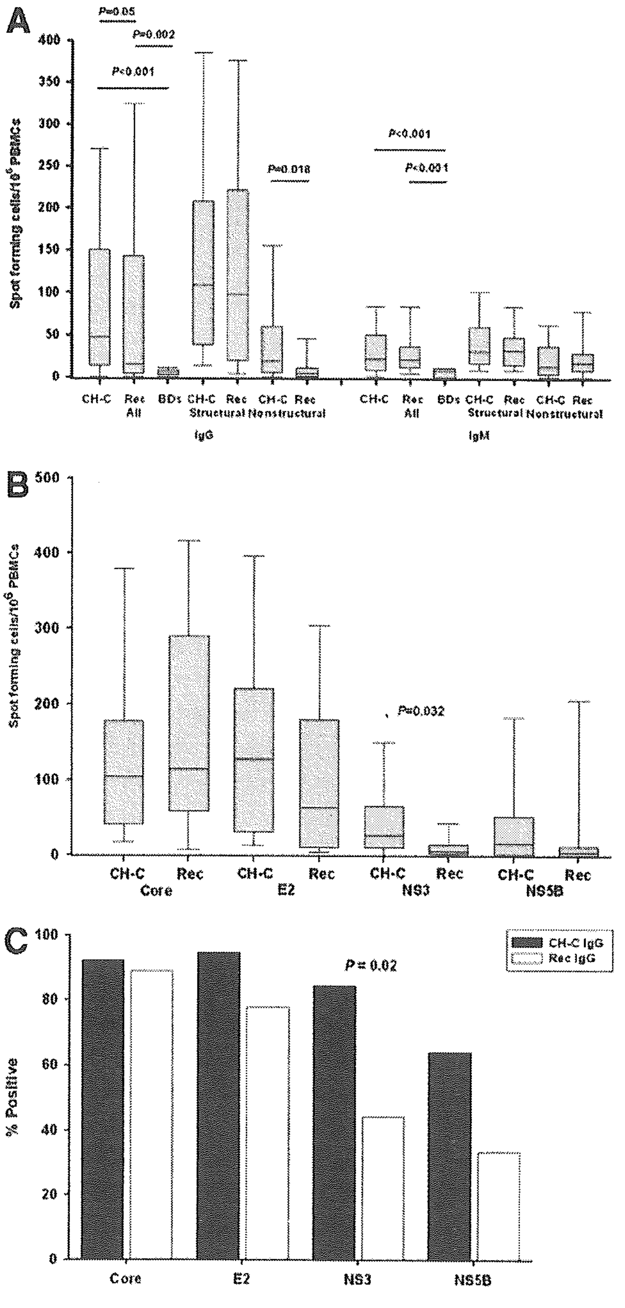

Fig. 4. Detection of anti-HCV antibody-secreting B cells in patients with chronic hepatitis C and in patients who had recovered from HCV. (A) Circulating anti-HCV IgG-secreting B cells were detected in 39 patients with chronic hepatitis C, 9 patients who had recovered from HCV infection, and 11 volunteer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

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

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

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

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

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

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

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

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

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

References

1. Alter HJ, Purcell RH, Shih JW, Melpolder JC, Houghton M, Choo Q-L, et al. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N Engl J Med 1989;321:1494-1500.
2. Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. HEPATOLOGY 1990;12:671-675.
3. Alter HJ, Seeff LB. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. Semin Liver Dis 2000;20: 17-35.
4. Beld M, Penning M, van Putten M, Lukashov V, van den Hoek A, McMorrow M, et al. Quantitative antibody responses to structural (Core) and nonstructural (NS3, NS4, and NS5) hepatitis C virus proteins among seroconverting injecting drug users: impact of epitope variation and relationship to detection of HCV RNA in blood. HEPATOLOGY 1999;29:1288-1298.
5. Chen M, Sallberg M, Sonnerborg A, Weiland O, Mattsson L, Jin L, et al. Limited humoral immunity in hepatitis C virus infection. Gastroenterology 1999;116:135-143.
6. Baumert TF, Wellnitz S, Aono S, Satoi J, Herion D, Tilman Gerlach J, et al. Antibodies against hepatitis C virus-like particles and viral clearance in acute and chronic hepatitis C. HEPATOLOGY 2000;32:610-617.

7. Takaki A, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, et al. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat Med 2000;6:578-582.
8. Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY, et al. Analysis of a successful immune response against hepatitis C virus. Immunity 1999;10:439-449.
9. Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, et al. Analysis of successful immune responses in persons infected with hepatitis C virus. J Exp Med 2000;191:1499-1512.
10. Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. J Exp Med 2001;194:1395-1406.
11. Grakoui A, Shoukry NH, Woollard DJ, Han JH, Hanson HL, Ghrayeb J, et al. HCV persistence and immune evasion in the absence of memory T cell help. Science 2003;302:659-662.
12. Czerkinsky CC, Nilsson LA, Nygren H, Ouchterlony O, Tarkowski A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J Immunol Methods 1983;65:109-121.
13. Sedgwick JD, Holt PG. A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. J Immunol Methods 1983;57:301-309.
14. Arvilommi H. ELISPOT for detecting antibody-secreting cells in response to infections and vaccination. APMIS 1996;104:401-410.
15. Besancon-Watelet C, De March AK, Renoult E, Kessler M, Bene MC, Faure GC, et al. Early increase of peripheral B cell levels in kidney transplant recipients with CMV infection or reactivation. Transplantation 2000;69:366-371.
16. Brown KA, Kriss JA, Moser CA, Wenner WJ, Offit PA. Circulating rotavirus-specific antibody-secreting cells (ASCs) predict the presence of rotavirus-specific ASCs in the human small intestinal lamina propria. J Infect Dis 2000;182:1039-1043.
17. Niewiesk S, Gotzelmann M, ter Meulen V. Selective in vivo suppression of T lymphocyte responses in experimental measles virus infection. Proc Natl Acad Sci U S A 2000;97:4251-4255.
18. Rahman F, Dahmen A, Herzog-Hauff S, Bocher WO, Galle PR, Lohr HF. Cellular and humoral immune responses induced by intradermal or intramuscular vaccination with the major hepatitis B surface antigen. HEPATOLOGY 2000;31:521-527.
19. Bauer T, Weinberger K, Jilg W. Variants of two major T cell epitopes within the hepatitis B surface antigen are not recognized by specific T helper cells of vaccinated individuals. HEPATOLOGY 2002;35:455-465.
20. Conry-Cantilena C, VanRaden M, Gibble J, Melpolder J, Shakil AO, Viladomiu L, et al. Routes of infection, viremia, and liver disease in blood donors found to have hepatitis C virus infection. N Engl J Med 1996;334:1691-1696.
21. Alter HJ, Conry-Cantilena C, Melpolder J, Tan D, Van Raden M, Herion D, et al. Hepatitis C in asymptomatic blood donors. HEPATOLOGY 1997; 26(Suppl):29S-33S.
22. Chen Z, Berkower I, Ching WM, Wang RYH, Alter HJ, Shih JWK. Identification of a murine CD4$^+$ T-lymphocyte response site in hepatitis C virus core protein. Mol Immunol 1996;33:703-709.
23. Jiao X, Wang RYH, Qiu Q, Alter HJ, Shih JWK. Enhanced hepatitis C virus NS3 specific Th1 immune responses induced by co-delivery of protein antigen and CpG with cationic liposomes. J Gen Virol 2004;85:1545-1553.
24. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. J Exp Med 2003;197:633-642.
25. Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, et al. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc Natl Acad Sci U S A 2003;100:7271-7276.
26. Bartosch B, Bukh J, Meunier JC, Granier C, Engle RE, Blackwelder WC, et al. In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. Proc Natl Acad Sci U S A 2003;100:14199-14204.
27. Logvinoff C, Major ME, Oldach D, Heyward S, Talal A, Balfe P, et al. Neutralizing antibody response during acute and chronic hepatitis C virus infection. Proc Natl Acad Sci U S A 2004;101:10149-10154.
28. Kuwana M, Okazaki Y, Kaburaki J, Ikeda Y. Detection of circulating B cells secreting platelet-specific autoantibody is useful in the diagnosis of autoimmune thrombocytopenia. Am J Med 2003;114:322-325.
29. Kajihara M, Kato S, Okazaki Y, Kawakami Y, Ishii H, Ikeda Y, et al. A role of autoantibody-mediated platelet destruction in thrombocytopenia in patients with cirrhosis. HEPATOLOGY 2003;37:1267-1276.
30. Mattsson A, Lonroth H, Quiding-Jarbrink M, Svennerholm AM. Induction of B cell responses in the stomach of Helicobacter pylori-infected subjects after oral cholera vaccination. J Clin Invest 1998;102:51-56.
31. Slifka MK, Ahmed R. Limiting dilution analysis of virus-specific memory B cells by an ELISPOT assay. J Immunol Methods 1996;199:37-46.
32. Corcoran A, Mahon BP, Doyle S. B cell memory is directed toward conformational epitopes of parvovirus B19 capsid proteins and the unique region of VP1. J Infect Dis 2004;189:1873-1880.
33. Ni J, Hembrador E, Di Bisceglie AM, Jacobson IM, Talal AH, Butera D, et al. Accumulation of B lymphocytes with a naive, resting phenotype in a subset of hepatitis C patients. J Immunol 2003;170:3429-3439.
34. Diepolder HM, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, Jung MC, et al. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 1995;346;1006-1007.
35. Wertheimer AM, Miner C, Lewinsohn DM, Sasaki AW, Kaufman E, Rosen HR. Novel CD4+ and CD8+ T-cell determinants within the NS3 protein in subjects with spontaneously resolved HCV infection. HEPATOLOGY 2003;37:577-589.
36. Chen PJ, Wang JT, Hwang LH, Yang YH, Hsieh CL, Kao JH, et al. Transient immunoglobulin M antibody response to hepatitis C virus capsid antigen in posttransfusion hepatitis C: putative serological marker for acute viral infection. Proc Natl Acad Sci U S A 1992;89:5971-5975.
37. Lohr H, Nagel C, Dienes HP, Simpson B, Michel G, Goergen B, et al. Significance of IgG and IgM HCV antibody secretion in vitro in patients with chronic hepatitis C: correlation with disease activity and response to interferon-alpha. HEPATOLOGY 1994;20:1383-1389.
38. Yuki N, Hayashi N, Ohkawa K, Hagiwara H, Oshita M, Katayama K, et al. The significance of immunoglobulin M antibody response to hepatitis C virus core protein in patients with chronic hepatitis C. HEPATOLOGY 1995; 22:402-406.
39. Quiroga JA, van Binsbergen J, Wang CY, Pardo M, Navas S, Trines C, et al. Immunoglobulin M antibody to hepatitis C virus core antigen: correlations with viral replication, histological activity, and liver disease outcome. HEPATOLOGY 1995;22:1635-1640.
40. Pawlotsky JM, Roudot-Thoraval F, Bastie A, Darthuy F, Remire J, Metreau JM, et al. Factors affecting treatment responses to interferon-alpha in chronic hepatitis C. J Infect Dis 1996;174:1-7.
41. Crespo J, Carte B, Lozano JL, Casafont F, Rivero M, de la Cruz F, et al. Hepatitis C virus recurrence after liver transplantation: relationship to anti-HCV core IgM, genotype, and level of viremia. Am J Gastroenterol 1997;92:1458-1462.
42. Bizollon T, Ahmed SN, Guichard S, Chevallier P, Adham M, Ducerf C, et al. Anti-hepatitis C virus core IgM antibodies correlate with hepatitis C recurrence and its severity in liver transplant patients. Gut 2000;47:698-702.
43. Hoofnagle JH. Course and outcome of hepatitis C. HEPATOLOGY 2002; 36(Suppl 1):S21-S29.
44. Yeo AE, Ghany M, Conry-Cantilena C, Melpolder JC, Kleiner DE, Shih JW, et al. Stability of HCV-RNA level and its lack of correlation with disease severity in asymptomatic chronic hepatitis C virus carriers. J Viral Hepat 2001;8:256-263.
45. Gane E. The natural history and outcome of liver transplantation in hepatitis C virus-infected recipients. Liver Transpl 2003;9:S28-S34.
46. Thomas DL, Astemborski J, Vlahov D, Strathdee SA, Ray SC, Nelson KE, et al. Determinants of the quantity of hepatitis C virus RNA. J Infect Dis 2000;181:844-851.
47. Farci P, Shimoda A, Coiana A, Diaz G, Peddis G, Melpolder JC, et al. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science 2000;288:339-344.

Journal of Medical Virology 78:68–73 (2006)

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

Noriko Misawa,[1] Akihiro Matsumoto,[1] Eiji Tanaka,[1]* Akinori Rokuhara,[1] Kaname Yoshizawa,[1] Takeji Umemura,[1] Noboru Maki,[2] Tatsuji Kimura,[2] and Kendo Kiyosawa[1,3]
[1]*Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan*
[2]*Advanced Life Science Institute, Inc., Wako, Japan*
[3]*Shinshu University Graduate School of Medicine, Institute of Organ Transplants, Reconstructive Medicine and Tissue Engineering, Matsumoto, Japan*

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

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

INTRODUCTION

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

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

---

Grant sponsor: Ministry of Health, Labour and Welfare of Japan; Grant number: 1640013-41.

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

Accepted 29 September 2005
DOI 10.1002/jmv.20505

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

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

MATERIALS AND METHODS

Patients

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

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

Serological Markers for HBV

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

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

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

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

Statistical Analyses

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

RESULTS

Grouping of Seroconverters According to HBV DNA Outcome

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

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

Changes in HBV DNA and HBV Core-Related Antigen Concentration

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

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

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

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

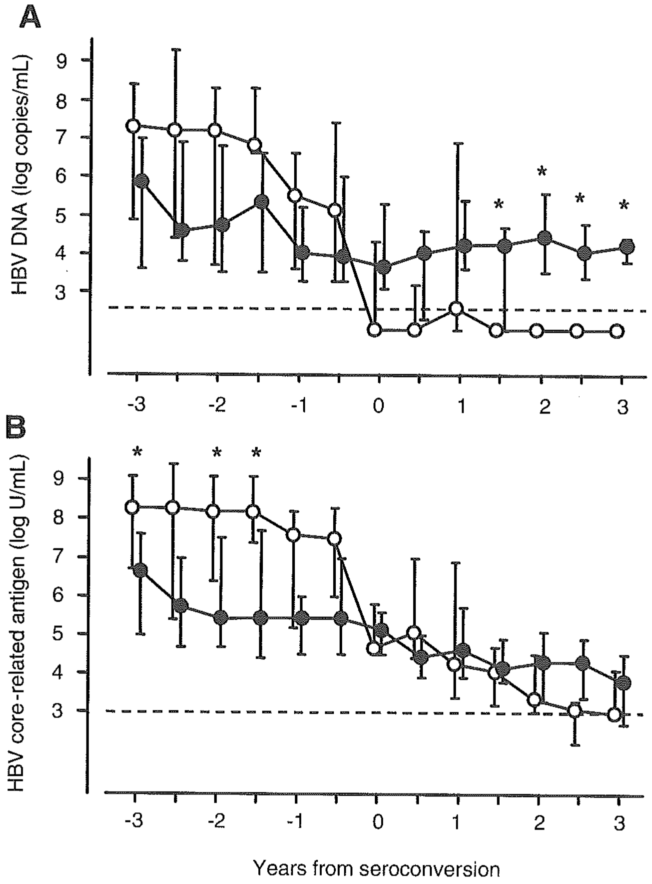

Fig. 1. Comparison of changes in HBV DNA (**A**) and HBV core-related antigen (**B**) levels between the inactive and active replication groups. Data are shown as the median ±25% ranges. The broken lines indicate the detection limits of the HBV DNA and HBV core-related antigen assays, respectively. Open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

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

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

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

Comparison of Pre-C and CP Mutations

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

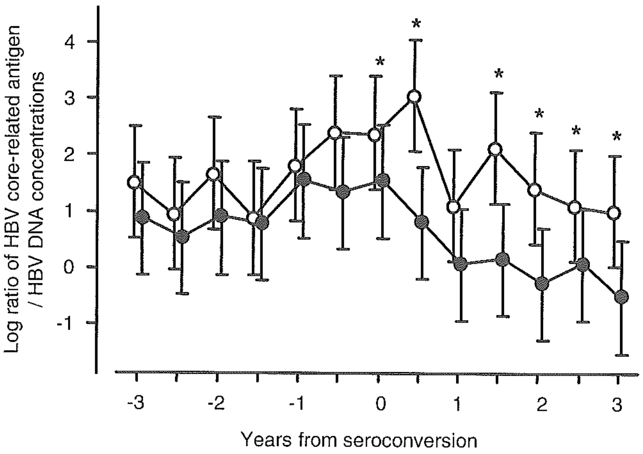

Fig. 2. Comparison of changes in the log ratio of HBV core-related antigen/HBV DNA levels between the inactive and active replication groups. Data are shown as the median ±25% ranges. Open circles indicate inactive replication group and closed circles indicate active replication group. *$P < 0.05$ between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