

et al., 1999; Auernhammer and Melmed, 2001; Alexander, 2002; Fujimoto and Naka, 2003; Lalvakolanu, 2003; Vlotides et al., 2004]. Previous studies reported that the HCV core region might be associated with resistance to the antiviral actions of IFN therapy involving the Jak-STAT signaling cascade [Blindenbacher et al., 2003; Bode et al., 2003; Melén et al., 2004; de Lucas et al., 2005]. The present study identified amino acid substitutions in the HCV core as a predictor of virological non-response to IFN/ribavirin combination therapy. This result suggests that substitutions of amino acids in the HCV core region might be associated with resistance to the antiviral actions of IFN therapy involving the Jak-STAT signaling cascade. Further studies that examine the structural and functional impact of core amino acid 70 and/or 91 substitutions during IFN/ribavirin combination therapy should be conducted in the future to confirm the above finding.

In the present study, virological non-response was noted in 26.3% of patients with high viral load of genotype 1b who received IFN/ribavirin combination therapy. This rate is worse than that of only 2.0% in patients with high viral load of genotype 2a treated with IFN alone [Akuta et al., 2002]. Akuta et al. [2002] examined patients infected with genotype 2a and reported that virological non-responders had higher viral load and one or more of other negative predictive factors associated with sustained virological response (i.e., lower total dose of IFN, moderate-to-severe grade of hepatocyte steatosis, lower levels of albumin, and ALT). Based on the above findings, it was concluded that a complex of negative predictive factors, including viral, host, and treatment-related factors, was the underlying cause of resistance to IFN treatment [Akuta et al., 2002]. Using multivariate analysis, the present study of patients with high viral load of genotype 1b who were treated with IFN/ribavirin, also identified lower ribavirin dose (as treatment-related factor), moderate-to-severe grade of hepatocyte steatosis (as host factor), and substitutions of aa 70 and/or 91 in the core region (as viral factor) as independent and significant factors associated with virological non-response. In this regard, another recent study did not identify ribavirin dose as an independent and significant predictor of virological non-response [Akuta et al., 2005b]. This discrepant finding may be due to the non-uniform dose of ribavirin used in the treatment of patients, which was not strictly adjusted according to body weight (e.g., 600 mg for weight \leq 60 kg, and 800 mg for weight $>$ 60 kg). Thus, the response to combination therapy of IFN/ribavirin is based on a dynamic tripartite interaction of the virus, host, and treatment-related factors. Further understanding of the complex interactions between these factors should facilitate the development of more effective therapeutic regimens.

Akuta et al. [2005b] reported that virological response to 48-week combination therapy of IFN/ribavirin was significantly influenced as negative predictive factor by the presence of pretreatment hypoalbuminemia, which might reflect liver function, based on multivariate

analysis. However, the same analysis in the present study did not identify serum albumin concentration as a significant predictor of virological non-response, although univariate analysis identified it as one of the parameters that tended to influence virological non-response. This discrepant finding could be due to one or more factors. The first is probably related to the design of the present study based on a case-control study matched for age and sex. The second is probably related to the relatively small number of patients in the previous study. A large-scale prospective study should be conducted in the future to establish the role of pretreatment hypoalbuminemia in virological non-response to 48-week IFN/ribavirin combination therapy.

In conclusion, the present study demonstrated that amino acid substitution patterns in the core region is a potentially useful predictor of virological non-response. One limitation of this study was that it did not examine other viral factors, such as amino acid substitutions in areas other than the core region and ISDR of HCV genome, as well as other host factors such as IFN-inducible protein kinase, MxA, and 2',5'-OAS protein [Gale et al., 1997; Wang and Floyd-Smith, 1997; Ronni et al., 1998; Antonelli et al., 1999; Akuta et al., 2003; Vlotides et al., 2004]. These factors should be investigated together with other factors in future studies. Moreover, further large-scale prospective studies are necessary to investigate whether the present results also explain resistance to combination therapy of IFN/ribavirin.

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REFERENCES

- Akuta N, Suzuki F, Tsubota A, Suzuki Y, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2002. Efficacy of interferon monotherapy to 394 consecutive naive cases infected with hepatitis C virus genotype 2a in Japan: Therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. *J Hepatol* 37:831–836.
- Akuta N, Suzuki F, Tsubota A, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2003. Association of amino acid substitution pattern in nonstructural protein 5A of hepatitis C virus genotype 2a low viral load and response to interferon monotherapy. *J Med Virol* 69:376–383.
- Akuta N, Suzuki F, Suzuki Y, Sezaki H, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kobayashi M, Kumada H. 2005a. Long-term follow-up of interferon monotherapy in 454 consecutive naive patients infected with hepatitis C virus. Multi-course interferon therapy may reduce the risk of hepatocellular carcinoma and increase survival. *Scand J Gastroenterol* 40:688–696.
- Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2005b. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon–ribavirin combination therapy. *Intervirology* 48:372–380.
- Alexander WS. 2002. Suppressors of cytokine signaling (SOCS) in the immune system. *Nat Rev Immunol* 2:410–416.
- Antonelli G, Simeoni E, Turriziani O, Tesoro R, Redaelli A, Roffi L, Antonelli L, Pistello M, Dianzani F. 1999. Correlation of interferon-induced expression of MxA mRNA in peripheral blood mononuclear

- cells with the response of patients with chronic hepatitis C to IFN- α therapy. *J Interferon Cytokine Res* 19:243–251.
- Auernhammer CJ, Melmed S. 2001. The central role of SOCS-3 in integrating the neuro-immunoendocrine interface. *J Clin Invest* 108:1735–1740.
- Blindenbacher A, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, Moradpour D, Blum HE, Alonzi T, Tripodi M, La Monica N, Heim MH. 2003. Expression of hepatitis C virus proteins inhibits interferon α signaling in the liver of transgenic mice. *Gastroenterology* 124:1465–1475.
- Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, Heinrich PC, Häussinger D. 2003. IFN- α antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. *FASEB J* 17:488–490.
- Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Ikeda K, Matsumoto T, Kobayashi M, Iwasaki S, Koyama S, Morinaga T, Kumada H. 1993. Genotypic subtyping of hepatitis C virus. *J Gastroenterol Hepatol* 8:150–156.
- D'Alessandro AM, Kalayouglu M, Sollinger HW, Hoffmann RM, Reed A, Knechtle SJ, Pirsch JD, Hafez GR, Lorentzen D, Belzer FO. 1991. The predictive value of donor liver biopsies for the development of primary nonfunction after orthotopic liver transplantation. *Transplantation* 51:157–163.
- de Lucas S, Bartolome J, Carreno V. 2005. Hepatitis C virus core protein down-regulates transcription of interferon-induced antiviral genes. *J Infect Dis* 191:93–99.
- Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. 1994. Classification of chronic hepatitis: Diagnosis, grading and staging. *Hepatology* 19:1513–1520.
- Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C. 1995. Comparison of full-length sequences of interferon sensitive and resistant hepatitis C virus 1b. *J Clin Invest* 96:224–230.
- Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 334:77–81.
- Fried MW, Shiffman ML, Reddy R, Smith C, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 347:975–982.
- Fujimoto M, Naka T. 2003. Regulation of cytokine signaling by SOCS family molecules. *Trends Immunol* 24:659–666.
- Gale MJ, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 230:217–227.
- Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Nakamura I, Murashima N, Kumada H, Kawanishi M. 1999. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: A long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. *Hepatology* 29:1124–1130.
- Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci USA* 87:9524–9528.
- Kwok S, Higuchi R. 1989. Avoiding false positives with PCR. *Nature* 339:237–238.
- Lalvakolanu DV. 2003. Alternate interferon signaling pathways. *Pharmacol Ther* 100:1–29.
- Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling MH, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomized trial. *Lancet* 358:958–965.
- Melén K, Fagerlund R, Nyqvist M, Keskinen P, Julkunen I. 2004. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. *J Med Virol* 73:536–547.
- Ronni T, Matikainen S, Lehtonen A, Palvimo J, Dellis J, Van Eylen F, Goetschy JF, Horisberger M, Content J, Julkunen I. 1998. The proximal interferon-stimulated response elements are essential for interferon responsiveness: A promoter analysis of the antiviral MxA gene. *J Interferon Cytokine Res* 18:773–781.
- Song MM, Shuai K. 1998. The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. *J Biol Chem* 273:35056–35062.
- Stoiber D, Kovarik P, Cohnhey S, Johnston JA, Steinlein P, Decker T. 1999. Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN- γ . *J Immunol* 163:2640–2647.
- Vlotides G, Sörensen AS, Kopp F, Zitzmann K, Cengic N, Brand S, Zachoval R, Auernhammer CJ. 2004. SOCS-1 and SOCS-3 inhibit IFN- α -induced expression of the antiviral 2,5-OAS and MxA. *Biochem Biophys Res Commun* 320:1007–1014.
- Wang Q, Floyd-Smith G. 1997. The p69/71 2-5A synthetase promoter contains multiple regulatory elements required for interferon- α -induced expression. *DNA Cell Biol* 16:1385–1394.

Virological Outcomes in Patients Infected Chronically With Hepatitis B Virus Genotype A in Comparison With Genotypes B and C

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In a single hospital in Tokyo, the 87 patients infected persistently with hepatitis B virus (HBV) genotype A, the 413 with B, and the 3,389 with C were compared for virological outcome. Hepatitis B surface antigen (HBsAg) was cleared from the serum in 12% (3/26), 2% (2/112), and 3% (23/826) of patients with genotypes A, B, and C, respectively, at 5 years of follow-up ($P=0.0395$). Hepatitis B e antigen (HBeAg) was cleared from serum more frequently in patients with genotype B than those with A or C (78% [32/41] vs. 58% [11/19] or 45% [251/562], $P=0.00001$) at 5 years. Of the 45 individuals infected with genotype A and followed for 3 years or longer, HBeAg was more frequent (16% [3/19] vs. 73% [19/26], $P=0.0002$) and levels of HBV DNA higher (median <2.6 [range: <2.6–5.6] vs. >7.6 [<2.6–>7.6] log copies/ml, $P=0.001$) in the 26 patients with biopsy-proven chronic hepatitis than the 19 asymptomatic carriers. Among the 26 hepatitis patients infected with HBV genotype A, decreases in HBV DNA were less frequent (20% [1/5] vs. 93% [13/14] or 86% [6/7], $P=0.0095$) and increases in serum levels of hyaluronic acid ≥ 10 ng/ml commoner (80% [4/5] vs. 14% [2/14] or 14% [1/7], $P=0.017$) in the patients who kept HBeAg than in those who seroconverted or who remained HBeAg-negative. In conclusion, patients persistently infected with HBV genotype A fare better than those with genotype B or C. However, high levels of HBV DNA continue in those in whom HBeAg persists along with fibrosis in the liver. *J. Med. Virol.* 78:60–67, 2006. © 2005 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis; cirrhosis; hepatitis B e antigen; hepatitis B surface antigen; hepatocellular carcinoma; sexual transmission

INTRODUCTION

There are an estimated 350 million people in the world who are persistently infected with hepatitis B virus (HBV), some of whom develop a spectrum of chronic liver disease ranging from chronic hepatitis through cirrhosis to hepatocellular carcinoma [Lee, 1997]. New HBV infections have been prevented by mass vaccination of neonates [Tsen et al., 1991; Chen et al., 1996] and immunoprophylaxis of babies born to mothers carrying HBV [Noto et al., 2003]. However, there are individuals who have been infected, and they need to be identified for receiving treatment as required. Clinical outcomes and the response to antiviral treatment are influenced by many host factors, such as ethnicity, gender, and the age at infection, as well as viral factors represented by HBV genotypes.

HBV has a partially double-stranded DNA genome of approximately 3,200 nucleotides (nt) [Tiollais et al., 1981]. Eight HBV genotypes have been classified by a sequence divergence in the entire genome exceeding 8% [Okamoto et al., 1988], and they are named by capital Alphabet letters from A to H [Okamoto et al., 1988; Norder et al., 1992; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. Recently, HBV genotypes have attracted an increasing attention because they influence the clinical outcome and treatment response in patients with chronic liver disease [Tsubota et al., 2001; Kao, 2002; Miyakawa and Mizokami, 2003; Schaefer, 2005; Yu et al., 2005]. Due to their uneven geographical

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distribution, however, only two HBV genotypes prevail in most countries; the United States is the only exception with seven (A–G) genotypes [Chu et al., 2003; Westland et al., 2003]. Thus, genotypes A and D are common in Europe and India, while genotypes B and C are frequent in Asia [Magnius and Norder, 1995; Miyakawa and Mizokami, 2003]. Therefore, comparison has been restricted between patients infected with genotypes A and D, as well as those with B and C [Zhang et al., 1996; Mayerat et al., 1999; Kao et al., 2000; Orito et al., 2001; Chu et al., 2002; Thakur et al., 2002].

During 31 years from 1973 to 2003, 4,121 patients visited Toranomon Hospital in the Metropolitan Tokyo, and HBV genotypes were determined in them. There were 128 patients with genotype A, of whom 87 were chronically infected with HBV at the presentation. They were followed along with the 413 patients chronically infected with genotype B and the 3,389 with genotype C for seroclearance of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg). Furthermore, patients with genotype A were grouped by the presence or absence of HBeAg at the presentation, as well as seroconversion during the follow-up, and they were compared for virological and clinical outcomes.

MATERIALS AND METHODS

Patients Chronically Infected With HBV

During 31 years from April 1973 to December 2003, genotypes of HBV DNA were determined in 4,121 patients with HBsAg in the Department of Gastroenterology at Toranomon Hospital in the Metropolitan Tokyo. Genotypes were A in 128 (3.11%) patients, B in 431 (10.46%), C in 3,434 (83.32%), D in 4 (0.97%), E in 1 (0.02%), and F in 3 (0.07%); they were not classifiable in the remaining 120 (2.91%) patients.

Of the 128 patients infected with HBV genotype A, 41 (32%) presented with acute hepatitis B as diagnosed by high-titered IgM antibody to hepatitis B core antigen. The remaining 87 (68%) patients were chronically infected with HBV genotype A when they visited our hospital. Their diagnoses were asymptomatic carriers with persistently normal ALT levels in 38 (44%) and chronic hepatitis in 39 (45%). In addition, nine (10%) patients presented with cirrhosis and one (1%) with hepatocellular carcinoma. Chronic hepatitis was diagnosed by liver biopsies performed under laparoscopy, and liver cirrhosis by liver biopsy and/or ultrasonographic images plus laparoscopic findings. Hepatocellular carcinoma was diagnosed by imaging modalities, such as ultrasonography, computed tomography, and magnetic resonance imaging, and by liver biopsy if necessary.

The 87 patients infected chronically with HBV genotype A had the median age of 34 years (range: 11–67 years), included 72 (83%) men and were followed for the median of 5.0 years (0.1–22 years). Only two (2%) had a history of blood transfusion, and three (3%) were co-infected with hepatitis C virus. They had the median

serum HBV DNA level at 4.2 log copies/ml, and HBeAg was detected in sera from 32 (37%). Subgenotypes of A [Bowyer et al., 1997; Sugauchi et al., 2004] were Aa (Asian or African type) in 5 (6%) and Ae (European type) in 65 (75%); they were not classifiable in the remaining 17 (19%).

Serological Markers of HBV Infection

HBsAg was determined by hemagglutination (MyCell; Institute of Immunology Co., Ltd., Tokyo, Japan) or enzyme-linked immunosorbent assay (ELISA) (ELISA, F-HBsAg; Sysmex, Kobe, Japan), and HBeAg by ELISA (ELISA, F-HBe; Sysmex). HBV DNA was determined by quantitative polymerase chain reaction (PCR) (Amplicor HBV Monitor Test; Roche Molecular Systems, Inc., New Jersey) and the results were expressed in log copies/ml within a detection range from 2.6 to 7.6.

Genotypes of HBV

The six major genotypes (A–F) were determined serologically by ELISA (HBV GENOTYPE EIA; Institute of Immunology). The method utilizes the combination of epitopes on preS2-region products that is specific for each genotype [Usuda et al., 1999, 2000]. Genotype G was determined by preS2 serotype for genotype D and HBsAg subtype adw, and H was recognized by serotype for genotype C and subtype adw, respectively; these combinations were specific for genotypes G and H, respectively [Kato et al., 2001, 2004].

Subgenotypes of A designated Ae prevalent in Europe and Aa frequent in Africa as well as Asia [Sugauchi et al., 2004] (corresponding to A' originally reported by Bowyer et al. [1997]), were determined by the nucleotide sequence in the S gene [Sugauchi et al., 2004]. Briefly, nucleic acids were extracted from serum and a sequence of the large S gene was amplified by PCR with nested primers. The first-round PCR was performed with BGF1 (sense, 5'-CTG TGG AAG GCT GGC ATT CT-3' [nt 2757–2776]) and BGR2 (antisense, 5'-GGC AGG ATA GCC GCA TTG TG-3' [nt 1050–1079]) primers, and the second-round PCR with PLF5Bm (sense, 5'-TGT GGA TCC TGC ACC GAA CAT GGA GAA-3' [nt 136–162]) and BR112 (antisense, 5'-TTC CGT CGA CAT ATC CCA TGA AGT TAA GGG A-3' [nt 865–895]) as well as BGF5 (sense, 5'-TGC GGG TCA CCA TAT TCT TG-3' [nt 2811–2830]) and BGR6 (antisense, 5'-AGA AGT CCA CCA CGA GTC TA-3' [nt 249–268]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). Amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, California), purified by Qquick PC purification kit (Qiagen, Hilden, Germany) and then sequenced in AGI Prism 310 Genetic Analyzer (Applied Biosystems). The large S-gene sequences were analyzed phylogenetically along with reference Aa and Ae sequences by six-parameter and neighbor-joining methods [Gojobori et al., 1982; Saitou and Nei, 1987].

Determination of Hyaluronic Acid in Serum

Hyaluronic acid was determined by the agglutination of microparticles coated with proteins that specifically bind with it (Elpia-Ace HA, Fujirepio, Tokyo, Japan).

Statistical Analysis

Frequencies were compared between groups by the Mann-Whitney *U*-test and Fisher's exact test, and means by the Wilcoxon signed rank test. Loss of HBeAg or HBsAg was compared in the Kaplan-Meier life table, and differences were evaluated by log-rank test after the production limit method. A *P*-value less than 0.05 was considered significant.

RESULTS

Patients Infected Chronically With HBV Genotype A

There were 45 patients who were infected chronically with HBV genotype A and had been followed for 3 years or longer. Of them, 19 had persistently normal ALT levels (asymptomatic carriers), while the remaining 26 with elevated ALT levels possessed biopsy-proven chronic hepatitis. Table I compares demographic and virological characteristics at the baseline between the 19 asymptomatic carriers and 26 patients with chronic hepatitis. HBeAg was more frequent and the median HBV DNA level higher in patients with chronic hepatitis than asymptomatic carriers. The majority of asymptomatic carriers (79% [15/19]) and patients with chronic hepatitis (73% [19/26]) were infected with subgenotype Ae. There were three (12%) patients infected with subgenotype Aa and two of them had chronic hepatitis. Subgenotypes were not classifiable in the remaining four (21%) asymptomatic carriers and four (15%) patients with chronic hepatitis. Liver disease worsened in a single patient with chronic hepatitis. He was 47 years old at the presentation and infected with subgenotype Ae. Cirrhosis developed followed by hepatocellular carcinoma in him.

HBsAg and HBeAg in Patients With Chronic Hepatitis Infected With HBV Genotype A

Of the 26 patients infected with HBV genotype A, 4 (15%) lost HBsAg during follow-up, in comparison with

16 of the 116 (14%) patients with genotype B and 68 of the 862 (8%) with genotype C. Figure 1 compares seroclearance of HBsAg among patients with genotype A, B, or C. The loss of HBsAg at 5 years was significantly more frequent in patients with genotype A than B or C (12% vs. 2% or 3%, *P* = 0.0395).

Of the 26 hepatitis patients with genotype A, 19 (75%) possessed HBeAg at the presentation. HBeAg was cleared from serum in 14 (74%) of them during follow-up, in comparison with the seroclearance in 36 of the 41 (88%) patients with genotype B and in 347 of the 562 (62%) with genotype C. Figure 2 compares seroclearance of HBeAg among patients with genotype A, B, or C. At 5 years of follow-up, HBeAg was cleared more frequently in patients with genotype B than in those with genotype A or C (78% vs. 58% or 45%, *P* = 0.00001).

Development of Cirrhosis and Hepatocellular Carcinoma in Patients Infected With HBV of Various Genotypes

Figure 3 compares the development of cirrhosis in patients infected with genotype A, B, or C. Of the patients with genotype A, cirrhosis developed in only one at 5 years, but not any more during follow-up for 20 years. In contrast, cirrhosis increased steadily in patients with genotype B or C; it developed twice more often in patients with genotype C than B (30% vs. 14%).

Hepatocellular carcinoma developed in the single cirrhotic patient with genotype A, but did not in any others with genotype A during follow up for 20 years (Fig. 4). It increased with time, however, in patients with genotype B or C. Hepatocellular carcinoma tended to develop more frequently in patients with genotype C than B at 20 years (15% vs. 11%).

Changes in HBV DNA Levels and Hyaluronic Acid in the Patients Infected With HBV Genotype A

Of the 26 patients with genotype A, 14 (54%) seroconverted for the loss of HBeAg, while 5 (19%) kept it throughout follow-up longer than 3 years; the remaining 7 (27%) patients were without HBeAg at the presentation and thereafter. Table II compares demographic and virological characteristics of the three

TABLE I. Baseline Characteristics of the 45 Patients Infected With HBV Genotype A Who Were Followed for Longer Than 3 Years

Feature	Asymptomatic carriers (n = 19)	Chronic Hepatitis (n = 26)	Differences
Age (years) ^a	29 (11-48)	32 (13-59)	NS ^c
Male	15 (79%)	24 (92%)	NS
Follow-up (years) ^a	6.5 (3.4-17.7)	6.8 (3.5-18.6)	NS
History of transfusion	0 (0%)	1 (4%)	NS
Anti-HCV	0 (0%)	1 (4%)	NS
HBeAg positive	3 (16%)	19 (75%)	<i>P</i> = 0.0002
HBV DNA (log copies/ml)	<2.6 (<2.6-5.9)	>7.6 (<2.6->7.6)	<i>P</i> = 0.001
Subgroups (Aa/Ae/ND ^b)	0%/79%/21%	12%/73%/15%	NS

^aMedian values are shown with the range in parentheses.

^bNot determined.

^cNot significant.

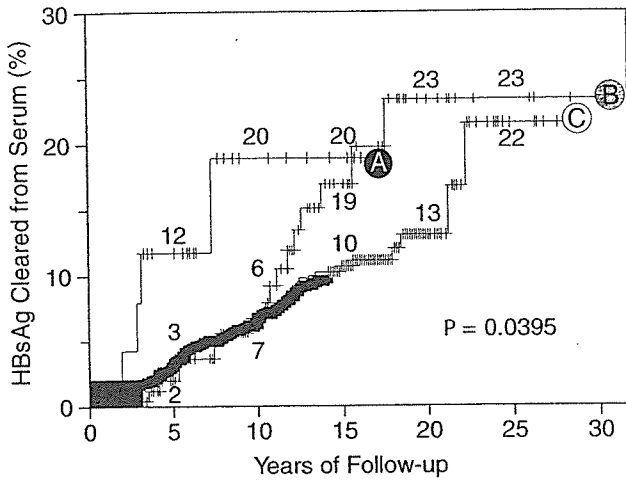


Fig. 1. Seroclearance of HBsAg during follow-up. Clearance rates of HBsAg are compared among patients with chronic hepatitis B who were infected with genotypes A, B, or C by the Kaplan–Meier life table. Differences are significant between genotype A and genotypes B and C at 5 and 10 years, as well as between genotypes B and C at 20 years by the log-rank test. Seroclearance of HBsAg did not spontaneously occur in all of them.

groups of patients at the baseline. Levels of HBV DNA were significantly lower in the patients without HBeAg than in those whom HBeAg persisted or who seroconverted within 3 years ($P = 0.03$).

Figure 5 compares changes in HBV DNA levels among patients infected with genotype A in whom HBeAg persisted, who seroconverted and who had remained negative for HBeAg. HBV DNA levels >7.6 log copies/ml continued for longer than 3 years in four of the five (80%) patients with persistent HBeAg. HBV DNA levels decreased in 13 of the 14 (93%) patients with seroconversion; they slightly changed from 6.7 to 7 log copies/ml in the remaining one patient. HBV DNA decreased to

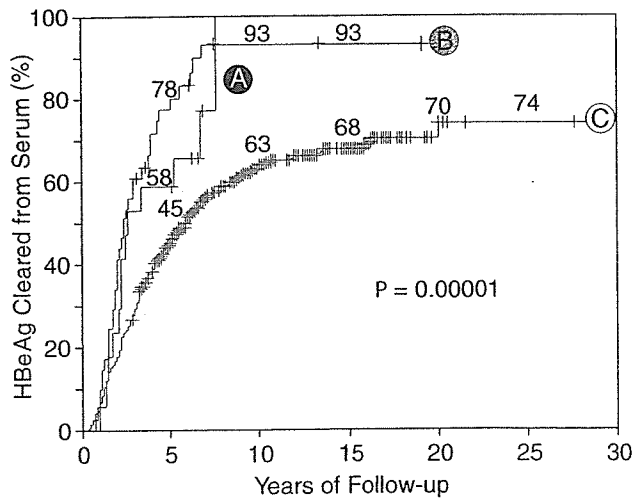


Fig. 2. Seroclearance of HBeAg during follow-up. Clearance rates of HBeAg are compared among patients with chronic hepatitis B who were infected with genotypes A, B, or C by the Kaplan–Meier life table. Differences are significant among genotypes A–C at 5 years as well as between genotypes B and C since 10 years or later by the log-rank test. Seroclearance of HBeAg did not spontaneously occur in all of them.

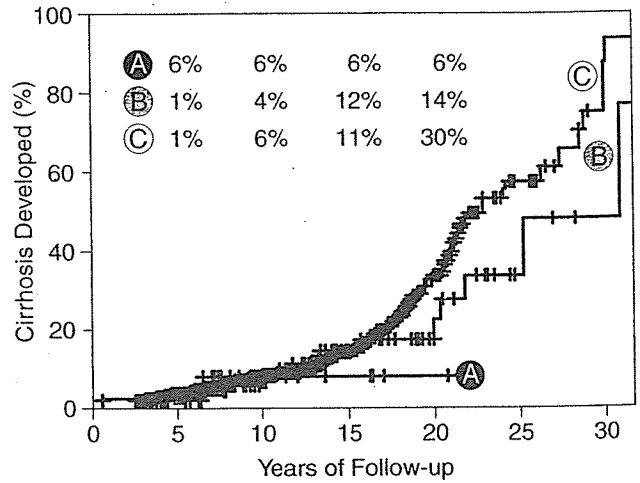


Fig. 3. Development of cirrhosis in patients infected with HBV genotype A, B, or C.

levels below the detection limit in 4 of the 14 (29%) patients with seroconversion and 1 of the 7 (14%) without HBeAg at the baseline. Of the 7 patients without HBeAg, 4 (57%) kept HBV DNA in detectable levels, comparable to 9 of the 14 (64%) patients with seroconversion. Decreases in HBV DNA during follow-up for 3 years or longer were significantly more frequent in the patients with seroconversion and those without HBeAg than in those with persistent HBeAg (93% [13/14] and 86% [6/7] vs. 20% [1/5], $P = 0.0095$ by the Fisher's exact test).

Figure 6 compares serum levels of hyaluronic acid among patients infected with genotype A in whom HBeAg persisted, who seroconverted and who had remained HBeAg-negative. Hyaluronic acid increased in four of the five (80%) patients in whom HBeAg persisted in contrast to only one of the seven (14%) patients without HBeAg. Increases in serum levels of hyaluronic acid ≥ 10 ng/ml was more frequent in the

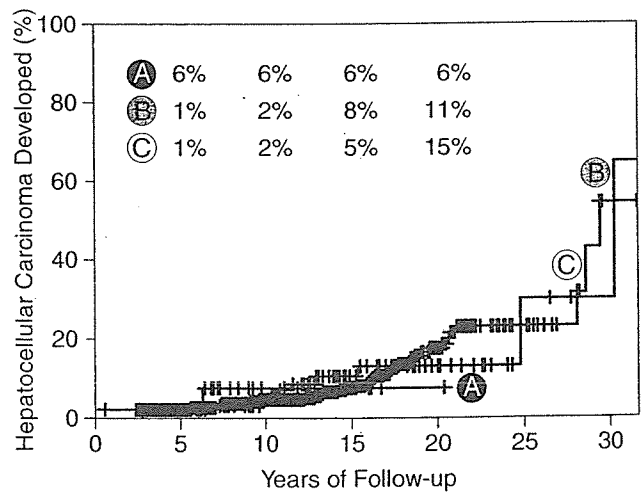


Fig. 4. Development of hepatocellular carcinoma in patients infected with HBV genotype A, B, or C.

TABLE II. Baseline Characteristics of the 26 Patients Infected With HBV Genotype A in Whom HBeAg Persisted, Who Seroconverted and Who Were Without HBeAg at the Presentation

Feature	HBeAg persisted (n = 5)	Seroconverted (n = 14)	Without HBeAg (n = 7)	Differences
Age (years) ^a	49 (24–59)	30 (13–60)	33 (14–41)	NS ^c
Male	5 (100%)	14 (100%)	5 (71%)	NS
Follow-up (years) ^a	6.2 (3.7–7.4)	9.2 (3.0–21)	8.1 (3.9–17)	NS
History of transfusion	0	1 (7%)	0	NS
Anti-HCV	0	0	1 (14%)	NS
HBV DNA (log copies/ml)	>7.6 (all patients)	>7.6 (6.7–>7.6)	4.1 (<2.6–7.1)	<i>P</i> = 0.03
Subgroups (Aa/Ae/ND ^b)	(0%/80%/20%)	(7%/79%/14%)	(29%/57%/14%)	NS

^aMedian values are shown with the range in parentheses.

^bNot determined.

^cNot significant.

patients with persistent HBeAg than in those with seroconversion and those without HBeAg (80% [4/5] vs. 14% [2/14] and 14% [1/7], *P* = 0.017 by the Fisher's exact test).

Of the 19 hepatitis patients presenting with serum HBeAg, 16 received antiviral and/or steroid withdrawal therapies, and 11 (69%) responded by the loss of HBeAg, while the remaining 4 failed to do so (Table III). There were three patients in whom HBeAg disappeared without receiving treatments. In total, therefore, seroconversion was accomplished in 14 of the 19 (74%) patients with genotype A.

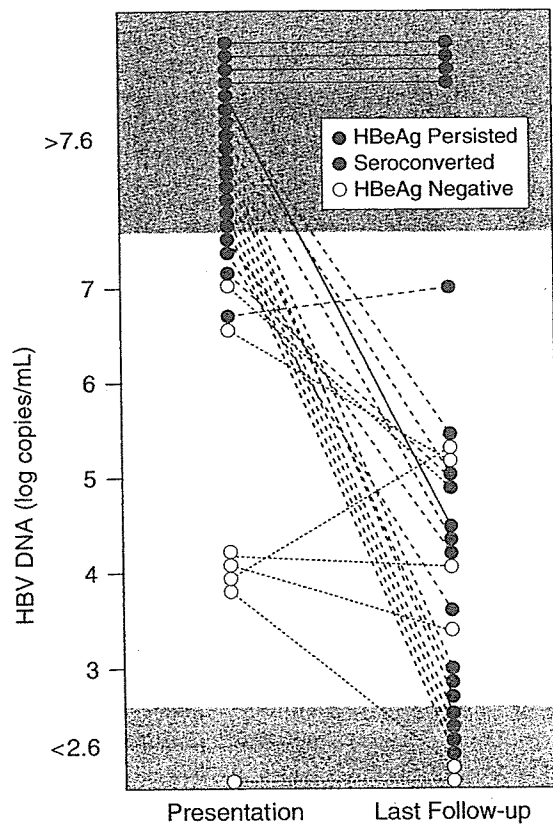


Fig. 5. Changes in serum levels of HBV DNA from the baseline to the last follow-up. Patients in whom HBeAg persisted, who seroconverted and who were without HBeAg at the baseline are compared.

DISCUSSION

Of the eight genotypes of HBV, E, and F are local, and confined to Central Africa and Central/South America, respectively [Magnius and Norder, 1995; Miyakawa and Mizokami, 2003]. Genotype H is genetically close to F and distributes in Central America [Arauz-Ruiz et al., 2002]. Genotype G occurs very rarely [Stuyver et al., 2000; Chu et al., 2003; Kato et al., 2004], and is always

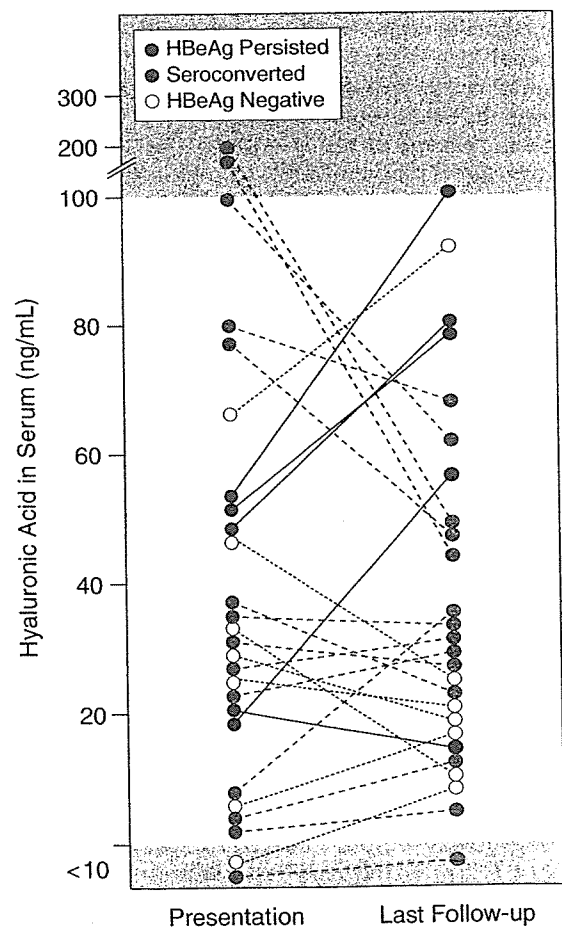


Fig. 6. Changes in serum levels of hyaluronic acid from the baseline to the last follow-up. Patients in whom HBeAg persisted, who seroconverted and who were without HBeAg at the baseline are compared.

TABLE III. Loss of HBeAg in the 19 Hepatitis Patients Infected With HBV Genotype A Who Had Been Followed for Longer Than 3 Years

Case No.	Sex/age	Pathology	Sub-group	Treatment	HBeAg Lost
1	M23	F1/A1	Ae	Interferon	Yes
2	M33	F2/A1	Ae	Interferon	Yes
3	M44	F3/A1	Ae	Interferon	Yes
4	M57	F2/A1	Ae	Interferon	Yes
5	M13	F1/A1	Ae	Steroid withdrawal	Yes
6	M16	F1/A1	Ae	Steroid withdrawal	Yes
7	M28	F1/A1	ND	Steroid withdrawal	Yes
8	M47	F2/A1	Aa	Steroid withdrawal	Yes
9	M17	F1/A1	Ae	Steroid/Interferon	Yes
10	M29	F1/A1	Ae	Lamivudine	Yes
11	M38	F1/A1	Ae	Lamivudine	Yes
12	M30	F1/A0	Ae	None	Yes
13	M39	F1A1	Ae	None	Yes
14	M47	F3/A2	Ae	None	Yes
15	M24	F2/A2	Ae	Interferon and others ^b	No
16	M43	F2/A1	Ae	Steroid/Interferon	No
17	M48	F1/A2	Ae	Interferon/Lamivudine	No
18	M49	F1/A1	ND ^a	Steroid withdrawal	No
19	M59	F1A1	Ae	Interferon	No

^aNot determined.^bThe patient received interferon, lamivudine interferon/lamivudine, and then lamivudine plus entecavir.

co-infected with HBV of the other genotypes [Kato et al., 2002, 2003]. Thus, only four genotypes (A–D) are left for comparison in epidemiological and clinical studies in most countries of the world. Since even these four genotypes have distinct geographical distributions, comparison with respect to severity of liver disease or response to antiviral treatment is hardly feasible among them, except in multi-national studies on patients of diverse ethnicities [Westland et al., 2003; Janssen et al., 2005].

In the Toranomon Hospital in Tokyo, by far the most patients presenting with HBsAg were infected with HBV of genotype B (10.5%) or C (83.3%), and genotype A infected only a minority (3.10%) of them. During 31 years, 128 patients with genotype A visited there. Unlike most infections with genotype B and C transmitted perinatally from carrier mothers with HBeAg [Okada et al., 1976], genotype A infection in Japan is often acquired in the adulthood by men having extra-marital sexual contacts either with men or women; there has been no evidence for maternal transmission of HBV genotype A in Japan [Kobayashi et al., 2002, 2003; Ogawa et al., 2002; Suzuki et al., 2005]. HBV infection prevails among homosexuals in Western countries where genotype A is frequent, who poorly respond to vaccines [Goilav and Piot, 1989]. Genotype A infection in Japan has a propensity to become chronic and tends to respond to antiviral therapies better than genotype B or C infection [Kobayashi et al., 2002, 2003; Suzuki et al., 2005].

In the present study, we have compared the virological outcome among infections with HBV genotypes A, B, and C, and found substantial differences. Patients with genotype A fared better than those with genotype B or C in that they cleared HBsAg and HBeAg faster during follow-up (Figs. 1 and 2). It is not certain, however, whether or not the observed differences are influenced

by the duration of HBV infection. HBV genotype A is contracted predominantly by men in the adulthood and genotypes B or C had been transmitted perinatally until 1986 when the national immunoprophylaxis started. It needs to be pointed out that this study is retrospective in nature, and most patients with HBeAg had received interferon, lamivudine or steroid withdrawal, or combination thereof. Of the 16 patients with genotype A who received treatment, 11 (69%) responded and cleared HBeAg from serum. In addition, three patients lost HBeAg spontaneously. Hence seroconversion was achieved in 14 of the 19 (74%) patients with genotype A. In view of lamivudine, adefovir dipivoxil, and pegylated interferon that are reported efficacious in treatment of chronic hepatitis B [Perrillo et al., 2000; Hadziyannis et al., 2003; Kumada, 2003; Janssen et al., 2005], it would be unethical to evaluate genotype-dependent differences in the natural course of persistent HBV infection.

Of the 45 individuals chronically infected with HBV genotype A and had been followed for 3 years or longer, HBeAg was more frequent and HBV DNA levels higher in the 26 patients with biopsy-proven chronic hepatitis than in the 19 asymptomatic carriers. Among the 26 patients with genotype A, HBeAg persisted throughout the observation in 5 (19%) and disappeared in 14 (54%); HBeAg remained negative in the other 7 (27%) patients. HBV DNA stayed in high levels more frequently ($P=0.0095$) in the patients with persistent HBeAg (80% [4/5]) than in those who seroconverted (7% [1/14]) or remained HBeAg-negative (29% [2/7]). Furthermore, increases in serum hyaluronic acid ≥ 10 ng/ml were more frequent ($P=0.017$) in the patients with persistent HBeAg (80% [4/5]) than in those with seroconversion (14% [2/17]) or HBeAg-negative (14% [1/7]). Although the patients with genotype A fare better than those with genotype B or C, persistent HBeAg refractory to

treatment would predict ongoing liver disease with fibrosis in progress.

Recently, subgenotypes have been recognized and they may influence the biology of HBV and liver disease. For instance, a subgenotype of B having the recombination with genotype C (Ba) induces more severe liver disease with poorer response to lamivudine than that without the recombination (Bj) [Sugauchi et al., 2002, 2003; Akuta et al., 2003]. As for genotype A, there are two subgenotypes with different geographical distributions. Subgenotype Ae is common in Europe and the United States, while Aa is prevalent in Asia and Africa [Bowyer et al., 1997; Sugauchi et al., 2004]. In a case-control study, HBeAg was more frequent and HBV DNA levels higher in carriers of Ae than Aa [Tanaka et al., 2004]. The majority of genotype A strains from our patients (86%) were found to be Ae; they were probably introduced to Japan by immigrants and visitors from foreign countries [Kobayashi et al., 2004]. Cirrhosis and hepatocellular carcinoma developed in only one of the 19 (5%) patients infected with subgenotype Ae, in remarkable contrast to frequent hepatocellular carcinoma in Africa where infection with subgenotype Aa is common during the infancy [Kew et al., 2005].

Although there have been accumulating lines of evidence for virological and clinical influence of HBV genotypes, there are conflicting views on them. Differences between genotypes B and C in Asia [Kao et al., 2000; Orito et al., 2001; Tsubota et al., 2001; Chan et al., 2004; Yu et al., 2005] have not been reproduced, probably due to selection bias for the patients with severe disease [Sumi et al., 2003] or subgenotypes of B different between Japan (Bj) and Hong Kong (Ba) [Yuen et al., 2004]. Liver disease, once advanced beyond a certain severity, will progress spontaneously irrespective of HBV genotypes. Subgenotype Ba having the recombination with genotype C may be endowed with a higher disease-inducing capacity than subgenotype Bj without the recombination [Sugauchi et al., 2002].

Of patients infected with three different genotypes in Japan, the virological outcome of persistent HBV infection was more favorable for those with genotype A than B and C in that order. It is not known where genotype D stands, although it fares worse than genotype A in chronic HBV infection [Thakur et al., 2002; Janssen et al., 2005]. In ranking the four major genotypes (A–D) in disease-inducing capacity and response to antiviral therapies, perinatals, or adulthood transmission, as well as subgenotypes inherent to countries, would have to be taken into considerations [Sugauchi et al., 2002, 2004; Norder et al., 2004].

REFERENCES

- Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, Someya T, Saitoh S, Arase Y, Ikeda K, Kumada H. 2003. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. *J Hepatol* 38:315–321.
- Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. 2002. Genotype H: A new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 83:2059–2073.

- Bowyer SM, van Staden L, Kew MC, Sim JG. 1997. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* 78:1719–1729.
- Chan HL, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, Sung JJ. 2004. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. *Gut* 53:1494–1498.
- Chen HL, Chang MH, Ni YH, Hsu HY, Lee PI, Lee CY, Chen DS. 1996. Seroepidemiology of hepatitis B virus infection in children: Ten years of mass vaccination in Taiwan. *JAMA* 276:906–908.
- Chu CJ, Hussain M, Lok AS. 2002. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. *Gastroenterology* 122:1756–1762.
- Chu CJ, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, Carey W, Brown RS, Jr., Luketic VA, Terrault N, Lok AS. 2003. Hepatitis B virus genotypes in the United States: Results of a nationwide study. *Gastroenterology* 125:444–451.
- Goilav C, Piot P. 1989. Vaccination against hepatitis B in homosexual men. A review. *Am J Med* 87:S21–S25.
- Gojobori T, Ishii K, Nei M. 1982. Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. *J Mol Evol* 18:414–423.
- Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, Marcellin P, Lim SG, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL. 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. *N Engl J Med* 348:800–807.
- Janssen HL, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y, Simon C, So TM, Gerken G, de Man RA, Niesters HG, Zondervan P, Hansen B, Schalm SW. 2005. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: A randomised trial. *Lancet* 365:123–129.
- Kao JH. 2002. Hepatitis B viral genotypes: Clinical relevance and molecular characteristics. *J Gastroenterol Hepatol* 17:643–650.
- Kao JH, Chen PJ, Lai MY, Chen DS. 2000. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 118:554–559.
- Kato H, Orito E, Sugauchi F, Ueda R, Gish RG, Usuda S, Miyakawa Y, Mizokami M. 2001. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. *J Virol Methods* 98:153–159.
- Kato H, Orito E, Gish RG, Sugauchi F, Suzuki S, Ueda R, Miyakawa Y, Mizokami M. 2002. Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). *J Virol* 76:6131–6137.
- Kato H, Orito E, Sugauchi F, Ueda R, Koshizaka T, Yanaka S, Gish RG, Kurbanov F, Ruzibakiev R, Kramvis A, Kew MC, Ahmad N, Khan M, Usuda S, Miyakawa Y, Mizokami M. 2003. Frequent coinfection with hepatitis B virus strains of distinct genotypes detected by hybridization with type-specific probes immobilized on a solid-phase support. *J Virol Methods* 110:29–35.
- Kato H, Gish RG, Bzowej N, Newsom M, Sugauchi F, Tanaka Y, Kato T, Orito E, Usuda S, Ueda R, Miyakawa Y, Mizokami M. 2004. Eight genotypes (A–H) of hepatitis B virus infecting patients from San Francisco and their demographic, clinical, and virological characteristics. *J Med Virol* 73:516–521.
- Kew MC, Kramvis A, Yu MC, Arakawa K, Hodgkinson J. 2005. Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-saharan Africans. *J Med Virol* 75: 513–521.
- Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Takagi K, Miyakawa Y, Kumada H. 2002. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. *J Med Virol* 68:522–528.
- Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Hosaka T, Saitoh S, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Kumada H. 2003. Clinical features of hepatitis B virus genotype A in Japanese patients. *J Gastroenterol* 38:656–662.
- Kobayashi M, Suzuki F, Arase Y, Akuta N, Suzuki Y, Hosaka T, Saitoh S, Kobayashi M, Tsubota A, Someya T, Ikeda K, Matsuda M, Sato J, Kumada H. 2004. Infection with hepatitis B virus genotype A in Tokyo, Japan during 1976 through 2001. *J Gastroenterol* 39:844–850.
- Kumada H. 2003. Continued lamivudine therapy in patients with chronic hepatitis B. *Intervirology* 46:377–387.
- Lee WM. 1997. Hepatitis B virus infection. *N Engl J Med* 337:1733–1745.

- Magnius LO, Norder H. 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 38:24–34.
- Mayerat C, Mantegani A, Frei PC. 1999. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepat* 6:299–304.
- Miyakawa Y, Mizokami M. 2003. Classifying hepatitis B virus genotypes. *Intervirology* 46:329–338.
- Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. 1992. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* 73:1201–1208.
- Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO. 2004. Genetic diversity of hepatitis B virus strains derived worldwide: Genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 47:289–309.
- Noto H, Terao T, Ryou S, Hirose Y, Yoshida T, Ookubo H, Mito H, Yoshizawa H. 2003. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980–1994. *J Gastroenterol Hepatol* 18:943–949.
- Ogawa M, Hasegawa K, Naritomi T, Torii N, Hayashi N. 2002. Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. *Hepatol Res* 23:167–177.
- Okada K, Kamiyama I, Inomata M, Imai M, Miyakawa Y. 1976. e antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. *N Engl J Med* 294:746–749.
- Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. *J Gen Virol* 69:2575–2583.
- Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. 2001. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. *Hepatology* 33:218–223.
- Perrillo R, Schiff E, Yoshida E, Statler A, Hirsch K, Wright T, Gutfreund K, Lamy P, Murray A. 2000. Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. *Hepatology* 32:129–134.
- Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425.
- Schaefer S. 2005. Hepatitis B virus: Significance of genotypes. *J Viral Hepat* 12:111–124.
- Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. 2000. A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness. *J Gen Virol* 81:67–74.
- Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Ueda R, Miyakawa Y, Mizokami M. 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. *J Virol* 76:5985–5992.
- Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Gish RG, Ueda R, Miyakawa Y, Mizokami M. 2003. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology* 124:925–932.
- Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, Gish RG, Tanaka Y, Kato T, Orito E, Ueda R, Miyakawa Y, Mizokami M. 2004. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J Gen Virol* 85:811–820.
- Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, Kanda T, Fukai K, Kato M, Saisho H. 2003. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 37:19–26.
- Suzuki Y, Kobayashi M, Ikeda K, Suzuki F, Arase Y, Akuta N, Hosaka T, Saitoh S, Kobayashi M, Someya T, Matsuda M, Sato J, Watabiki S, Miyakawa Y, Kumada H. 2005. Persistence of acute infection with hepatitis B virus genotype A and the treatment in Japan. *J Med Virol* 76:33–39.
- Tanaka Y, Hasegawa I, Kato T, Orito E, Hirashima N, Acharya SK, Gish RG, Kramvis A, Kew MC, Yoshihara N, Shrestha SM, Khan M, Miyakawa Y, Mizokami M. 2004. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* 40:747–755.
- Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. 2002. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. *J Gastroenterol Hepatol* 17:165–170.
- Tiollais P, Charnay P, Vyas GN. 1981. Biology of hepatitis B virus. *Science* 213:406–411.
- Tsen YJ, Chang MH, Hsu HY, Lee CY, Sung JL, Chen DS. 1991. Seroprevalence of hepatitis B virus infection in children in Taipei, 1989: Five years after a mass hepatitis B vaccination program. *J Med Virol* 34:96–99.
- Tsubota A, Arase Y, Ren F, Tanaka H, Ikeda K, Kumada H. 2001. Genotype may correlate with liver carcinogenesis and tumor characteristics in cirrhotic patients infected with hepatitis B virus subtype adw. *J Med Virol* 65:257–265.
- Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M. 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J Virol Methods* 80:97–112.
- Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, Mayumi M. 2000. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. *J Virol Methods* 87:81–89.
- Westland C, Delaney Wt, Yang H, Chen SS, Marcellin P, Hadziyannis S, Gish R, Fry J, Brosgart C, Gibbs C, Miller M, Xiong S. 2003. Hepatitis B virus genotypes and virologic response in 694 patients in phase III studies of adefovir dipivoxil. *Gastroenterology* 125:107–116.
- Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, Shih WL, Kao JH, Chen DS, Chen CJ. 2005. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: A prospective study in men. *J Natl Cancer Inst* 97:265–272.
- Yuen MF, Fung SK, Tanaka Y, Kato T, Mizokami M, Yuen JC, Wong DK, Yuan HJ, Sum SM, Chan AO, Wong BC, Lai CL. 2004. Longitudinal study of hepatitis activity and viral replication before and after HBeAg seroconversion in chronic hepatitis B patients infected with genotypes B and C. *J Clin Microbiol* 42:5036–5040.
- Zhang X, Zoulim F, Habersetzer F, Xiong S, Trepo C. 1996. Analysis of hepatitis B virus genotypes and pre-core region variability during interferon treatment of HBe antigen negative chronic hepatitis B. *J Med Virol* 48:8–16.

Changes in Viral Loads of Lamivudine-Resistant Mutants and Evolution of HBV Sequences During Adefovir Dipivoxil Therapy

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The addition of adefovir dipivoxil (ADV) to ongoing lamivudine therapy is effective against lamivudine-resistant virus in patients with hepatitis B virus (HBV) infection. We studied 39 patients who received ADV added to lamivudine for breakthrough hepatitis. We determined early viral changes (12 weeks) in YMDD mutants (rtM204I [YIDD sequence], rtM204V [YVDD]) and rtL180M in all 39 patients as well as amino acid changes in the polymerase reverse transcriptase (rt) region and precore/core promoter mutations in 15 patients who received long-term treatment (more than 1 year). Changes in rtM204I and rtL180M viral loads were greater than that of the rtM204V, albeit statistically insignificant. Moreover, the greatest change in viral load was seen for rtM204I without hepatitis B e antigen (HBeAg). The precore mutant was replaced with wild-type virus in three of eight patients after 1 year of added ADV therapy. Compared to baseline with lamivudine therapy only, new amino acid mutations were seen in the rt region at baseline with ADV in seven patients. At 1 year after ADV coadministration, the YMDD motif was replaced with wild-type (rt204M) in two patients, in whom mutations were fewer and of a different type. We conclude that the rtM204I may be more sensitive to ADV in vivo. ADV tended to select wild-type virus from precore mutants. Moreover, viruses that were wild-type in the rt region reappeared after 1 year of ADV coadministration in some patients. *J. Med. Virol.* 78:1025–1034, 2006. © 2006 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus; breakthrough hepatitis; YMDD mutant; precore; core promoter; reverse transcriptase

INTRODUCTION

The goal of therapy in patients with hepatitis B virus (HBV) is to limit or reverse progression of the disease through the sustained suppression of HBV replication [Conjeevaram and Lok, 2003]. Several studies have reported that various nucleoside analogues such as lamivudine are effective in suppressing HBV replication, improving transaminase levels and liver histology, and enhancing the rate of loss of hepatitis B e antigen (HBeAg) [Dienstag et al., 1995, 1999; Lai et al., 1998; Suzuki et al., 1999]. A major problem with the long-term use of lamivudine, however, is its potential to induce viral resistance, with associated increases in HBV DNA and serum transaminases [Honkoop et al., 1997; Chayama et al., 1998; Suzuki et al., 2003].

Adefovir dipivoxil (ADV) is a potent suppressor of both wild-type and lamivudine-resistant HBV in vitro and a suppressor of wild-type HBV in vivo [Hadziyannis et al., 2003; Marcellin et al., 2003]. Clinical trials to date show that the addition of ADV to ongoing lamivudine therapy in lamivudine-resistant patients, or its administration as monotherapy, produces virologic and biochemical improvements [Perrillo et al., 2000, 2004; Hosaka et al., 2004; Peters et al., 2004].

Recently, a rapid, highly sensitive and reproducible method for quantifying mutant HBV virus in lamivudine-treated patients was reported [Punia et al., 2004]. Using a real-time polymerase chain reaction (PCR; LightCycler) with a ResonSense probe, this method detects as little as 0.01% of YMDD mutant DNA among

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10^5 – 10^9 copies of wild-type DNA. However, there are few reports of changes in YMDD mutant (rtM204I [YIDD sequence], rtM204V [YVDD]) viral loads during ADV treatment added to ongoing lamivudine therapy [Punia et al., 2004].

During the course of chronic HBV infection, natural seroconversion to antibody to HBeAg (anti-HBe) usually correlates with the resolution of viremia and clinical recovery. Mutation in the precore region (nucleotide [nt] 1896) is related to the absence of HBeAg secretion [Carman et al., 1989] and may enhance the stability of the secondary structure of pregenome encapsidation signals, ensuring perpetuation of viral replication and thus contributing to viral persistence [Lok et al., 1994]. Buckword et al. [1996] showed that HBV genome carrying core promoter mutations (nt G1762A and A1764T) influenced viral replication. Cho et al. [2000] and our group [Suzuki et al., 2002] reported that lamivudine therapy resulted in reversion from precore and core promoter mutants to wild-type, but that these mutants reappeared during long-term therapy. However, it is not clear at this stage how ADV influences precore and core promoter mutants of lamivudine-resistant virus.

Analysis of mutations of the reverse transcriptase (rt) domain of HBV polymerase in patients who had received long-term (48 or 60 weeks) ADV monotherapy revealed the presence of several amino acid substitutions [Yang et al., 2002; Westland et al., 2003]. Other studies showed that selection of the rtN236T polymerase mutant is associated with resistance to ADV [Angus et al., 2003; Villeneuve et al., 2003]. Further elucidation of this process requires the analysis of amino acid substitutions during coadministration of ADV with ongoing lamivudine therapy for lamivudine-resistant virus.

The aims of this prospective study were (1) to determine changes in YMDD mutant (rtM204I, rtM204V) and rtL180M viral loads during coadministration of ADV with ongoing lamivudine therapy in

patients with HBV, and (2) to determine viral polymerase (rt region), precore and core promoter mutants during treatment with ADV by analyzing serial serum samples from patients with lamivudine resistance.

PATIENTS AND METHODS

Patients

The subjects were 39 consecutive adult Japanese patients who commenced ADV treatment between November 2002 and June 2004 at the Department of Gastroenterology, Toranomon Hospital. At entry, all 39 patients were being treated with lamivudine for chronic HBV infection when the emergence of YMDD motif mutations indicated the development of breakthrough hepatitis. They had not received other nucleoside analogue drugs before lamivudine and were therefore treated by the addition of ADV to the ongoing lamivudine therapy (Group 1). Moreover, viral load data for another group of nine patients who were previously cotreated with interferon (IFN) in addition to ongoing lamivudine against breakthrough hepatitis (before ADV therapy was instituted in Japan) were compared with the viral load of patients treated with ADV (Group 2). These nine patients received IFN therapy daily for 4 weeks, and then three times a week for 20 weeks (Table I). All patients were negative for hepatitis C serologic markers. Lamivudine and ADV were administered orally at 100 and 10 mg/day, respectively. Chronic hepatitis or cirrhosis was confirmed before lamivudine treatment by peritoneoscopy and/or needle biopsy ($n = 23$), or clinical features ($n = 16$) [Suzuki et al., 2003].

Blood Tests and Serum Viral Markers

Routine biochemical tests were performed using standard procedures before and during therapy at least once each month. HBsAg, HBeAg, and anti-HBe were determined by radioimmunoassay kits (Abbot Diagnostics, Chicago, IL) according to the instructions provided by

TABLE I. Patient Characteristics at the Start of Therapy for Lamivudine Breakthrough Hepatitis

	ADV (Group 1)	IFN (Group 2)
Total number	39	9
Sex (female/male)	4/35	2/7
Age (years) ^a	48 (26–58)	46 (23–56)
Aspartate aminotransferase (IU/L) ^a	118 (37–478)	158 (42–495)
Alanine aminotransferase (IU/L) ^a	188 (24–858)	234 (72–727)
Bilirubin (mg/dl) ^a	0.8 (0.3–13.7)	0.8 (0.2–2.3)
Albumin (g/dl) ^a	3.7 (2.6–4.5)	3.9 (3.4–4.3)
Liver histology (CH/LC) ^b	23/16	7/2
Serum HBV DNA ^c (Amplicor: log copy/ml) ^a	7.3 (4.4–> 7.6)	>7.6 (5.9–> 7.6)
HBeAg (positive/negative)	24/15	7/2
HBV genotype (A/B/C)	2/3/34	0/0/9

^aData are median (range).

^bLiver histology: CH, chronic hepatitis; LC, liver cirrhosis.

^cHBV DNA levels were measured by Amplicor HBV Monitor assay. HBV DNA values below the lower limit of detection are listed as 2.6 Log copy/ml and those over the upper limit of detection as 7.6 Log copy/ml. For statistical analysis, all HBV DNA values over the upper limit of detection (>7.6 Log copy/ml) were set to 8.0.

the manufacturer. Serum HBV DNA was quantified using the Roche Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN), a PCR-based assay with a lower limit of detection of 400 copies of HBV DNA/ml (2.6 Log copy/ml).

Quantitation of Lamivudine-Resistant Mutants by Real-Time Amplification Refractory Mutation System (ARMS) PCR

DNA was extracted from 100 μ l of serum. The assay was performed using a sensitive, real-time PCR-based assay for the detection of lamivudine resistance-associated mutations in the presence of high levels of wild-type virus, as reported recently [Punia et al., 2004]. Briefly, this method is based on ARMS PCR for the detection of single base mutations [Newton et al., 1989] and uses the same ARMS primers, reactions, and cycling conditions on the LightCycler. To prepare the standards (rt204M, rtM204I, and rtM204V), the first PCR product amplified using primers P1 and P2, as reported previously [Günther et al., 1995], was cloned into the plasmid vector pBluescript (Stratagene, La Jolla, CA). The concentration of purified plasmids was based on absorbance at 260 nm (GeneQuant II; Amersham Pharmacia Biotech, Tokyo, Japan). The standards for real-time PCR were prepared by serial dilution of a plasmid of known concentration. DNA values of those mutants below the lower limit of detection were expressed as 2.0 Log copy and those over the upper limit of detection as 9.0 Log copy. Selectivity of this assay was tested as described previously [Punia et al., 2004] using reactions containing 10^9 copies of wild-type DNA (rt204M) template and from 10^9 to 0 copies of mutant virus (rtM204I or rtM204V) template. Under these conditions, the mutant primers (for rtM204I and rtM204V) detected the number of copies of mutant template present within the range of 10^9 – 10^4 copies. Moreover, one primer (rtM204I or rtM204V) detected the number of copies of mutant template present within the range of 10^9 – 10^4 copies (mixed with 10^9 copies of the other mutant virus [rtM204V DNA or rtM204I DNA], respectively). The detection limit for mutation of rtL180 (rtL180M) was the same. Total HBV DNA levels were measured by real-time PCR as described previously [Punia et al., 2004]. Serum samples were assayed at five time points, namely before (baseline) and at 2, 4, 8, and 12 weeks after the start of coadministration of ADV with ongoing lamivudine therapy. Moreover, in some patients, serum samples were also assayed at two other time points; at 24 and 52 weeks. Data for the time-dependent decline in viral load relative to baseline were log transformed, and thus all results for quantitative HBV level are expressed as Log₁₀ copy.

Determination of Nucleotide Sequences of HBV DNA

We determined the nucleotide sequences of HBV DNA of the initial 15 patients who received ADV treatment. Among these 15 patients, 4 received combination

therapy of lamivudine, ADV, and IFN because of severe hepatitis. The remaining 11 patients were cotreated with ADV in addition to the ongoing lamivudine therapy and belonged to Group 1. DNA was extracted from 100 μ l of serum. PCR reactions for detection of the rt region (nt 130–1161) of HBV DNA were performed in two parts. The first and second PCR reactions for detection of the first part of the rt region were performed using primers BGF1 (sense: 5'-CTGTGGAAGGCTGGCATTCT-3') and BGR2 (antisense: 5'-GGCAGGATAGCCGCATTGTG-3'), and PreSBamH1 (sense: 5'-CTTGGGATCCAGAGC-TACAGCATGG-3') and BR112 (antisense: 5'-TTCCGTCGACATATCCCATGAAGTTAAGGGA-3'), respectively, under conditions of initial denaturation for 4 min, 35 cycles of amplification with 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and final extension at 72°C for 7 min. The first and second PCR reactions for detection of the second part of the same region were performed using primer pairs B11F (sense: 5'-GGCCAAGTCTGTACAACATC-3') and B12R (antisense: 5'-TGCA-GAGGTGAAGCGAAGTG-3'), and B11F and B14R (antisense: 5'-GATCCAGTTGGCAGCACACC-3'), respectively, under the same conditions. The amplified PCR products were used for direct sequencing. Measurement of sequences in the rt region was performed at three time points; at the start of lamivudine, start of ADV, and 1 year after the start of ADV therapy. Nucleotide sequences of the core promoter and precore regions were determined as described previously [Suzuki et al., 2002], with measurements taken at the same three time points. All HBV genomes analyzed in detail by sequencing were found to be of genotype C. All sequence alignments were performed in comparison with genotype C wild-type sequences (accession no. AB014378, AB014394, AB033550, AB033551, AB033556, AB042283).

Mutation of the HBV DNA polymerase gene (rtM204I/V) was determined using PCR and restriction fragment length polymorphism (PCR-RFLP) as described previously [Chayama et al., 1998].

Statistical Analysis

Differences between groups were examined for statistical significance using the χ^2 and Mann–Whitney test (*U*-test) where appropriate. A two-tailed *P*-value less than 0.05 was considered significant.

RESULTS

Changes in Viral Loads of Lamivudine-Resistant Mutants During ADV Therapy

Changes in rtM204I, rtM204V, and rtL180M viral loads were measured in all 39 patients. At the start of ADV coadministration, the number of patients with detectable rtM204I alone, rtM204V alone and mixed-type (rtM204I and rtM204V) among the 39 patients was 17, 4, and 18, respectively. Viral load of rtL180M was detected in 36 patients. Figure 1 shows the median log changes from baseline in rtM204I, rtM204V, and

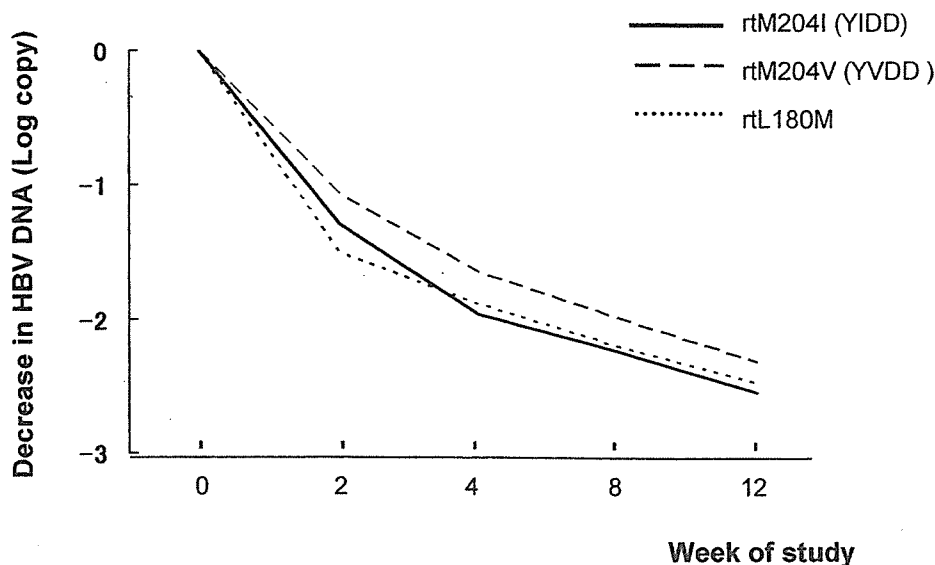


Fig. 1. Median log changes in rtM204I, rtM204V, and rtL180M viral loads from baseline during the initial 12 weeks of coadministration of ADV added to ongoing lamivudine therapy. HBV DNA levels of rtM204I, rtM204V, and rtL180M were measured by real-time PCR. rt, HBV polymerase reverse transcriptase.

rtL180M viral loads during the initial 12 weeks of ADV and lamivudine coadministration. The changes in viral load of rtM204I and rtL180M were greater than that of rtM204V, although the difference was not statistically significant. The rate of decrease of all mutants at 12 weeks was about one-hundredth (1/100) that at baseline. Moreover, the change of viral load of HBV DNA in HBeAg-negative patients was greater than that in HBeAg-positive patients at 12 weeks (median log changes in viral load; HBeAg-positive vs. -negative = -2.14 : -2.71 ; $P=0.077$). The numbers of rtM204I and rtM204V with HBeAg, and rtM204I and rtM204V without HBeAg were 24, 13, 11, and 9,

respectively. The change of viral load of rtM204I without HBeAg was the greatest among the groups.

Among the nine patients coadministered IFN with ongoing lamivudine therapy, rtM204I only was detected in three and mixed-type was detected in six patients. Viral load of rtL180M was detected in eight patients. Log changes in rtM204I, rtM204V, and rtL180M viral loads under IFN coadministration are shown in Figure 2. The log viral load change for the rtM204V was greater than that for the rtM204I, although the difference was not statistically significant.

Normalization of alanine aminotransferase (ALT) level at 1 year was noted in 35 of 39 patients of Group

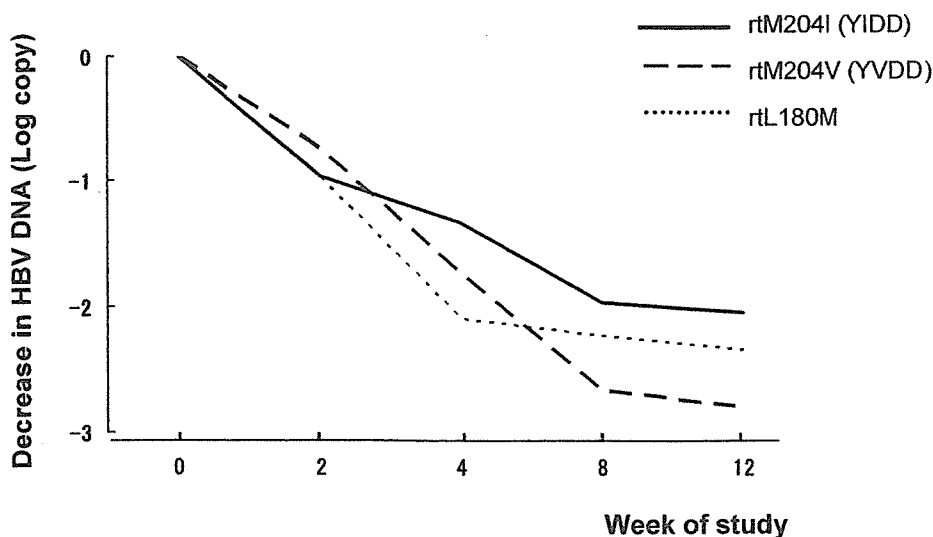


Fig. 2. Median log changes in rtM204I, rtM204V, and rtL180M viral loads from baseline and during the initial 12 weeks of coadministration of IFN added to ongoing lamivudine therapy. HBV DNA levels of rtM204I, rtM204V, and rtL180M were measured by real-time PCR.

1. Moreover, HBV DNA levels in 11 of 39 patients of Group 1 were more than 2.6 Log copy/ml by Amplicor HBV Monitor assay at 52 weeks. Those 11 patients were persistently HBeAg-positive and had mutant viral loads that were over 10^6 copies at the commencement of ADV and lamivudine coadministration. The number of

patients with detectable rtM204I alone and mixed-type (rtM204I and rtM204V) was five and six, respectively. The rtM204I and rtM204V viral loads in these 11 patients were also measured at 24 and 52 weeks (Fig. 3). Viral loads of five patients with rtM204I alone gradually decreased but were still detectable at 52 weeks

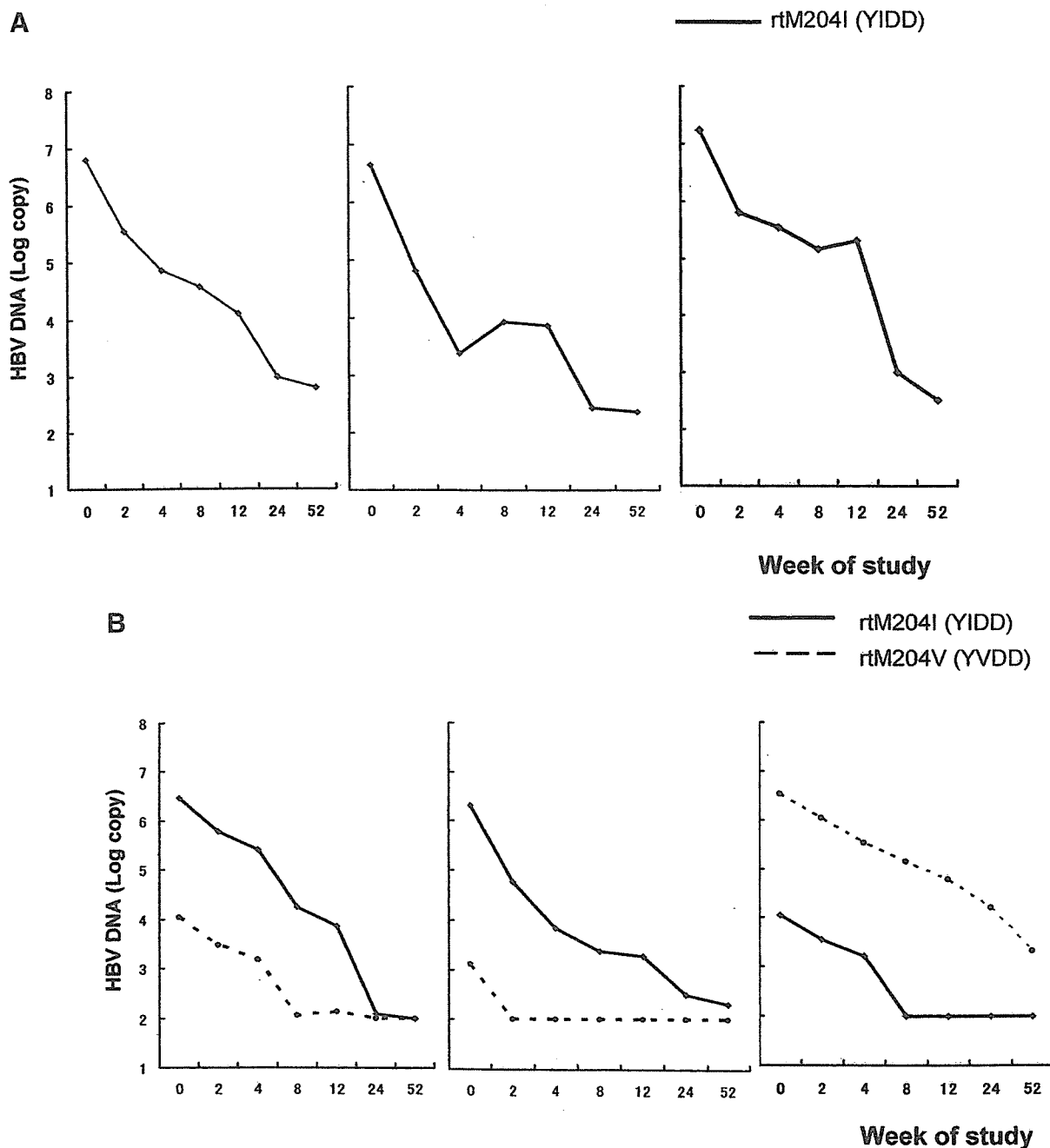


Fig. 3. Changes in rtM204I and/or rtM204V viral loads in nine patients with HBV DNA levels of >2.6 Log copy/ml as determined by Amplicor HBV Monitor assay at 52 weeks during ADV and lamivudine coadministration. HBV DNA levels of rtM204I and rtM204V were measured by real-time PCR. **A:** Viral loads in three of the five patients with rtM204I alone at commencement of ADV plus lamivudine combination therapy. Similar changes in viral loads were noted in the other two patients. Viral loads of these five patients with rtM204I alone

gradually decreased but were still detectable at 52 weeks. **B:** Viral loads in three patients in whom either rtM204I or rtM204V was the major mutant (viral load of major mutant was over 10-fold that of minor mutant). Only the major mutant was detected at 52 weeks. **C:** Viral loads in three patients with two similar mutants (viral load of major mutant was within 10-fold that of minor mutant). Mutant rtM204V predominated over rtM204I at 52 weeks.

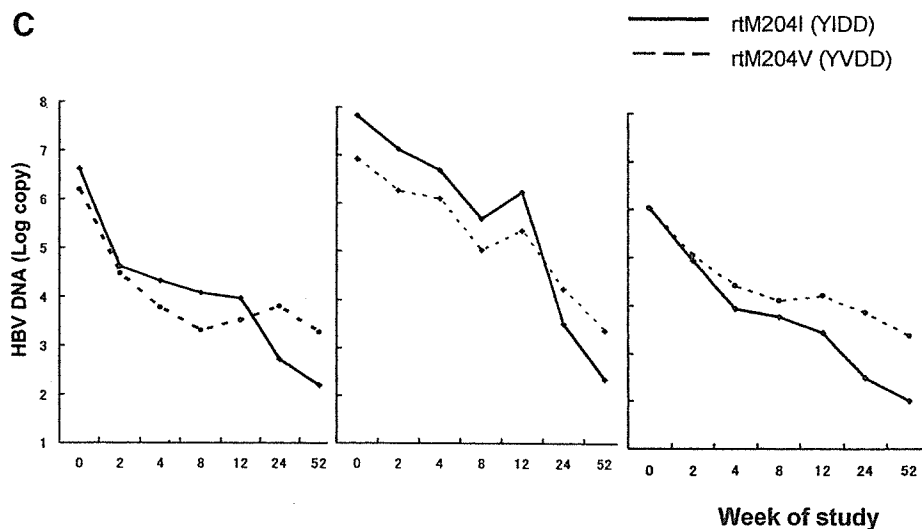


Fig. 3. (Continued)

(Fig. 3A). In three patients where the viral load of either rtM204I or rtM204V was major (i.e., viral load of the major mutant was more than 10-fold that of the minor mutant) at commencement of therapy, only the major mutant was detected at 52 weeks (Fig. 3B). However, in three patients where the viral loads of both mutants were similar (i.e., viral load of major mutant was within 10-fold that of minor mutant) at commencement of therapy, rtM204V predominated over rtM204I at 52 weeks (Fig. 3C).

Changes in Precore and Core Promoter Sequences Before and During Therapy

Precore and core promoter sequences in 15 patients were analyzed over 1 year of treatment with coadministered ADV in addition to ongoing lamivudine therapy for lamivudine breakthrough hepatitis. There was no clinical or virological evidence of breakthrough during lamivudine and ADV combination treatment in all 15 patients on ongoing lamivudine therapy. Precore sequences at baseline for lamivudine were the same as those at baseline for ADV in 9 of 10 patients (excluding 5 lacking lamivudine baseline data) (Table II). Analysis of serum samples obtained at ADV baseline revealed a precore stop codon mutation (A1896) in 9 of 15 patients, among whom A1896 occurred as a mixed population with wild-type virus (G1896) in 6 and as a pure population in 3 patients. After coadministration of ADV, A1896 was replaced with wild-type virus in three patients at 1 year and with mixed-type virus in one patient. In particular, among five patients without HBeAg at 1 year, including 2 HBeAg-seronegative patients, A1896 was replaced with wild-type virus in two and by mixed-type virus in one patient. Thus, A1896 was observed in three of eight patients, excluding seven PCR-negative patients, at 1 year.

The core promoter sequences at baseline for lamivudine therapy were the same as those at baseline for ADV

in 9 of 10 patients (Table II). Among 15 patients, all had core promoter mutations in samples collected at baseline for ADV therapy. During treatment, the core promoter mutations were replaced with the wild-type in one patient (Patient 6) at 1 year. In this patient, a precore stop codon mutation was also replaced with a wild-type sequence.

Changes in Viral Sequences of Polymerase Reverse Transcriptase Before and During ADV Therapy

Hepatitis B virus DNA levels in all 15 patients coadministered ADV during ongoing lamivudine therapy were below 3,000 copy/ml at 1 year. Analysis of the rt region sequences (amino acid 1–344) of HBV polymerase in seven patients, excluding eight patients who were PCR-negative after ADV for 1 year, showed amino acid substitutions in the rt region in all seven (Fig. 4). Compared with baseline for lamivudine, there were new substitutions at baseline for ADV in all patients. Substitutions after 1 year of ADV, however, were very similar to those at ADV baseline in five patients (Patients 2, 3, 4, 7, 9). Interestingly, the YMDD motif in two patients (Patients 5 and 6) was replaced with wild-type (rt204M/YMDD) after 1 year of ADV. Substitutions in these two patients were fewer and of a different type than those at ADV baseline. Furthermore, Amplicor HBV Monitor assay showed that their HBV DNA levels were negative at 12 weeks after the start of ADV and fell to a greater extent than those of the other patients. This finding suggests that ADV may suppress YMDD mutants more than wild-type virus in some patients.

DISCUSSION

Mutations leading to lamivudine resistance are generally detected by conventional DNA sequencing after PCR amplification of a selected portion of the viral

TABLE II. Serial Precore and Core Promoter Sequences of Patients Treated With Lamivudine and Adefovir Dipivoxil

Patient	Genotype	Lamivudine				Adefovir dipivoxil				1 Year					
		Baseline		CP nt		Baseline		CP nt		Baseline		CP nt			
		YMDD motif	Precore nt 1896	1762	1764	YMDD motif	Precore nt 1896	1762	1764	YMDD Motif	Precore nt 1896	1762	1764		
1	C	ND	ND	ND	ND	+	I+V	G/A	T	A	+	I	A	T	A
2	C	+	G	A	A	+	I+V	G	T	A	+	I+V	G	T	A
3 ^a	C	+	G/A	A	A	+	I+V	G/A	T	A	-	V	G	T	A
4	C	+	G/A	A	A	+	I+V	G/A	T	A	+	I+V	G	T	A
5	C	+	G	A	T	+	I+V	G	T	A	+	M	G/A	T	A
6 ^a	C	-	G/A	A	T	-	I+V	G/A	T	A	-	M	G	A	G
7	C	-	G	A	T	-	I	A	T	A	-	I	G/A	T	A
8	C	+	ND	ND	ND	+	I+V	G	T	A	+	V	N	N	N
9	C	+	G	A	T	+	I	G	T	A	+	I	N	N	N
10	A	-	G	A	T	-	V	G	T	A	-	N	G	T	A
11	C	ND	ND	ND	ND	+	I+V	G/A	T	A	+	N	N	N	N
12	C	ND	ND	ND	ND	+	I+V	G	T	A	+	N	N	N	N
13 ^a	C	+	G/A	A/T	G/A	+	I	G/A	T	A	+	N	N	N	N
14 ^a	C	+	A	T	A	-	I	A	T	A	-	N	N	N	N
15	C	-	ND	ND	ND	-	I+V	A	T	A	-	N	N	N	N

Baseline, time of the beginning of therapy; PCR, core promoter; ND, not done; N, PCR-negative; eAg, HBeAg; YMDD motif, M, rt204M; I, rtM204I; V, rtM204V; I+V, mixed type (rtM204I + rtM204V).

^aReceived lamivudine, adefovir dipivoxil, and interferon therapy.

aa.1		Reverse transcriptase										344	
		YMDD motif											
Pat. 2	(1)	I16T	S78T	D134E								V214A	
	(2)	N13Y	I16T	H55R	L80I							M204I	F221Y
	(3)	N13Y	I16T	H55R	L80I							M204I	F221Y
Pat. 3	(1)												H337N
	(2)			V84M	K154N	L180M	V191I					M204V	H337N
	(3)		G52E	V84M	K154N	L180M	V191I					M204V	H337N
Pat. 4	(1)					N139Q	Y141F	V142I	L145M				H337N
	(2)				L80V	N139Q	Y141F	V142I	L145M			M204I	H337N
	(3)				L80V	N139Q	Y141F	V142I	L145M			M204I	H337N
Pat. 5	(1)							F195I					
	(2)				L80I	F151L	L180M					M204I	
	(3)					S106C							S256C H337N
Pat. 6	(1)			T118A	D134N								C303W H337N
	(2)			A96V	T118A		L180M					M204V	S219A L229F H337N
	(3)												N238H
Pat. 7	(1)	T7A											P325S
	(2)	T7A		H55R	S106C							M204I	T222A S223A P325S
	(3)	T7A		H55R	S106C							M204I	
Pat. 9	(1)			S78T									H337N
	(2)			L80I								M204I	H337N
	(3)			L80I								M204I	I265M H337N

Fig. 4. Changes in viral sequences of polymerase reverse transcriptase before and during therapy. Measurements were taken at three time points: (1) start of lamivudine therapy, (2) start of coadministration of ADV with ongoing lamivudine therapy against breakthrough hepatitis, and (3) after coadministration with ADV for 1 year. Patient numbers are the same as in Table II. L180M denotes the substitution of leucine with methionine at amino acid position 180 in the reverse transcriptase region of HBV polymerase.

polymerase gene. The sensitivity of sequencing for minority quasispecies is low, however, with detection in most cases limited to no more than 20% of the total viral population [Gunthard et al., 1999]. Other molecular techniques developed to detect changes associated with lamivudine resistance include PCR-RFLP, a 5' nuclease assay, and line probe assay technology [Chayama et al., 1998; Stuyver et al., 2001; Whalley et al., 2001].

Punia et al. [2004] first reported that rtM204I, rtM204V, and rt180M viral loads could be measured by real-time ARMS-PCR. However, their report included data from only a few cases. Here, we measured sequential viral loads of mutants during coadministration of ADV in addition to established treatment with lamivudine and showed that the viral loads of rtM204I, especially without HBeAg, decreased at the most rapid rate. This finding indicates that ADV therapy has a more suppressive effect against rtM204I. Moreover, when viral loads of both mutants (rtM204I and rtM204V) were similar at commencement of ADV therapy in patients with mixed-type virus, rtM204V predominated over rtM204I at 52 weeks. Considering these findings, the rtM204I may be more sensitive to ADV in vivo. On the other hand, it was reported that ADV was an equally effective inhibitor of rtM204I and rtM204V replication in vitro, and suppressed the

rtL180M to an even greater extent [Chin et al., 2001; Ono et al., 2001]. With respect to the effectiveness of ADV against rtM204I and rtM204V, our data (in vivo) differ from that of previous studies (in vitro). Moreover, suppression of the rt180M was linked to that of the rtM204I or rtM204V and the rt180M viral load was influenced by those of rtM204I or rtM204V in vivo. However, it is not clear why ADV was apparently more effective against the rtM204I in vivo, and further studies are necessary to investigate this issue.

On the other hand, the log viral load change for rtM204V was greater than that for rtM204I during IFN coadministration with ongoing lamivudine, although the difference was not statistically significant. However, the number of patients undergoing IFN therapy was small and further studies of larger population samples are necessary to confirm this finding. On the other hand, our previous study showed that the suppression of lamivudine-resistant virus by long-term IFN + lamivudine therapy was clinically insufficient, with only 38% of patients achieving negative HBV DNA status [by branched DNA assay] after 6 months of IFN (unpublished data). On this basis, the long-term clinical efficacy of ADV added to ongoing lamivudine therapy is apparently superior to that of IFN coadministration.

During lamivudine therapy, precore mutants tend to be replaced with wild-type virus at 1 year, and this

change is unrelated to the emergence of YMDD motif mutations [Cho et al., 2000; Suzuki et al., 2002]. On the other hand, patients who showed mutations in the YMDD motif during long-term lamivudine therapy also exhibited the reappearance of precore mutants [Suzuki et al., 2002]. However, the addition of ADV to ongoing lamivudine therapy appeared to result in the preferential selection of wild-type virus, similar to the case of initial lamivudine therapy, although only a few cases were tested. This finding suggests that antiviral nucleoside analogues, such as lamivudine and ADV, selectively suppress precore mutants over wild-type virus. On the other hand, core promoter mutations at 1 year were replaced with wild-type in only one patient (Patient 6). It has been reported that core promoter mutations during lamivudine therapy also tended to be replaced at 1 year by wild-type virus [Cho et al., 2000; Suzuki et al., 2002], and more recently that three of five seroconverters of HBeAg harbored core promoter mutations at baseline that were progressively replaced with wild-type genome during ADV monotherapy [Werle et al., 2004]. However, our present study showed that, compared to initial lamivudine therapy or ADV monotherapy, coadministration of ADV with ongoing lamivudine therapy might be less effective against core promoter mutants than wild-type virus.

With regard to ADV monotherapy, several mutations in the HBV polymerase rt region have been observed during this treatment [Yang et al., 2002; Westland et al., 2003]. Moreover, selection of the rtN236T polymerase mutant was associated with resistance to ADV [Angus et al., 2003; Villeneuve et al., 2003]. Few data are available on sequencing of the rt region during coadministration of ADV with ongoing lamivudine therapy. Mutations after 1 year of coadministration of ADV and lamivudine were very similar to those at coadministration baseline. However, the YMDD motif mutation in two patients was replaced with wild-type (rt204M) at 1 year after coadministration, and another mutation pattern within the rt region was also changed. Moreover, in Patient 6, precore and core promoter mutations were replaced with wild-type at 1 year after coadministration. These findings suggest that ADV may selectively suppress lamivudine-resistant virus, and that wild-type virus may predominate in patients in whom drug efficacy is high, although the status of the rt region in eight patients whose PCR was negative at 1 year could not be ascertained.

In conclusion, we analyzed changes in rtM204I, rtM204V, and rtL180M viral loads in patients with HBV cotreated with lamivudine and ADV for lamivudine-resistant virus. The changes in rtM204I and rtL180M viral loads were greater than that of rtM204V, although the difference was not statistically significant. This finding was also clarified by analysis of serial changes in rtM204I and rtM204V viral loads. Moreover, the change in rtM204I viral load without HBeAg was greatest. Precore wild-type virus was apparently preferentially selected by the coadministration of ADV with lamivudine, in the same way that it was by initial

lamivudine therapy at 1 year. Moreover, analysis of the rt region showed that ADV may suppress lamivudine-resistant virus and that wild-type virus may predominate. A better efficacy of ADV was noted against HBeAg-negative (and/or precore mutant) and lamivudine-resistant virus. Further studies are necessary to correlate virological changes and clinical efficacy during longer coadministration of ADV with ongoing lamivudine therapy for lamivudine-resistant virus.

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REFERENCES

- Angus P, Vaughan R, Xiong S, Yang H, Delaney W, Gibbs C, Brosgart C, Colledge D, Edwards R, Ayres A, Bartholomeusz A, Locarnini S. 2003. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. *Gastroenterology* 125:292–297.
- Buckword VE, Xu Z, Chen M, Yen TS, Ou JH. 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on pre-core gene expression and viral replication. *J Virol* 70:5845–5851.
- Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. 1989. Mutation preventing formation of e antigen in patients with chronic HBV infection. *Lancet* ii:588–591.
- Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Murashima N, Ikeda K, Kumada H. 1998. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term Lamivudine therapy and re-takeover by wild type after cessation of therapy. *Hepatology* 27:1711–1716.
- Chin R, Shaw T, Torresi J, Sozzi V, Trautwein C, Bock T, Manns M, Isom H, Furman P, Locarnini S. 2001. In vitro susceptibilities of wild-type or drug-resistant hepatitis B virus to (–)-β-D-2,6-Diaminopurine dioxolane and 2'-Fluoro-5-Methyl-β-Arabinofuranosyluracil. *Antimicrob Agents Chemother* 45:2495–2501.
- Cho SW, Hahn K-B, Kim JH. 2000. Reversion from precore/core promoter mutants to wild-type hepatitis B virus during the course of lamivudine therapy. *Hepatology* 32:1163–1169.
- Conjeevaram HS, Lok ASF. 2003. Management of chronic hepatitis B. *J Hepatol* 38:S90–S103.
- Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. 1995. A preliminary trial of lamivudine for chronic hepatitis B infection. *N Engl J Med* 333:1657–1661.
- Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HWL, Goodman Z, Crowther L, Condrey LD, Woessner M, Rubin M, Brown NA, The U.S. Lamivudine Investigator Group. 1999. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 341:1256–1263.
- Gunthard HF, Frost SD, Leigh-Brown AJ, Ignacio CC, Kee K, Perelson AS, Spina CA, Havlir DV, Hezareh M, Looney DJ, Richman DD, Wong JK. 1999. Evolution of envelope sequences of human immunodeficiency virus type 1 in cellular reservoirs in the setting of potent antiviral therapy. *J Virol* 73:9404–9412.
- Günther S, Li BC, Miska S, Krüger DH, Meisel H, Will H. 1995. A novel method for efficient amplification of whole hepatitis B virus genomes permit rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* 69:5437–5444.
- Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, Marcellin P, Lim SG, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL, Adefovir Dipivoxil 438 Study Group. 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. *N Engl J Med* 348:800–807.
- Honkoop P, Niesters HG, de Man RA, Osterhaus AD, Schalm SW. 1997. Lamivudine resistance in immunocompetent chronic hepatitis B. Incidence and patterns. *J Hepatol* 26:1393–1395.

- Hosaka T, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, Someya T, Sezaki H, Akuta N, Tsubota A, Arase Y, Ikeda K, Kumada H. 2004. Adefovir dipivoxil for treatment of breakthrough hepatitis caused by lamivudine-resistant mutants of hepatitis B virus. *Intervirology* 47:362–369.
- Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF, The Asia Hepatitis Lamivudine Study Group. 1998. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 339:61–68.
- Lok ASF, Akarca US, Greene S. 1994. Mutations in pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pregenome encapsidation signal. *Proc Natl Acad Sci USA* 91:4077–4081.
- Marcellin P, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, Jeffers L, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL, Adefovir Dipivoxil 437 Study Group. 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 348:808–816.
- Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF. 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res* 17:2503–2516.
- Ono SK, Kato N, Shiratori Y, Kato J, Goto T, Schinazi RF, Carrilho FJ, Omata M. 2001. The polymerase L528M mutation correlates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. *J Clin Invest* 107:449–455.
- Perrillo R, Schiff E, Yoshida E, Statler A, Hirsch K, Wright T, Gutfreund K, Lamy P, Murray A. 2000. Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. *Hepatology* 32:129–134.
- Perrillo R, Hann HW, Mutimer D, Willems B, Leung N, Lee WM, Moorat A, Gardner S, Woessner M, Bourne E, Brosgart CL, Schiff E. 2004. Adefovir dipivoxil added to ongoing lamivudine in chronic hepatitis B with YMDD mutant hepatitis B virus. *Gastroenterology* 126:81–90.
- Peters MG, Hann HW, Martin P, Heathcote EJ, Buggisch P, Rubin R, Bourliere M, Kowdley K, Trepo C, Gray DF, Sullivan M, Kleber K, Ebrahimi R, Xiong S, Brosgart CL. 2004. Adefovir dipivoxil alone or in combination with lamivudine in patients with lamivudine-resistant chronic hepatitis B. *Gastroenterology* 126:91–101.
- Punia P, Cane P, Teo CG, Saunders N. 2004. Quantitation of hepatitis B lamivudine resistant mutants by real-time amplification refractory mutation system PCR. *J Hepatol* 40:986–992.
- Stuyver L, Van Geyt C, De Gendt S, Van Reybroeck G, Zoulim F, Leroux-Roels G, Rossau R. 2000. Line probe assay for monitoring drug resistance in hepatitis B virus-infected patients during antiviral therapy. *J Clin Microbiol* 38:702–707.
- Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, Tsubota A, Kobayashi M, Koike M, Ogawa N, Tanikawa K. 1999. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. *J Hepatol* 30:743–748.
- Suzuki F, Suzuki Y, Tsubota A, Akuta N, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2002. Mutations of polymerase, precore and core promoter gene in hepatitis B virus during 5-year lamivudine therapy. *J Hepatol* 37:824–830.
- Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Hosaka T, Kobayashi M, Saitoh S, Ikeda K, Kobayashi M, Matsuda M, Satoh J, Takagi K, Kumada H. 2003. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. *Intervirology* 46:182–189.
- Villeneuve JP, Durantel D, Durantel S, Westland C, Xiong S, Brosgart CL, Gibbs CS, Parvaz P, Werle B, Trepo C, Zoulim F. 2003. Selection of a hepatitis B virus strain resistant to adefovir in a liver transplantation patient. *J Hepatol* 39:1085–1089.
- Werle B, Cinquin K, Marcellin P, Pol S, Maynard M, Trepo C, Zoulim F. 2004. Evolution of hepatitis B viral load and viral genome sequence during adefovir dipivoxil therapy. *J Viral Hepatol* 11:74–83.
- Westland CE, Yang H, Delaney WE IV, Gibbs CS, Miller MD, Wulfsohn M, Fry J, Brosgart CL, Xiong S, 437 and 438 Study Teams. 2003. Week 48 resistance surveillance in two phase 3 clinical studies of adefovir dipivoxil for chronic hepatitis B. *Hepatology* 38:96–103.
- Whalley SA, Brown CG, Teo CG, Dusheiko GM, Saunders NA. 2001. Monitoring the emergence of hepatitis B virus polymerase gene variants during lamivudine therapy using LightCycler. *J Clin Microbiol* 39:1451–1459.
- Yang H, Westland CE, Delaney WE IV, Heathcote EJ, Ho V, Fry J, Brosgart C, Gibbs CS, Miller MD, Xiong S. 2002. Resistance surveillance in chronic hepatitis B patients treated with adefovir dipivoxil for up to 60 weeks. *Hepatology* 36:464–473.