stop codon, and the A to T mutation at nt 1762 and the G to A mutation at nt 1764 in the core promoter region are frequently found in patients with fulminant hepatitis [16–21]. These mutations in the precore and core promoter regions are related to a reduction of the HBeAg by the translational and transcriptional levels, respectively. The mechanisms involved with severe liver damage and these mutations are under investigation in relation to viral replication, viral gene expression and localization of viral proteins [22–27]. On the other hand, there are many reports from western countries showing that the frequencies of these mutations were not high among fulminant hepatitis patients and that they were not related to the severity of hepatitis [28–32]. It has been reported that the frequencies of these mutations are different among HBV genotypes [33–36]. The differences of frequency of these mutations in fulminant hepatitis in western and eastern countries may be related to the difference of HBV genotype distribution throughout the world, as genotypes B and C are major genotypes in East Asia, and genotypes A and D are major in the US and Europe [33, 34, 37–39].

Several investigators have studied infectious source factors in cases of infant patients with fulminant hepatitis B, and some results have indicated that anti-HBe positivity or mutant HBV with a precore mutation (nt 1896G to A) in the mother has a relation with fulminant hepatitis in the child; however, some reports are contradictory [40–44]. In cases of adult patients with acute HBV infection, only a few reports have discussed the virological aspects of the infectious sources. Aye et al. [45] studied 7 adult patients with acute HBV infection and 7 HBV carriers who were their infectious sources, and found that all 5 infectious sources with acute hepatitis B were positive for HBeAg without precore mutant HBV and 4 of the 5 were healthy carriers, whereas 2 of 2 infectious sources of fulminant hepatitis were patients with chronic hepatitis and 1 was positive for anti-HBe with precore mutated HBV. Further, Yotsumoto et al. [46] and Tanaka et al. [47] noted that infectious sources of adult patients with fulminant hepatitis were positive for anti-HBe with a precore mutation. However, there are no known reports that have performed statistical analysis of the risk factors in infectious sources in relationship to the severity of hepatitis in partner patients.

In the present study, we analyzed the clinical features and viral genomes of infectious sources infected with genotype C HBV, which is the main genotype found in our region. It has been proven that the infectious source factors that related to the transmission of SH were higher age, anti-HBe, chronic liver diseases, ALT abnormality and mutations in core promoter and precore regions. It is interesting that all of these factors were related to the immune pressure to HBV in the chronically infected host. Therefore, it is suspected that HBV strains that are able to replicate under host immune pressure may have a relationship to SH in the receivers. HBeAg-positive asymptomatic carriers are thought to be at the immune tolerance stage in the course of chronic HBV infection. All patients infected by an HBeAg-positive asymptomatic carrier were diagnosed with AH, and no infectious sources with SH were diagnosed as an HBeAg-positive asymptomatic carrier. One infectious source of SH was diagnosed as ASC; however, this source was positive for anti-HBe, and it may be better to diagnose this case as an inactive HBsAg carrier state [48]. These results also support the suspicion that replication-competent HBV strains under immune pressure have a relation with SH in receivers. These results of adult acute HBV infection were not inconsistent with reports of infant cases with fulminant hepatitis B, suggesting the importance of anti-HBe and the precore mutant in mothers.

The present results confirmed that core promoter or precore mutations are related to the severity of acute genotype C HBV infection, as seen from the analyses of HBV in patients with acute HBV infection and their infectious sources. However, all but one infectious sources in this study were infected with either mutant type HBV (nt 1762T, nt 1764A and nt 1896A) or wild type HBV (nt 1762A, nt 1764G and nt 1896G); therefore, it was not possible to analyze and compare the impact of the nature of these 3 mutations with the severity of hepatitis in infected patients.

Because of the restricted number of patients in this study, statistical analysis could only be done for genotype C. Risk factors relating to the severity of hepatitis might be different among different genotypes. This issue should be clarified in the future.

In conclusion, among the partners of patients chronically infected with genotype C HBV, those who were diagnosed with chronic liver diseases, positive for anti-HBe and infected with core promoter (nt 1762T, nt 1764A) or precore (nt 1986A) mutants were identified as risky infectious sources of sexually transmitted severe hepatitis.

Acknowledgement

This study was supported in part by a grant to the study group from the Ministry of Health, Labour and Welfare, Japan.

References

1 Eto T, Shiraki K: National project on the prevention of mother-to-infant infection by hepatitis B virus in Japan. Acta Paediatr Jpn 1989; 31:681–684.

2 Japanese Red Cross Non-A, Non-B Hepatitis Research Group: Effect of screening for hepatitis C virus antibody and hepatitis B core antibody on incidence of post-transfusion hepatitis. Lancet 1991;338:1040–1041.

3 NIH Consensus Development Panel on Infectious Disease Testing for Blood Transfusions: Infectious disease testing for blood transfusions. JAMA 1995;274:1374–1379.

4 Struve J, Giesecke J, Lindh G, Weiland O: Heterosexual contact as a major route for transmission of acute hepatitis B among adults. J Infect 1990;20:111–121.

5 Hou MC, Wu JC, Sheng WY, Chen TZ, Lee SD, Lo KJ: Heterosexual transmission as the most common route of acute hepatitis B virus infection among adults in Taiwan – the importance of extending vaccination to susceptible adults. J Infect Dis 1993;167:938–941.

6 Bath GE, Scott TG, Sibbald CJ, Upton PA, Ramsay CN, Willocks LJ: Acute hepatitis B in Edinburgh 1975–92: A retrospective study in a population where human immunodeficiency virus is highly prevalent. Epidemiol Infect 1997;119:85–89.

7 Massari V, Maison P, Desenclos JC, Flahault A: Six years of sentinel surveillance of hepatitis B in general practice in France. Eur J Epidemiol 1998;14:765–767.

8 Lindh M, Horal P, Norkrans G: Acute hepatitis B in Western Sweden – genotypes and transmission routes. Infection 2000;28:161–163.

9 Arima S, Michitaka K, Horiike N, Kawai K, Matsubara H, Nakanishi S, Abe M, Hasebe A, Tokumoto Y, Yamamoto K, Onji M: Change of acute hepatitis B transmission routes in Japan. J Gastroenterol 2003;38:772–775.

10 Bernuau J, Rueff B, Benhamou JP: Fulminant and subfulminant liver failure: Definitions and causes. Semin Liver Dis 1986;6:97–106.

11 Robert A, Chazouilleres O: Prothrombin time in liver failure: Time, ratio, activity percentage or international normalized ratio? Hepatology 1996;24:1392–1394.

12 Takahashi Y, Shimizu M, Study Group of Fulminant Hepatitis: Aetiology and prognosis of fulminant hepatitis in Japan: A multicentre study. J Gastroenterol Hepatol 1991;6:159–164.

13 Kaplan PM, Greenman RL, Gerin JL, Purcell RH, Robinson WS: DNA polymerase associated with human hepatitis B antigen. J Virol 1973;12:995–1005.

14 Chen Y, Michitaka K, Matsubara H, Yamamoto K, Horiike N, Onji M: Complete genome sequence of hepatitis B virus (HBV) from a patient with fulminant hepatitis without precore and core promoter mutations: Comparison with HBV from a patient with acute hepatitis infected from the same infectious source. J Hepatol 2003;38:84–90.

15 Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH: Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 1999;450:66–71.

16 Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M: Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 1991; 324:1699–1704.

17 Carman WF, Fagan EA, Hadziyannis S, Karayiannis P, Tassopoulos NC, Williams R, Thomas HC: Association of a precore genomic variant of hepatitis B virus with fulminant hepatitis. Hepatology 1991;14:219–222.

18 Liang TJ, Hasegawa K, Rimon N, Wands J, Ben-Porath E: A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med 1991;324:1705–1709.

19 Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshiba M, Tanaka S, Akahane Y, Okamoto H, Tsuda F, Miyazawa Y, Mayumi M: Fulminant hepatitis B: Induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. Gastroenterology 1991;100:1087–1094.

20 Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, Tsuda F, Tanaka T, Okamoto H, Miyakawa Y, Mayumi M: Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med 1995;122:241–248.

21 Teo EK, Ostapowicz G, Hussain M, Lee WM, Fontana R, Lok AS, US ALF Study Group: Hepatitis B infection in patients with acute liver failure in the United States. Hepatology 2001;33:972–976.

22 Lamberts C, Nassal M, Velhagen I, Zentgraf H, Schroder CH: Precore-mediated inhibition of hepatitis B virus progeny DNA synthesis. J Virol 1993;67:3756–3762.

23 Baumert TF, Rogers SA, Hasegawa K, Liang TJ: Two core promoter mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J Clin Invest 1996;98:2268–2276.

24 Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH: Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 1996;70:5845–5851.

25 Kidd-Ljunggren K, Oberg M, Kidd AH: Hepatitis B virus X gene 1751 to 1764 mutations: Implications for HBeAg status and disease. J Gen Virol 1997;78:1469–1478.

26 Sterneck M, Kalinina T, Gunther S, Fischer L, Santantonio T, Greten H, Will H: Functional analysis of HBV genomes from patients with fulminant hepatitis. Hepatology 1998;28: 1390–1397.

27 Kawai K, Horiike N, Michitaka K, Onji M: The effects of hepatitis B virus core promoter mutations on hepatitis B core antigen distribution in hepatocytes as detected by laser-assisted microdissection. J Hepatol 2003;38:635–641.

28 Feray C, Gigou M, Samuel D, Bernuau J, Bismuth H, Brechot C: Low prevalence of precore mutations in hepatitis B virus DNA in fulminant hepatitis type B in France. J Hepatol 1993;18:119–122.

29 Laskus T, Rakela J, Nowicki M, Persing DH: Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. Gastroenterology 1995;109:1618–1623.

30 Hsu HY, Chang MH, Lee CY, Hsieh KH, Ni YH, Chen PJ, Chen DS: Precore mutant of hepatitis B virus in childhood fulminant hepatitis B: An infrequent association. J Infect Dis 1995;171:776–781.

31 Sterneck M, Gunther S, Santantonio T, Fischer L, Broelsch CE, Greten H, Will H: Hepatitis B virus genomes of patients with fulminant hepatitis do not share a specific mutation. Hepatology 1996;24:300–306.

32 Karayiannis P, Alexopoulou A, Hadziyannis S, Thursz M, Watts R, Seito S, Thomas HC: Fulminant hepatitis associated with hepatitis B virus e antigen-negative infection: Importance of host factors. Hepatology 1995;22:1628–1634.

33 Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S: A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Hepatology 2001;33:218–223.

34 Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, Kanda T, Fukai K, Kato M, Saisho H: Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. Hepatology 2003;37:19–26.

35 Chan HL, Hussain M, Lok AS: Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology 1999;29:976–984.

36 Blackberg J, Kidd-Ljunggren K: Genotypic differences in the hepatitis B virus core promoter and precore sequences during seroconversion from HBeAg to anti-HBe. J Med Virol 2000; 60:107–112.

37 Mayerat C, Mantegani A, Frei PC: Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? J Viral Hepat 1999;6:299–304.

38 Swenson PD, Van Geyt C, Alexander ER, Hagan H, Freitag-Koontz JM, Wilson S, Norder H, Magnius LO, Stuyver L: Hepatitis B virus genotypes and HBsAg subtypes in refugees and injection drug users in the United States determined by LiPA and monoclonal EIA. J Med Virol 2001;64:305–311.

39 Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M: Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 2001;34:590–594.

40 Beath SV, Boxall EH, Watson RM, Tarlow MJ, Kelly DA: Fulminant hepatitis B in infants born to anti-HBe hepatitis B carrier mothers. BMJ 1992;304:1169–1170.
41 Terazawa S, Kojima M, Yamanaka T, Yotsumoto S, Okamoto H, Tsuda F, Miyakawa Y, Mayumi M: Hepatitis B virus mutants with precore-region defects in two babies with fulminant hepatitis and their mothers positive for antibody to hepatitis B e antigen. Pediatr Res 1991;29:5–9.
42 Vanclaire J, Cornu C, Sokal EM: Fulminant hepatitis B in an infant born to a hepatitis Be antibody positive, DNA negative carrier. Arch Dis Child 1991;66:983–985.
43 Komatsu H, Inui A, Morinishi Y, Sogo T, Fujisawa T: Sequence analysis of hepatitis B virus genomes from an infant with acute severe hepatitis and a hepatitis B e antigen-positive carrier mother. J Med Virol 2001;65:457–462.
44 Cacciola I, Cerenzia G, Pollicino T, Squadrito G, Castellaneta S, Zanetti AR, Mieli-Vergani G, Raimondo G: Genomic heterogeneity of hepatitis B virus (HBV) and outcome of perinatal HBV infection. J Hepatol 2002;36:426–432.
45 Aye TT, Uchida T, Becker SO, Shikata T, Mima S: Completely or nearly identical hepatitis B virus strains replicate between patients with acute or fulminant hepatitis B and their respective infectious sources. J Med Virol 1994;42:60–65.
46 Yotsumoto S, Kojima M, Shoji I, Yamamoto K, Okamoto H, Mishiro S: Fulminant hepatitis related to transmission of hepatitis B variants with precore mutations between spouses. Hepatology 1992;16:31–35.
47 Tanaka S, Yosiba M, Iino S, Fukuda M, Nakao H, Tsuda F, Okamoto H, Miyakawa Y, Mayumi M: A common-source outbreak of fulminant hepatitis B in hemodialysis patients induced by precore mutant. Kidney Int 1995;48:1972–1978.
48 Lok AS, McMahon BJ: Chronic hepatitis B. Hepatology 2001;34:1225–1241.
49 Kobayashi M, Koike K: Complete nucleotide sequence of hepatitis B virus DNA of subtype adr and its conserved gene organization. Gene 1984;30:227–232.

Original Paper

Intervirology

Intervirology 2005;48:262–267
DOI: 10.1159/000084604

Received: September 21, 2004
Accepted after revision: October 29, 2004

Co-Infection with Hepatitis B Virus Genotype D and Other Genotypes in Western Japan

Kojiro Michitaka[a, b] Norio Horiike[a] Yan Chen[a] Tran Nhu Duong[a] Kana Matsuura[a] Yoshio Tokumoto[a] Yoichi Hiasa[a] Fazle S.M. Akbar[a] Morikazu Onji[a]

[a]Third Department of Internal Medicine, and [b]Endoscopy Center, Ehime University School of Medicine, Ehime, Japan

Key Words
Hepatitis B virus · Co-infection · Genotype · Hepatitis B surface antigen · Subtype

Abstract
Objective: Genotypes B and C are the prevalent hepatitis B virus (HBV) genotypes in eastern Asia. Although very rare in this region of the world, genotype D was found to be prevalent in a small area of western Japan. In this study, we confirm the frequency and clinical significance of co-infection with different genotypes among patients from that area infected with genotype D. ***Methods:*** Twenty-three patients from the same area of western Japan infected with HBV genotype D, determined using a genotyping enzyme immunoassay, were studied. Cloning was done using DNA extracted from serum samples, and polymerase chain reaction assays with the restriction fragment length polymorphism for HBV genotyping were performed with 10 clones from each patient. ***Results:*** Four (17.4%) of the 23 patients were found to be co-infected with HBV genotype C, and the HB surface antigen subtype was *ayw* in both mono- and co-infected patients. No clinical differences were found between mono-infected and co-infected patients carrying genotype D. ***Conclusion:*** A significant number of patients from the study area found to be infected with HBV genotype D were co-infected with genotype C. Additional study with a larger number of patients is needed to elucidate the possible clinical significance.

Introduction

Hepatitis B virus (HBV) is a DNA virus, with genomic length of approximately 3,200 bases, and is an important causative agent of acute and chronic liver diseases. Approximately 350 million people overall are chronically infected with HBV in the world, and 8 genotypes (A through H), classified according to the divergence of the entire genome, have been reported [1–4]. HBV genotypes have a distinct geographic distribution among various regions of the world. Genotypes A and D are dis-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0300–5526/05/0484–0262$22.00/0

Accessible online at:
www.karger.com/int

Morikazu Onji, MD
Third Department of Internal Medicine, Ehime University School of Medicine
Shigenobu-cho
Onsen-gun, Ehime 791-0295 (Japan)
Tel. +81 89 960 5308, Fax +81 89 960 5310, E-Mail onjimori@m.chime-u.ac.jp

tributed mainly in Europe, West Asia, Africa and America, while genotypes B and C are predominant in East Asian countries [5–7]. Further, many reports have indicated that there are some differences in the clinical features of patients infected with the various genotypes [8–13]. The rate of persistence of HBV genotype A infection following acute infection is reported to be high as compared with genotype D, and genotype C has been reported to have a relationship with advanced liver diseases and delayed HBeAg seroconversion, in contrast to genotype B.

Several studies have reported recombination with different genotypes, and a few reports have indicated the clinical relevance of such recombination [14–18]. On the other hand, co-infection with genotypes B and C, and with genotypes A and D have been reported [19–22], with clinical and virological significance of co-infection studied. In contrast, there are no known reports that have provided detailed data of other combinations, such as genotypes B and D or genotypes C and D, because of their different geographical distribution.

Genotype C is the most prevalent HBV genotype in Japan, followed by genotype B, whereas genotype D is very rare [5]. However, we have reported that approximately 10% of the HBV carriers in a small area in western Japan were infected with genotype D [23]. In the present study, we attempted to clarify the frequency and clinical features of co-infection with other genotypes in patients infected with genotype D in this area. The rate of prevalence of genotypes A, B, C, and D in this area were found to be 2.7, 3.4, 84.7 and 9.2%, respectively, among chronic HBV carriers [23].

Patients and Methods

Patients

Among all patients positive for the HB surface antigen (HBsAg) who consulted our hospital from 1999 to 2002, 23 (14 males, 9 females; median age 26 years old, ranging from 13 to 88 years of age; 20 with chronic HBV infection, 3 with acute infection) were confirmed to be infected with genotype D and included in this study. All were negative for anti-hepatitis C virus (HCV). The infectious route was not determined in 21 patients, whereas sexual transmission was suspected in 2 patients with acute HBV infection. None were drug abusers or homosexuals. Among the 20 patients with chronic infection, none had a history of acute hepatitis. The purpose of the study was explained to each patient beforehand, and written informed consent was obtained from each.

Serological Testing

The presence of HBsAg was assayed using a chemiluminescent immunoassay (Architect HBsAg, Dainabot, Tokyo, Japan), while hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) were checked with an enzyme immunoassay (EIA) (AxSYM HBeAg and AxSYM HBeAb assays, Dainabot), and the subtype of HBsAg (serotype) was tested using a commercially available EIA kit (HBsAg subtype EIA, Institute of Immunology, Tokyo).

Genotype Testing

The HBV genotypes of the 23 serum subjects were determined using an HBV-genotyping EIA kit (Institute of Immunology) [24]. Genotype of clones described below was assayed using a restriction fragment length polymorphism method on the small-S gene sequence amplified by a polymerase chain reaction (PCR-RFLP) [25].

Cloning

Serum samples were stored at –80°C. DNA was extracted from 50 μl of serum with lysis buffer containing proteinase K, and the S region of HBV-DNA was amplified using PCR. The details of the present method were reported in our previous study [26]. Briefly, a semi-nested PCR assay was performed using sense primers SS1 (5′-CTCCACCACATTCCACCAA, nt1–20), SS3 (5′-TCCTGCTGGTGGCTCCAGTTC, nt55–75) and anti-sense primer SS2 (5′-GAGGAGCCACAAAGGTTCCGC, nt1258–1238). SS1 and SS2 were used for the first-round of PCR, and SS2 and SS3 for the second-round. The amplified HBV-DNA was inserted into a plasmid vector (pT7BBlue-vector) and transformed into competent cells (NovaBlue Singles™) using a commercially available cloning kit (Perfectly Blunt™ cloning kits, Novagen, Darmstadt, Germany), with 10 clones obtained from each serum sample. Each clone was analyzed for genotype using the PCR-RFLP method described above. Sequencing was done with a commercially available kit (BigDye Terminator Cycle Sequencing FS Ready Reaction Kit, Applied Biosystems, Alameda, Calif., USA).

Statistical Analyses

Statistical analyses were performed using a Mann-Whitney test and Fisher's exact test. $p < 0.05$ was considered statistically significant.

Results

Frequency of Co-Infection with Different Genotypes

All of the 23 subjects with HBV were shown to be genotype D by the EIA method. PCR-RFLP findings of the cloned samples revealed that the genotype of all 10 clones from 19 patients was D, while some in each group of 10 clones from the other 4 (17.4% of all patients) were genotype C (table 1; fig. 1). The HBsAg subtype was also determined in 15 patients and all, including 3 patients who were co-infected with genotype C and D, were shown to be serotype *ayw*. To ensure the accuracy of the PCR-RFLP genotyping results for each clone, sequencing was performed for all 10 clones from a single patient

who was co-infected with genotypes C and D (patient 4 in table 1), and the PCR-RFLP findings were confirmed (fig. 2).

Clinical Features of Co-Infected Patients

We compared the clinical features between 4 patients co-infected with genotypes C and D and the 19 patients who showed mono-infection with genotype D, however, no significant differences were found for age, sex, diagnosis, level of ALT, and HBeAg/Ab status (table 2).

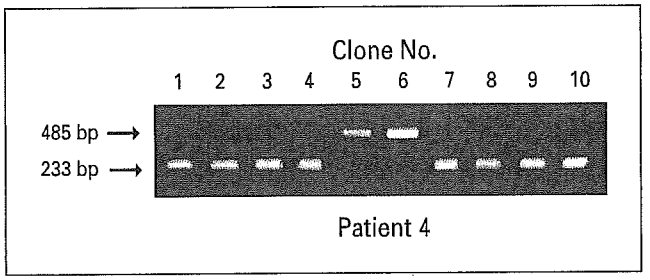

Fig. 1. Restriction fragment polymorphism patterns from genotyping 10 clones from patient 4. Shown are the electrophoresis results of the 10 clones from patient 4 digested with *Alw I*, which was the first genotyping step used in the PCR-RFLP method. DNA with 485 bp corresponds to genotype C, while that with 233 bp corresponds to genotype A, B, D, E, or F [25]. Clones with 233 bp were proven to be genotype D by the final results of the entire PCR-RFLP procedure.

Table 1. Patient clinical backgrounds, HBsAg subtypes, and number of clones with each genotype

No.	Age	Sex	Diagnosis	ALT	HBeAg	Anti-HBe	HBsAg subtype	Clones with each genotype	
1	28	F	ASC*	12	–	+	NE	D: 9	C: 1
2	29	F	ASC*	19	–	+	*ayw*	D: 7	C: 3
3	88	F	CH	41	+	–	*ayw*	D: 7	C: 3
4	26	M	CH	43	–	+	*ayw*	D: 8	C: 2
5	16	M	ASC	15	+	–	NE	D: 10	
6	64	F	ASC	33	+	–	*ayw*	D: 10	
7	70	M	ASC	21	+	–	*ayw*	D: 10	
8	23	F	ASC*	21	–	+	NE	D: 10	
9	63	F	ASC*	17	–	+	*ayw*	D: 10	
10	26	M	ASC*	39	–	+	*ayw*	D: 10	
11	26	M	ASC*	20	–	+	NE	D: 10	
12	24	M	ASC*	14	–	+	*ayw*	D: 10	
13	25	M	ASC*	20	–	+	NE	D: 10	
14	28	M	ASC*	28	–	+	*ayw*	D: 10	
15	27	M	ASC*	24	–	+	*ayw*	D: 10	
16	28	M	ASC*	26	–	+	*ayw*	D: 10	
17	18	F	FH	7,620	+	–	NE	D: 10	
18	20	F	AH	189	+	–	*ayw*	D: 10	
19	13	F	AH	1,452	–	+	*ayw*	D: 10	
20	21	M	CH	46	+	–	NE	D: 10	
21	27	M	CH	88	+	–	*ayw*	D: 10	
22	64	M	CH	39	+	–	*ayw*	D: 10	
23	26	M	CH	50	–	+	NE	D: 10	

AH = Acute hepatitis; FH = fulminant hepatitis; ASC = asymptomatic HBV carrier; CH = chronic hepatitis; ALT = alanine aminotransferase; NE = not examined.

* These cases were also diagnosed to be an inactive HBsAg carrier state, though histological examinations were not performed [38].

```
                          nt 287          493          630          963
                            |             |            |            |
Genotype C (AB033550) GCACCCAC----  --AACAACTAC---  --CAAGAT--  --AAATAGAC-
Genotype D (M32138)   A-CA--GT----  --TT----A-----  --G--A----  --T--C--G--

Clone 1               A-CA--GT----  --TT----A-----  --G--A----  --T--C--G--
Clone 2               A-CA--GT----  --TT----A-----  --G--A----  --T--C--G--
Clone 3               A-CA--GT----  --TT----A-----  --G--A----  --T--C--G--
Clone 4               A-CA--GT----  --TT----A-----  --G--A----  --T--C--G--
Clone 5               ------------  -------------  ----------  ----------
Clone 6               ------------  -------------  ----------  ----------
Clone 7               A-CA--GT----  --TT----A-----  --G--A----  --T--C--G--
Clone 8               A-CA--GT----  --TT----A-----  --G--A----  --T--C--G--
Clone 9               A-CA--GT----  --TT----A-----  --G--A----  --T--C--G--
Clone 10              A-CA--GT----  --TT----A-----  --G--A----  --T--C--G--
```

Fig. 2. Nucleotide sequences of S regions in 10 clones from patient 4. The 10 clones (clone 1–10) are the same as in figure 1. DNA from clone 5 and 6 were confirmed to be genotype C, while that from the other 8 clones was shown to be genotype D by their nucleotide sequences. The nucleotide sequences of strains AB033550 and M32138 are shown as prototype sequences of genotypes C and D, respectively [1, 39].

Table 2. Comparison of clinical features between co-infected (genotype D + C) and mono-infected (D) subjects

	Co-infection (D+C)	Mono-infection (D)	p
Number	4	19	
Age, years, median (range)	28.5 (26–88)	32 (13–70)	NS
Sex (M/F)	1/3	13/6	NS
Diagnosis			NS
FH,AH	0	3	
ASC	2	12	
CH	2	4	
ALT, IU/l, median (range)	30 (12–43)	28 (15–7,620)	NS
HBcAg/anti-HBc			NS
HBeAg+	1	8	
Anti-HBe+	3	11	

AH = Acute hepatitis; FH = fulminant hepatitis; ASC = asymptomatic HBV carrier; CH = chronic hepatitis; ALT = alanine aminotransferase; NS = not significant.

Discussion

The samples examined in the present study came from subjects living in the small area in western Japan, where Gianotti-Crosti syndrome caused by infection with HBV subtype *ayw* was endemic in the 1970s [27, 28]. Recently, we reported that the infectious agent that caused the outbreak in this area was suspected to be HBV genotype D [23, 29]. It is convincing that genotype C was the common genotype involved in the co-infected cases in the present study, because C is the most prevalent genotype in this area.

In all of the present subjects infected with genotype D, the serotype of HBsAg was *ayw*. On the other hand, most of patients infected with genotype C in Japan show serotype *adr* [24] and the same result was obtained in the study area (data, not shown). We expected that patients co-infected with genotypes C and D would show combined serotypes of *ayw* and *adr*, however, all of those assayed for serotypes in the present study were found to be serotype *ayw*. Thus, we suspected that the major serotype in the cases of co-infection was *ayw*, while the sensitivity for detection of HBsAg serotype *adr* in the serum samples was not adequate using the present method. As a result, the present serotyping method may not be sensitive enough to screen patients co-infected with different serotypes. On the other hand, several other reports have described compound serotypes, such as *adwr* or *adywr* in infected patients [30–33]. Further study is needed to determine whether patients with compound subtypes are co-infected with different genotypes.

Several studies regarding co-infection with different genotypes of HBV have been reported. Chen et al. found that 17.5% of intravenous drug abusers co-positive for HBsAg, anti-HCV and anti-HDV were co-infected with genotypes B and C [19]. Kato et al. [20] reported that 2 distinct HBV genotypes were found in 10.9% of the samples from HBV carriers collected from several countries throughout the world. In another study in which 40% of the subjects were homosexual, Hannoun et al. [21] showed that 67% of chronic hepatitis B patients were co-infected with genotype A and other genotypes. In the present study, 4 (17.4%) of the 23 patients were found to have co-infection, which is comparable to that among drug abuser reported by Chen et al. described above. Although none of the present subjects were known to be drug abusers or homosexual, a considerable ratio of genotype D HBV carriers were co-infected with genotype C.

The possible route of co-infection with genotypes C and D was considered to be superinfection with genotype C in a genotype D carrier, superinfection with genotype D in a genotype C carrier, or simultaneous infection with genotypes C and D. No history of acute hepatitis was observed in any of the subjects with chronic infection, thus the process remains unidentified. The high prevalence of co-infection with genotype A and other genotypes in the report of Hannoun et al. [21] is intriguing because the rate of progression to chronic carrier state in adult patients with acute genotype A infection is known to be high and many of their subjects were homosexuals. Recently, the most important infectious route for acute HBV infection in Japanese adults is sexual transmission [34], and it has been reported that acute hepatitis B with genotype A is increasing in the Tokyo area [35, 36], whereas acute hepatitis B with genotype D is prevalent in the present area [26]. The rate of chronicity following acute HBV infection of each genotype via sexual transmission must be clarified, as well as that following acute HBV superinfection in chronic carrier state patients with other genotypes.

Little is known regarding the significance of co-infection with the different genotypes, though Kato et al. reported the virological significance of co-infection with genotypes G and A in regard to the existence of HBeAg in serum [37]. We have reported previously that in comparison to genotype C, infection with genotype D was related to normal levels of ALT, low frequency of advanced liver diseases (liver cirrhosis and hepatocellular

carcinoma), and early seroconversion of HBeAg to anti-HBe in contrast to genotype C [23]. In the present study, we attempted to know the differences of clinical features between patients co-infected with genotype C and D and those infected with genotype D only, but the significant differences were not found. However, the number of patients was too few to form a conclusion.

In conclusion, we confirmed that a considerable number of patients in the study area infected with genotype D were co-infected with genotype C. No clinical significance of such co-infection was found, however, a larger study group is needed.

Acknowledgement

This study was supported by a grant to the study group from the Ministry of Health, Labour and Welfare, Japan.

References

1 Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M: Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69:2575–2583.

2 Norder H, Courouce AM, Magnius LO: Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 1994;198:489–503.

3 Stuyver L, Gendt SD, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R: A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67–74.

4 Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO: Genotype H: A new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;83:2059–2073.

5 Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M: Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 2001;34:590–594.

6 Miyakawa Y, Mizokami M: Classifying hepatitis B virus genotypes. Intervirology 2003;46:329–338.

7 Lindh M, Andersson AS, Gusdal A: Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus-large scale analysis using a new genotyping method. J Infect Dis 1997;175:1285–1293.

8 Mayerat C, Mantegani A, Frei PC: Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? J Viral Hepat 1999;6:299–304.

9 Kao JH, Chen PJ, Lai MY, Chen DS: Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000;118:554–559.

10 Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, Kanda T, Fukai K, Kato M, Saisho H: Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. Hepatology 2003;37:19–26.

11 Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikkawa K, Ichida T, Okanouc T, Yotsuyanagi H, Iino S: A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Hepatology 2001 33:218–223.

12 Chu CJ, Hussain M, Lok AS: Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. Gastroenterology 2002;122:1756–1762.

13 Sanchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodes J: Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. Gastroenterology 2002;123:1848–1856.

14 Bollyky PL, Rambaut A, Harvey PH, Holmes EC: Recombination between sequences of hepatitis B virus from different genotypes. J Mol Evol 1996;42:97–102.

15 Morozov V, Pisareva M, Groudinin M: Homologous recombination between different genotypes of hepatitis B virus. Gene 2000;260:55–65.

16 Cui C, Shi J, Hui L, Xi H, Zhuoma, Quni, Tsedan, Hu G: The dominant hepatitis B virus genotype identified in Tibet is a C/D hybrid. J Gen Virol 2002 ;83:2773–2777.

17 Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Ueda R, Miyakawa Y, Mizokami M: Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 2002;76:5985–5992.

18 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: Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124:925–932.

19 Chen BF, Kao JH, Liu CJ, Chen DS, Chen PJ: Genotypic dominance and novel recombinations in HBV genotype B and C co-infected intravenous drug users. J Med Virol 2004;73:13–22.

20 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: 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 2003;110:29–35.

21 Hannoun C, Krogsgaard K, Horal P, Lindh M: Interpred trial group. Genotype mixtures of hepatitis B virus in patients treated with interferon. J Infect Dis 2002;186:752–759.

22 Kao JH, Chen PJ, Lai MY, Chen DS: Acute exacerbations of chronic hepatitis B are rarely associated with superinfection of hepatitis B virus. Hepatology 2001;34:817–823.

23 Duong TN, Horiike N, Michitaka K, Yan C, Mizokami M, Tanaka Y, Jyoko K, Yamamoto K, Miyaoka H, Yamashita Y, Ohno N, Onji M: Comparison of genotypes C and D of the hepatitis B virus in Japan: a clinical and molecular biological study. J Med Virol 2004;72:551–557.

24 Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M: Serological detection of hepatitis B genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 1999;80:97–112.

25 Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Roberson BH: Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 1999;450:66–71.

26 Chen Y, Michitaka K, Matsubara H, Yamamoto K, Horiike N, Onji M: Complete genome sequence of hepatitis B virus (HBV) from a patient with fulminant hepatitis without precore and core promoter mutations: comparison with HBV from a patient with acute hepatitis infected from the same infectious source. J Hepatol 2003;38:84–90.

27 Ishimaru Y, Ishimaru H, Toda G, Baba K, Mayumi M: An epidemic of infantile papular acrodermatitis (Gianotti's disease) in Japan associated with hepatitis-B surface antigen subtype *ayw*. Lancet 1976;i:707–709.

28 Toda G, Ishimaru Y, Mayumi M, Oda T: Infantile papular acrodermatitis (Gianotti's disease) and intrafamilial occurrence of acute hepatitis B with jaundice: age dependency of clinical manifestations of hepatitis B virus infection. J Infect Dis. 1978;138:211–216.
29 Michitaka K, Horiike N, Chen Y, Duong TN, Konishi I, Mashiba T, Tokumoto Y, Hiasa Y, Tanaka Y, Mizokami M, Onji M: Gianotti-Crosti syndrome caused by acute hepatitis B virus genotype D infection. Intern Med, 2004; 43:696–699.
30 Okamoto H, Imai M, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M: Point mutation in the S gene of hepatitis B virus for a d/y or w/r subtypic change in two blood donors carrying a surface antigen of compound subtype adyr or adwr. J Virol 1987;61:3030–3034.
31 Yamanaka T, Akahane Y, Suzuki H, Okamoto H, Tsuda F, Miyakawa Y, Mayumi M: Hepatitis B surface antigen particles with all four subtypic determinants: point mutations of hepatitis B virus DNA inducing phenotypic changes or double infection with viruses different subtypes. Mol Immunol 1990;27:443–449.
32 Kanagawa H, Takai E, Tsuda F, Machida A, Kojima M, Ishijima A, Tanaka T, Okamoto H, Miyakawa Y, Mayumi M: Hepatitis B surface antigen particles of subtypes adw and adr, and compound subtype (adwr) in symptom-free carriers in Japan. J Med Virol 1992;37:288–293.
33 Sakai T, Shiraki K, Inoue H, Okano H, Deguchi M, Sugimoto K, Ohmori S, Murata K, Fujioka H, Takase K, Tameda Y, Nakano T: HBV subtype as a marker of the clinical course of chronic HBV infection in Japanese patients. J Med Virol 2002;68:175–181.
34 Arima S, Michitaka K, Horiike N, Kawai K, Matsubara H, Nakanishi S, Abe M, Hasebe A, Tokumoto Y, Yamamoto K, Onji M: Change of acute hepatitis B transmission routes in Japan. J Gastroenterol 2003;38:772–775.
35 Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, Kobayashi M, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Takagi K, Miyakawa Y, Kumada H: Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 2002; 68:522–528.
36 Ogawa M, Hasegawa K, Naritomi T, Torii N, Hayashi N: Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. Hepatol Res 2002;23:167–177.
37 Kato H, Orito E, Gish RG, Bzowej N, Newsom M, Sugauchi F, Suzuki S, Ueda R, Miyakawa Y, Mizokami M: Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. Hepatology 2002;35:922–929.
38 Lok AS, McMahon BJ: Practice Guidelines Committee, American Association for the Study of Liver Diseases. Chronic hepatitis B. Hepatology 2001;34:1225–1241.
39 Tong SP, Li JS, Vitvitski L, Trepo C: Active hepatitis B virus replication in the presence of anti-HBe is associated with viral variants containing an inactive pre-C region. Virology 1990;176:596–603.

Efficacy of lamivudine therapy for decompensated liver cirrhosis due to hepatitis B virus with or without hepatocellular carcinoma

ATSUSHI HIRAOKA[1], KOJIRO MICHITAKA[1], TERU KUMAGI[1], KIYOTAKA KUROSE[1], TAKAHIDE UEHARA[1], MASASHI HIROOKA[1], YOSHIMASA YAMASHITA[2], YOSHIKAZU KUBO[3], HIROAKI MIYAOKA[4], HIDEHITO IUCHI[5], SHINICHI OKADA[5], MASAKI OHMOTO[6], KAZUHISA YAMAMOTO[7], NORIO HORIIKE[1] and MORIKAZU ONJI[1]

[1]Third Department of Internal Medicine, Ehime University School of Medicine; [2]Internal Medicine, Ehime Prefecture Central Hospital; [3]Internal Medicine, Ehime National Hospital; [4]Internal Medicine, Saiseikai Matsuyama Hospital; [5]Internal Medicine, Saiseikai Saijo Hospital; [6]Internal Medicine, Saiseikai Imabari Hospital; [7]Internal Medicine, Matsuyama Shimin Hospital, Ehime, Japan

Received January 11, 2005; Accepted February 28, 2005

Abstract. The prognosis for patients with decompensated hepatitis B virus (HBV) related liver cirrhosis (LC-B), especially for those with LC-B complicated with hepatocellular carcinoma (HCC), is poor. We investigated the effects of lamivudine in patients with decompensated LC-B, with and without HCC. Decompensated LC-B patients (n=55) with Child-Pugh classification scores (CPS) >7 points were enrolled. All were admitted to the hospitals of the authors between January 1997 and December 2004. Decompensated cases due to a severe exacerbation of hepatitis with CH-B and patients with HCC showing an extra hepatic metastasis or portal vein tumor thrombus were excluded. Some 19 cases (including 5 cases complicated with HCC at the start of therapy) were treated with lamivudine at 100 mg/day (L group), and 36 (including 7 cases with HCC at time of admittance) were treated without lamivudine (non-L group). The median of CPS points in the L group was higher than that of non-L group (11 points versus 9 points, $p<0.02$). Prothrombin time (%), albumin, ascites, CPS, and HBV-DNA quantity were each significantly improved after 6 months in the L group ($p<0.05$). A mutation in the YMDD motif was observed in 5 patients in the L group, however liver function did not deteriorate. Further, the survival rate was significantly higher in the L group ($p<0.05$). HCC was found in 3 L group and 4 non-L group patients during the study. In the L group, all patients complicated with HCC were treated repeatedly or until cured, whereas 91% of those in the non-L group could not be treated ($p<0.01$). Our results suggest that lamivudine is a useful and important therapy for patients with decompensated LC-B with and without HCC, as well as those who are restricted from having liver transplantation.

Correspondence to: Dr Morikazu Onji, Third Department of Internal Medicine, Ehime University School of Medicine, Ehime 791-0295, Japan
E-mail: onjimori@m.ehime-u.ac.jp

Key words: hepatic decompensation, lamivudine, hepatocellular carcinoma, liver cirrhosis, hepatitis B virus

Introduction

In patients with hepatitis B virus (HBV)-related liver cirrhosis (LC-B), hepatic decompensation is a major clinical problem. Further, in those cases complicated with hepatocellular carcinoma (HCC), hepatic decompensation decreases quality of life and limits the available treatments for HCC. Liver transplantation is recommended for liver cirrhosis (1,2) and HCC (3,4), however many patients are unable to receive a transplant with the lack of donors available in Japan. Therefore, it is hoped that liver cirrhosis and HCC can be controlled non-surgically in many cases.

Interferon therapy is usually difficult in cases of LC-B with severe liver dysfunction because of the low levels of platelets, though lamivudine has been widely used for chronic hepatitis due to HBV (CH-B) (5), and also reported to be useful for decompensated LC-B (6-8). However, data regarding the efficacy of lamivudine for patients with decompensated LC-B with HCC is scarce, even though cases of liver cirrhosis with HCC have been increasing at a high rate. We previously reported on a decompensated LC-B patient classified as grade C in the Child-Pugh classification, in whom hepatic resection of an HCC that emerged 3 years after lamivudine therapy was enabled (9). In the present study, we retrospectively analyzed the usefulness of lamivudine therapy for patients with decompensated LC-B and in those complicated with HCC.

Patients and methods

Patients. Decompensated LC-B patients (n=55) with Child-Pugh classification score (CPS) >7 points were enrolled in the present study, and their results were analyzed retrospectively. All patients were admitted to the hospitals of the authors

Table I. Clinical data at entry and observation period for all patients.

	L group	Non-L group	p-value
Number of patients	19	36	-
Age in years			
Median	53	57	NS
Range	28-68	40-82	
Sex (males:females)	15:4	19:17	NS (0.06)
Total-bilirubin (mg/dl)	4.3±3.1	2.0±1.5	<0.001
Prothrombin time (%)	47.1±14.2	54.3±13.4	NS
ALT (IU/l)	95.7±119.4	79.1±120.0	NS
Albumin (g/dl)	2.8±0.6	3.0±0.4	NS
Platelets ($x10^4$/ml)	8.7±4.1	9.1±4.4	NS
Child-Pugh classification score (CPS)			
Median	11	9	<0.020
Range	8-15	7-12	
Patients with Child-Pugh class C	15 (79%)	18 (50%)	<0.050
Ascites	15 (79%)	29 (81%)	NS
Hepatic encephalopathy	1 (5%)	2 (6%)	NS
Patients positive for HBeAg	10 (53%)	10 (42%) (12:NE)	NS
HBV-DNA (TMA: Meq/ml)	6.2±1.1	6.2±1.5	NS
Patients with emergence of YMDD mutant	5 (26%)	-	-
Patients with breakthrough hepatitis	0 (0%)	-	-
Patients with HCC diagnosed at the entry	5 (26%)	7 (19%)	NS
Observation period (months)	28.0±16.0	29.0±19.0	NS

NS, not significant; NE, not examined. Analyses were conducted with the Student's t-test and Mann-Whitney U test.

between January 1997 and December 2004, and were positive for hepatitis B surface antigen (HBsAg), negative for anti-hepatitis C virus antibody second generation, and not alcoholic. Those cases with an HCC diagnosed within 6 months of admittance were classified as HCC complicated at entry. Decompensated cases due to a severe exacerbation of hepatitis with CH-B and patients with HCC showing an extra hepatic metastasis or portal vein tumor thrombus were excluded.

Methods. Of the 55 patients, 19 were treated orally with lamivudine at 100 mg/day (L group) (median age, 53 years old; 15 males and 4 females). Fifteen had Child-Pugh class C liver disease (79%), and 5 had known HCC at the start of lamivudine therapy, of whom 2 were HCC recurrence cases. Also, 2 were tumor node metastasis (TNM) stage II (10) and 3 were stage III. Of 19 patients, 17 had no past history of HCC.

A total of 36 patients were treated conservatively without lamivudine (non-L group) (median age, 57 years; 19 males and 17 females). Eighteen had Child-Pugh class C liver disease (50%), and 7 patients were diagnosed with HCC within 6 months after entry to this study, of which 4 were TNM stage II and 3 were stage III. None of the patients in the non-L group had a past history of therapy for HCC.

The diagnosis of liver cirrhosis was made by a histological study of past hospitalization in 8 cases, and past history of treated esophageal varices or existence of esophageal varices was determined using biochemical data combined with characteristic findings of abdominal ultrasonography (US) in the other 47 cases (11). Informed consent was received from all patients.

Evaluation. During the follow-up period, serial serum samples were measured for HBV-DNA quantity (TMA method), prothrombin time (PT%), aspartate aminotransferase, alanine aminotransferase (ALT), total bilirubin, albumin, and hepatitis B e antigen (HBeAg). Further, US and physical examinations were also performed. Data from examinations at 6, 12, 24, 36, and 48 months after entry to the study were analyzed.

Statistical analysis. Results are shown as the mean ± standard deviation (SD). Analyses were conducted using the Student's t-test, Mann-Whitney U test, paired t-test, Wilcoxon signed-rank test, Fisher's exact probability test, log-rank test, and Kaplan-Meier method. p-values <0.05 were considered statistically significant. All statistical analyses were carried

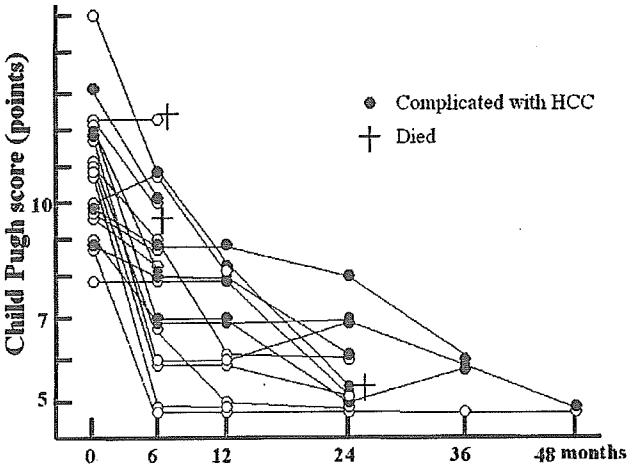

Figure 1. Serial changes of CPS in L group. After starting lamivudine, an improvement in CPS points was observed. CPS points following complication with HCC (●).

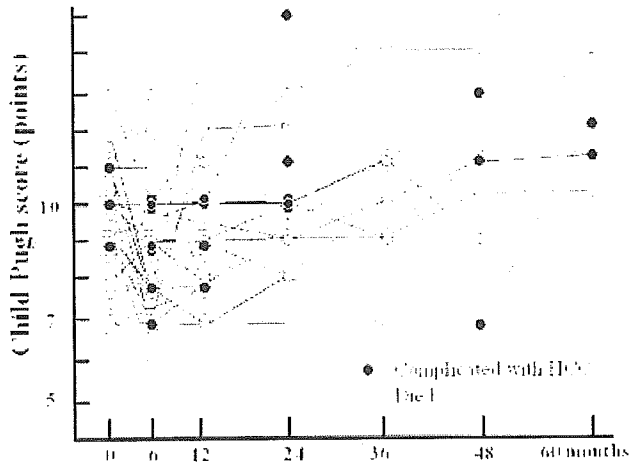

Figure 2. Serial changes of CPS points in non-L group. Six months after beginning conservative therapy without lamivudine, the CPS point average was decreased ($p<0.05$), then finally rebounded in nearly all cases in the non-L group. CPS points following complication with HCC (●).

Table II. Changes of CPS points in both groups.

	Months					
	0	6	12	24	36	48
L group						
Median	11	8	8	6	5	5
Range	(8-15)	(5-12)	(5-9)	(5-8)	(5-6)	-
n	19	19	15	10	6	2
Non-L group						
Median	9	9	9	10	10	11
Range	(7-13)	(6-13)	(6-14)	(7-15)	(7-14)	(7-14)
n	36	30	25	17	9	7
p-value	<0.020	NS	<0.001	<0.001	0.001	<0.050

NS, not significant. Analyses were conducted using a Mann-Whitney U test. All data are expressed as CPS.

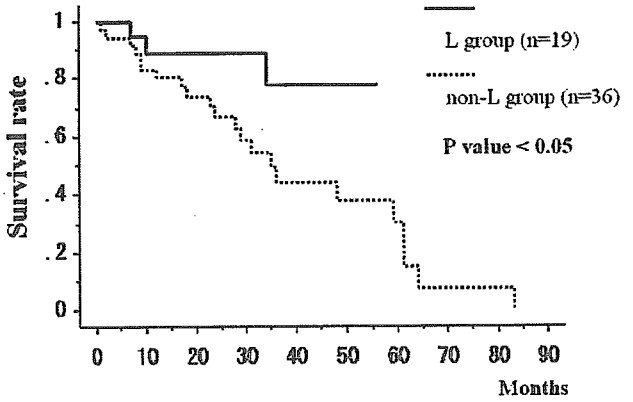

Figure 3. Survival rates of patients in L group and non-L group. The survival rate was lower in the non-L group ($p<0.05$).

out on a personal computer using StatView version 5.0 (SAS Institute, Inc., Berkeley, CA, USA).

Results

Clinical backgrounds and outcomes for the L group and non-L group are shown in Table I. The levels of total bilirubin and CPS points were worse in the L group than in the non-L group ($p<0.001$ and $p<0.02$, respectively) at study entry.

Fig. 1 shows the changes in CPS after the start of therapy in the L group. Of the 19 patients in that group, 16 showed a decrease in CPS points 6 months after beginning lamivudine (84%), and most cases showed a good response to therapy. Mutant HBV resistance to lamivudine appeared in 5 cases, though breakthrough hepatitis was not observed. Fig. 2 shows the changes in CPS in the non-L group. After 1 year, no significant improvement was observed in those patients regarding CPS points as compared with the time of entry.

CPS points in the L group were significantly lower than in the non-L group at 12, 24, 36, and 48 months after entry (Table II), and the survival rate in the L group was significantly longer (Fig. 3; $p<0.05$). In addition to the improvement of CPS, remarkable improvements in ALT (from 95.7±119.4 to 36.6±14.1 IU/l, $p<0.05$), and a decreasing quantity of HBV-DNA (TMA method) (from 6.2±1.1 to 3.3±0.8 Meq/ml, $p<0.01$) were observed after 6 months of lamivudine therapy in the L group.

HCC occurred in 3 patients in the L group, and 4 patients in the non-L group became complicated with HCC during the study. There were no significant differences between the clinical profiles of HCC-complicated cases in both groups (data not shown). Therapies against HCC [e.g. transcatheter hepatic arterial embolization (TAE), radiofrequency ablation (RFA), and surgery] could be performed without exacerbation of hepatic decompensation after recovery from a decompensated state in the L group patients. On the other hand, 5 of 11 cases with HCC in the non-L group could not receive any of the above treatments for HCC because of a decompensated state. In 5 cases, the decompensated state (ascites or icterus) deteriorated after therapy [TAE, percutaneous ethanol injection therapy (PEIT), or RFA]. Some 10 of the 11 non-L group patients with HCC died (91%) (Table III). The rate of patients

Table III. Clinical data for patients with HCC.

	Complicated with HCC at study entry		Complicated with HCC after study entry	
	L group	non-L group	L group	non-L group
No. of patients (males:females)	5[a] (3:2)	7 (3:4)	3 (3:0)	4 (3:1)
Outcome (survivor:non-survivor)	4:1	1:6	3:0	0:4
Performed therapies without exacerbation of decompensated state	5:0	1:6	3:0	0:4
CPS points at the entry (median:range)	12 (9-13)	10 (8-11)	12 (8-15)	10 (7-12)
CPS points at diagnosis of HCC	-	-	8 (7-11)	10 (7-12)
TNM classification (I:II:III)	0:2:3	0:4:3	2:1:0	2:1:1
Average size of HCC (mm)	25.2±5.7	29.1±6.0	17.7±6.4	19.8±8.2
Possible to perform radical treatment for HCC (n)	2	1	3	-
Possible to perform repeated treatment for HCC (n)	3	-	-	-
Impossible to perform therapy for HCC (n)[b]	-	6	-	4

[a]Of 5 cases, 2 were recurrence of known HCC cases. [b]Hepatic decompensation deteriorated after treatment for HCC in 5 cases.

with successful therapy for HCC was significantly higher in the L group ($p<0.001$). There was no significant difference in survival rate in the non-L group between cases with and without HCC (data not shown).

Discussion

Previous studies have shown that lamivudine is useful for patients with decompensated LC-B (6-8), however there are no known reports regarding the prognosis of patients with decompensated LC-B complicated with HCC treated with lamivudine. This is the first known report of lamivudine therapy for such cases.

The reserve function of the liver is an important factor in the prognosis of HCC patients (12) because therapy restrictions lead to a poor prognosis. As a result, some HCC staging scores that refer to the reserve function of the liver have been used as a means to predict the prognosis of HCC patients (13-16). In the present study, survival rates were not significantly different between cases with and without HCC in the non-L group, which indicated that severe hepatic failure might be a more important factor in the prognosis of decompensated LC-B patients with HCC.

Optional therapies against HCC include PEIT (17), RFA (18), TAE (19), surgery (20), and liver transplantation (3,4). When liver transplantation is restricted, decompensated liver cirrhosis patients with HCC usually cannot undergo radical therapy, such as surgery, PEIT or RFA, due to ascites or the tendency to bleed. In the present study, following recovery from hepatic decompensation, of 8 HCC complicated patients in the L group, 5 underwent therapies and were alive at the time of writing, with no recurrence or controlled with repeated TAE.

On the other hand, the appearance of mutant HBV resistance to lamivudine and breakthrough hepatitis are known as major problems that can occur during lamivudine therapy. Adefovir dipivoxil and entecavir have been introduced as drugs for breakthrough hepatitis (21,22), though it did not occur in any patients in the present study. Lamivudine therapy for decompensated LC-B requires careful follow-up, as breakthrough hepatitis can be fatal, and early therapy is required when it appears.

The prognosis of patients with hepatic decompensated LC-B is poor, with or without HCC complication. A liver transplantation is the most effective therapy, however, for cases that cannot undergo transplantation, the present results showed that lamivudine administration can improve the prognosis in those with decompensated LC-B complicated with HCC, though further investigation with a greater number of patients in a randomized controlled study is needed. Further, additional study with more cases and a longer period is needed regarding the frequency and severity of breakthrough hepatitis in patients undergoing lamivudine therapy.

In conclusion, lamivudine improved the survival rate of decompensated LC-B patients, and it is suggested that those with decompensated LC-B with or without HCC are indicated for lamivudine therapy.

References

1. Gane EJ, Portmann BC, Naoumov NV, *et al*: Long-term outcome of hepatitis C infection after liver transplantation. N Engl J Med 334: 815-820, 1996.
2. Sawyer RG, McGory RW, Gaffey MJ, *et al*: Improved clinical outcomes with liver transplantation for hepatitis B-induced chronic liver failure using passive immunization. Ann. Surg 227: 841-850, 1998.
3. Mazzaferro V, Regalia E, Doci R, *et al*: Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med 334: 693-699, 1996.
4. Tan KC, Rela M, Ryder SD, *et al*: Experience of orthotopic liver transplantation and hepatic resection for hepatocellular carcinoma of less than 8 cm in patients with cirrhosis. Br J Surg 82: 253-256, 1995.

5. Dienstag JL, Perillo RP, Schiff ER, Bartholomew M, Vicary C and Rubin M: A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 333: 1659-1661,1995.
6. Villeneuve JP, Condreary LD, Willems B, *et al*: Lamivudine treatment for decompensated cirrhosis resulting from chronic hepatitis B. Hepatology 31: 207-210, 2000.
7. Kapoor D, Guptan RC, Wakil SM, *et al*: Beneficial effects of lamivudine in hepatitis B virus-related decompensated cirrhosis. J Hepatol 33: 308-312, 2000.
8. Yao FY, Terrault NA, Freise C, Maslow L and Bass NM: Lamivudine treatment is beneficial in patients with severely decompensated cirrhosis and actively replicating hepatitis B infection awaiting liver transplantation: a comparative study using a matched, untreated cohort. Hepatology 34: 411-416, 2001.
9. Nakanishi S, Michitaka K, Miyake T, *et al*: Decompensated hepatitis B virus-related cirrhosis successfully treated with lamivudine allowing surgery for hepatocellular carcinoma. Intern Med 42: 416-420, 2003.
10. Liver Cancer Study Group of Japan: The general rules for the clinical and pathological study of primary liver cancer (in Japanese). 4th edition. Kanehara, Tokyo, p19, 2000.
11. Lin DY, Sheen IS, Chiu CT, Lin SM, Kuo YC and Liaw YF: Ultrasonographic changes of early liver cirrhosis in chronic hepatitis B: a longitudinal study. J Clin Ultrasound 21: 303-308, 1993.
12. Arii S, Yamamoto Y, Futagawa S, *et al*: Results of surgical and nonsurgical treatment for small-sized hepatocellular carcinomas: a retrospective and nationwide survey in Japan. The Liver Cancer Study Group of Japan. Hepatology 32: 1224-1229, 2000.
13. Okuda K, Ohtsuki T, Obata H, *et al*: Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Cancer 56: 918-928, 1985.
14. Llove JM, Bru C and Bruix J: Prognosis of Hepatocellular carcinoma: the BCLC staging classification. Semin Liver Dis 19: 329-337, 1999.
15. The Cancer of the Liver Italian Program (CLIP) Investigators. A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients. Hepatology 28: 751-755, 1998.
16. Kudo M, Chung H and Osaki Y: Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). J Gastroenterol 38: 207-215, 2003.
17. Seki T, Nonaka T, Kubota Y, Mizuno T and Sameshima Y: Ultrasonically guided percutaneous ethanol injection therapy for hepatocellular carcinoma. Am J Gastroenterol 84: 1400-1407, 1989.
18. Rossi S, Buscarni E, Garbagnati F, *et al*: Percutaneus treatment of small hepatic tumors by an expandable RF needle electrode. Am. J. Roentgenol 170: 1015-1022, 1998.
19. Llovet JM, Real ML, Montana X, *et al*: Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomized controlled trial. Lancet 359: 1734-1739, 2002.
20. Imamura H, Seyama Y, Kokudo N, *et al*: One thousand fifty-six hepatectomies without mortality in 8 years. Arch Surg 38: 1198-1206, 2003.
21. Perrillo R, Schiff E, Yoshida E, *et al*. Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. Hepatology 32: 129-134, 2000.
22. Liaw YF: Management of YMDD mutation during lamivudine therapy in patients with chronic hepatitis B. J Gastroenterol Hepatol 17: 333-337, 2002.

Liver International 2005: 25: 595–603
Printed in Denmark. All rights reserved

Liver International
DOI: 10.1111/j.1478-3231.2005.01043.x

Basic Studies

Early development of primary biliary cirrhosis in female C57BL/6 mice because of poly I:C administration

Okada C, Fazle Akbar SkMd, Horiike N, Onji, M. Early development of primary biliary cirrhosis in female C57BL/6 mice because of poly I:C administration.
Liver International 2005: 25: 595–603. © Blackwell Munksgaard 2005

Abstract: *Background:* Primary biliary cirrhosis (PBC) is one of the organ-specific autoimmune diseases characterized by destruction of the biliary epithelial cells, cholestasis, liver cirrhosis, and liver failure. With the postulation that induction of the autoimmune process might induce PBC-like cholangitis, here we used polyinosinic polycytidylic acid (poly I:C), an inducer of type-1 interferon (IFN), to generate an autoimmune cholangitis animal model. *Methods:* Female C57BL/6 mice were injected with 5 mg/kg of poly I:C twice a week for 28 consecutive weeks. Liver specimens were collected to evaluate the degree of cell infiltration. Autoantibodies, including antimitochondrial antibody (AMA), were assayed by immunofluorescence, enzyme-linked immunosorbent assay (ELISA) and immunoblotting. IFN-α was estimated in the sera by an ELISA method. Poly I:C injection induced IFN-α. *Results:* Mononuclear cells were detected at the portal areas 8 weeks after the start of poly I:C injection, which progressed up to 16 weeks. Autoantibodies, including AMA, were detected in the sera from all poly I:C-injected mice. *Conclusions:* In conclusion, we show an early development of a PBC-like cholangitis in a genetically susceptible mouse strain because of poly I:C administration. This model would be helpful to study PBC immunopathogenesis and to evaluate the effectiveness of newly developed therapeutic regimens for PBC.

Chizuko Okada, Sk. Md. Fazle Akbar, Norio Horiike and Morikazu Onji
Third Department of Internal Medicine, Ehime University School of Medicine, Ehime, Japan

Key words: AMA – animal model – poly I:C – primary biliary cirrhosis – type-1 interferon

Morikazu Onji, MD, Third Department of Internal Medicine, Ehime University School of Medicine, Ehime, Toon-shi,
Ehime 791-0295, Japan
Tel: +81-89-960-5308
Fax: +81-89-960-5310
e-mail: onjimori@m.ehime-u.ac.jp

Received 15 January 2004,
accepted 11 August 2004

Primary biliary cirrhosis (PBC) is one of the organ-specific autoimmune diseases characterized by the destruction of the biliary epithelial cells, cholestasis, liver cirrhosis, and liver failure (1). PBC diagnosis is comparatively easier because autoreactive antibodies (antimitochondrial antibody (AMA)) (2) are usually detected in PBC patients. The major antigens (M2 antigen: complex of three kinds of 2-oxo-acid dehydrogenase complexes) reactive to this antibody have also been detected (3–6). The disease remains mostly asymptomatic for prolonged periods and symptoms usually develop in patients with advanced stages of disease (7–9). Accordingly, the early cellular events underlying PBC pathogenesis are difficult to study in PBC patients because most of these patients attend the clinics during the later stages of the disease.

This prompted the development of PBC animal models by several investigators (summarized in Vierling (10)). Spontaneous development of PBC-like biliary lesions has been reported in some genetically susceptible mouse strains. PBC-like lesions were also developed by administration of the pyruvate dehydrogenase complexes (PDCs) or biliary epithelial cells in mice. Although PBC-like lesions might be developed in experimental animals, there are several limitations to these models in the context of understanding the pathogenesis of human PBC. However, PBC animal models might be useful to study the impact of newer drugs or novel therapeutic approaches. Hayashi et al. (11) and Kanda et al. (12) have reported the spontaneous development of PBC-like lesions in more than half of the female C57BL/6 mice at 18–24 months. However, the effect of newer drugs could not be tested in this model because mice became too old when PBC-like lesions were eminent. It was also uncertain which mice would and would not develop PBC-like lesions in this model.

We speculated that PBC-like lesions could be developed at an early time by inducing autoim-

mune processes in genetically susceptible mice. It is now evident that type-1 interferons (IFN) might be a useful agent for attaining this goal. Overproduction or increased levels of type-1 IFN have been documented either in the sera or in target organs from patients with systemic lupus erythematosus (13, 14) and rheumatoid arthritis (15). In addition, administration of type-1 IFN for therapeutic purposes has induced features of autoimmunity in patients with chronic viral hepatitis and renal carcinoma (16, 17). Moreover, there have been some reports about the exacerbation of PBC symptoms during therapy with type-1 IFN (18–20).

In this study, we speculated that type-1 IFN might cause the earlier onset of PBC-like cholangitis in female C57BL/6 mice. We used polyinosinic polycytidylic acids (poly I:C), a synthetic double stranded RNA (21), to induce type-1 IFN in female C57BL/6 mice. Type-1 IFN was produced in all mice because of poly I:C administration. Considerable numbers of mononuclear cells infiltrated into the liver starting at 8 weeks in all poly I:C-injected mice. These mice also showed autoantibodies, including AMA, a hallmark of PBC.

Material and methods

Mice

Adult 6–8 week-old C57BL/6J (H-2^b) mice were purchased from CLEA Japan, Inc. (Osaka, Japan). They were maintained separately at the Department of Biological Resources, Integrated Centre for Sciences (INCS), Ehime University, Ehime, Japan, under controlled conditions (22 °C, 55% humidity, and 12 h day/night), and were fed freely supplied standard laboratory chow and water. All animals received adequate care according to good laboratory practice guidelines. The Committee of Animal Experimentation, Ehime University School of Medicine, Japan approved this study protocol.

Poly I:C injection

Poly I:C injection was done according to the method of Qu et al. (22) with brief modifications. Poly I:C was obtained from Sigma Chemical Co. (St Louis, MO). It was dissolved in sterilized phosphate-buffered saline (PBS) at a concentration of 1 mg/ml and stored at −20 °C until needed. Female C57BL/6 mice were injected with graded doses of poly I:C (2, 5, 10, 15, 20, and 25 mg/kg of body weight) intraperitoneally twice a week for 28 consecutive weeks. As controls, a group of female C57BL/6 mice was injected with PBS according to the poly I:C injection protocol.

Mice were killed by cervical dislocation at different times after administration of poly I:C or PBS. Several tissues including liver, pancreas, salivary gland, and kidney were collected. Tissues were fixed in buffered formalin (10%) or snapped frozen for histological evaluation. Sera and tissue specimens were stored at −80 °C until used.

Histopathology and immunohistochemical examination

Formalin-fixed, paraffin-embedded tissues were used for histological evaluation. After deparafinization, various tissues were stained with hematoxylin and eosin. The extent of infiltrating cells in the liver tissues was evaluated by light microscopy. The total number of portal veins and portal veins with moderate cell infiltrations in the liver specimens were counted. The area of the liver specimens, the number of portal tracts, and the extent of cell infiltration were estimated using a combination of a digital camera and imaging tools. A digital camera system and Nikon ACT-1 software (NIKON Instech Co., Kanagawa, Japan) along with Scion image software (Scion Co., Frederick, MD) were used for this purpose. Periodic acid Schiff staining after digestion (d-PAS) was done in some liver specimens to see the relation between infiltrating cells and biliary epithelium in the liver.

Frozen sections of liver tissues were fixed in acetone and incubated with normal goat serum (1:100, Funakoshi, Osaka, Japan) to block binding with unrelated proteins. After adding optimum concentrations of primary antibodies, rat anti-murine Thy-1.2 [clone 53-2.1], biotin-conjugated antibodies to murine CD45/B220 [clone RA3-6B2], CD4 [clone H129.19], CD8α [clone 53-6.7], CD11b [clone M1/70] and CD11c [clone HL3] antibodies (all from BD Pharmingen, San Diego, CA), the specimens were incubated overnight at 4 °C. After washing in PBS, the specimens were incubated with rhodamine-conjugated anti-rat IgG or alkaline phosphatase-conjugated streptavadin (Dako Cytomation Co. Ltd, Kyoto, Japan) or Alexa Fluor 488 or Alexa Fluor 555-conjugated streptavadin (Molecular Probe Co. Eugene, OR).

The positive signals were visualized by Fuchsin (Dako Cytomation Co. Ltd) in specimens stained with alkaline phosphatase-conjugated streptavadin by light microscopy. Tissue specimens that were stained with Alexa Fluor 488 and Alexa Fluor 555 were visualized with a fluorescence microscope (Carl Zeiss Fluorescence Microscope, Gottingen, Germany). Signals from Alexa Fluor 488 were green, whereas Alexa Fluor 555 signals were red. Dual-positive cells stained yellow.

Biochemical analysis of the sera

The serum levels of alkaline phosphatase (ALP) and alanine amino-transferase (ALT) were measured by commercially available kit (WAKO Pure Chemical Industry, Osaka, Japan) exactly according to the manufacturer's protocol.

Detection of autoantibodies

Three methods were employed to detect various autoantibodies in the mouse serum. The existence of autoantibodies was firstly examined by staining HEp-2 cells (23) using the commercially available fluoroHEPANA test system (MBL, Nagoya, Japan). The sera were diluted 300 times in ice-chilled PBS. This dilution was estimated by measuring untreated young male mice serum as negative controls. Alexa Fluor 555-conjugated polyclonal goat anti-mouse immunoglobulin (Molecular Probes Inc.) was used as a secondary antibody. We also used frozen sections of rat kidney for immunofluorescence assay (IFA). Alexa Fluor 488-conjugated polyclonal goat anti-mouse immunoglobulin (Molecular Probes Inc.) was used.

Then, the levels of AMAs in the sera were measured semi-quantitatively by enzyme-linked immunosorbent assay (ELISA) (24–26) using the Immunis EIA kit (Institute of Immunology Co., Tochigi, Japan), according to the manufacturer's protocol with some modifications. Microtiter wells were coated with porcine heart M2 antigens. Diluted sera (1:1000) were added to the plates and incubated at room temperature for 30 min. Either horseradish peroxidase (HRP)-conjugated monoclonal goat anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary antibody. Color development was completed by tetramethylbenzidine. The optical density (OD) values of each of the wells were measured using a microplate reader (Nalge Nunc International K.K, Tokyo, Japan). Sera that were negative for autoantibodies by the immunofluorescence method were used as negative controls.

The existence of AMA in mouse sera was also confirmed by immunoblotting. Mitochondrial antigen (a kindly gift from Prof. Miyakawa) contained PDC-E2 (74 kDa), protein-X (52 kDa), branched chain 2-oxo dehydrogenase complex (BCOADC)-E2 (50 kDa), 2-oxoglutarate dehydrogenase complex (OGDC)-E2 (48 kDa) and PDC-E1 (41 kDa). This antigen was resolved by SDS-PAGE using commercially available gel (ATTO Co., Tokyo, Japan) and then transferred electrophoretically to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). After transfer, membranes were cut into strips and blocked with PBS-tween containing 10% skim milk. Then strips were incubated with diluted mouse sera (× 20) for 90 min and then washed with PBS-tween. The strips were then incubated with HRP-labeled goat anti mouse immunoglobulin (Sigma Chemical Co.), washed and visualized with diaminobenzidine (DAB) (Dako Cytomation Co. Ltd). Known AMA-positive human sera and sera from untreated male mouse were used as positive and negative control, respectively.

Serum IFN-α levels

IFN-α levels in the sera were measured by a commercially available ELISA Kit (PBL Biomedical Laboratories, Piscataway, NJ) according to the manufacturer's protocol. The OD values were estimated by a microplate reader (Nalge Nunc International K.K). The OD values of the samples were calibrated against the OD values of the standard containing known amounts of mouse IFN-α. The detectable levels of IFN-α are 10–500 pg/ml with this kit. Other cytokines were measured by commercially available cytometric beads assay (CBA) kit (BD Pharmingen).

Statistical analysis

Data are expressed as mean ± standard error (SE). The statistical analyses were done by the unpaired or paired *t*-test, χ^2 test, and Fischer's exact test, when indicated. Mann–Whitney's *U*-test was also utilized when the unpaired and paired *t*-test was not indicated, respectively. *P* values less than 0.05 were considered to indicate statistical significance. Statistical calculations were performed using the SPSS (version 10.0 J) statistical program on a Windows computer.

Results

IFN-α production in mouse serum because of poly I:C injection

The kinetics of IFN-α because of poly I:C injection were studied in female C57BL/6 mice. Although IFN-α could not be detected in the sera of untreated mice, IFN-α was induced in these mice by poly I:C injection. Kinetic studies showed that the IFN-α levels were highest within 3–6 h after poly I:C injection, then gradually decreased and became almost undetectable 24 h after injection (data not shown). In this study, poly I:C was injected twice a week. When poly I:C was subsequently injected 72 h after the first injection, the IFN-α levels were again elevated. The IFN-α levels in female C57BL/6 mice 3 h

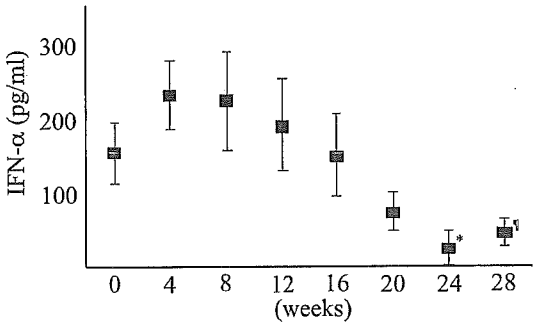

Fig. 1. Production of interferon-α (IFN-α) because of polyinosinic polycytidylic acid (poly I:C) administration. Female C57BL/6 mice were injected with 5 mg/kg of poly I:C intraperitoneally twice a week for 28 consecutive weeks. Sera were collected 3 h after injecting poly I:C for 0 (first injection), 4, 8, 12, 16, 20, 24, and 28 weeks. The IFN-α levels in the sera were estimated by enzyme-linked immunosorbent assay and expressed as pg/ml. Data are shown as the mean ± SE. The mean data of at least three mice are plotted. $*P = 0.036$ compared with 12 weeks, $¶P = 0.032$, compared with 12 weeks.

after poly I:C injection are shown in Fig. 1. Few sera from poly I:C-injected mouse were measured by CBA kit. Interleukin (IL)-12p70, IL-10, Monocyte Chemoattractant Protein (MCP)-1, IFN-γ levels were raised within 3 h after the injection. However, the levels of IL-6 remained higher until 24 h.

Induction of PBC-like lesions in the livers of female C57BL/6 mice treated with poly I:C

The mice were injected twice a week with different doses of poly I:C for 28 consecutive weeks. Preliminary studies indicated that injection with poly I:C at 5 mg/kg of body weight would be optimum because higher doses of poly I:C (20 or 25 mg/kg of body weight) did not flare up the PBC-like lesions in these mice. Representative staining patterns of mononuclear cell infiltration in the liver tissues because of injection with 5 mg of poly I:C/kg of body weight are shown in Fig. 2. Cellular infiltration in the liver tissues was extremely low 4 weeks after poly I:C injection (A); however, considerable numbers of infiltrating cells were detected at 8 weeks after poly I:C injection (B). After this time point, the extent of the infiltrating cells progressively increased (B–G). A representative staining pattern of the magnified versions of the portal areas at 16 weeks after the start of poly I:C injection is shown in Fig. 2H. Some infiltrating cells accumulated around the damaged bile ducts (indicated by the arrowhead). The localization of infiltrating cells near the basement membrane of bile duct was also confirmed by d-PAS staining (data not shown).

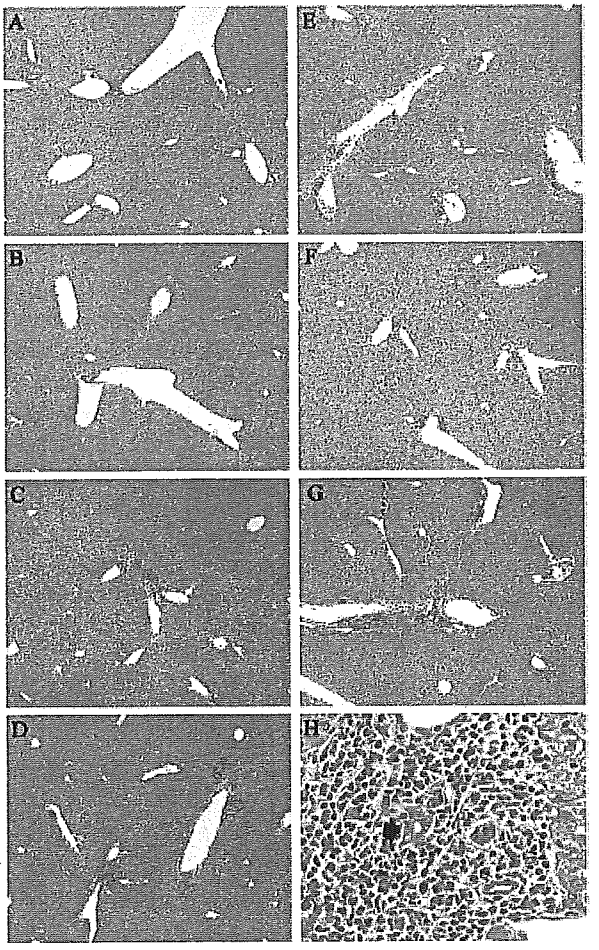

Fig. 2. Serial observation of infiltration of mononuclear cells in the liver tissue because of polyinosinic polycytidylic acid (poly I:C) administration. Female C57BL/6 mice were injected with 5 mg/kg of poly I:C twice a week for 28 consecutive weeks. Liver specimens were collected at 4 (A), 8 (B), 12 (C), 16 (D), 20 (E), 24 (F), and 28 (G) after the start of poly I:C injection and were stained with hematoxylin and eosin (H.E). Mononuclear cells are present mainly at and around the portal areas. A representative staining pattern from each period is shown. A representative magnified version of H.E staining shows distorted bile ductules in the portal areas along with many infiltrating cells at 16 weeks after the commencement of poly I:C administration (H). Magnification: (A–G), × 4; (H), × 40.

The number of portal tracts with infiltrating mononuclear cells was estimated for the beginning of the study at 4-week intervals. As shown in Fig. 3, the number of portal tracts with more than 100 infiltrating mononuclear cells progressively increased up to 16 weeks.

Serum ALT levels were raised among the poly I:C injected mouse compared with untreated mouse (35.0 ± 3.8 IU/l vs. 13.7 ± 1.7 IU/l, $P = 0.006$). Serum ALP levels also raised among the poly I:C injected mouse (70.7 ± 5.5 IU/l vs. 50.2 ± 5.9 IU/l, $P = 0.054$).

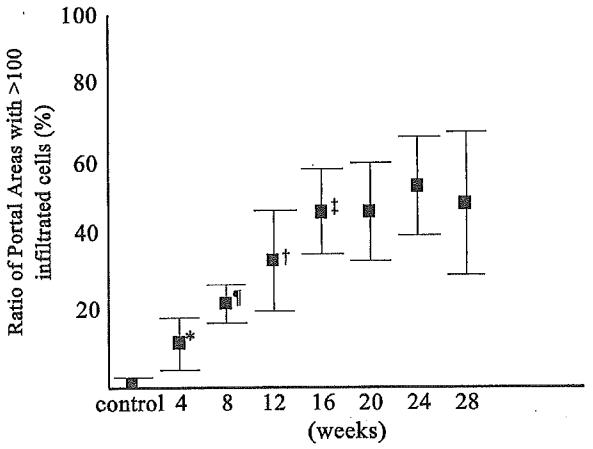

Fig. 3. Increased infiltration of mononuclear cells at the portal areas verses time because of polyinosinic polycytidylic acid (poly I:C) administration. Female C57BL/6 mice were injected with 5 mg/kg of poly I:C twice a week for 28 consecutive weeks. Liver specimens were collected at 4, 8, 12, 16, 20, 24, and 28 weeks after the start of poly I:C injection or from control mice and were stained with hematoxylin and eosin. The portal tracts with size of more than 0.01 mm^2 were counted as total portal areas in the liver specimens. Among them, the number of portal areas with more than 100 mononuclear cells was also counted as cell infiltrated portal areas. Data are shown as the mean ± SE of the ratio of cell infiltrated portal areas to that of the total portal areas. The mean data of at least five mice are plotted. $^*P<0.001$, compared with control; $¶P=0.017$, compared with 4 weeks, $†P=0.01$, compared with 8 weeks, $‡P=0.002$, compared with 12 weeks.

Development of autoantibodies in female C57BL/6 mice injected with poly I:C

Although female C57BL/6 mice injected with poly I:C (5 mg/kg of body weight) exhibited infiltration of mononuclear cells in the liver, the prevalence of autoantibodies, including AMA was examined in the sera from these mice. A representative staining pattern of autoantibodies of anti-nuclear type (all show speckled type) (A), anticytoplasmic type (suspected AMAs) (B), and mixed type (C) using HEp-2 cells by an immunofluorescence method is shown in Fig. 4. The IFA staining pattern of Rat kidney section was also shown in Fig. 4 (untreated mouse sera (D), AMA positive (E), and mixed type (F)).

The prevalence of the different autoantibodies has been compiled in Table 1. Autoantibodies were not detected in untreated mice. However autoantibodies were detectable from 4 weeks after the start of poly I:C injection. By 24 weeks, all of the poly I:C-injected mice exhibited one or both types of autoantibodies.

AMA in the mouse sera were measured by ELISA. As shown in Fig. 5A, 27.8% of female C57BL/6 mice injected with poly I:C developed AMA in the sera at 8 weeks. Eighty-seven percentage of female C57BL/6 mice administrated with poly I:C developed AMA in the sera at 24 weeks after injection commencement.

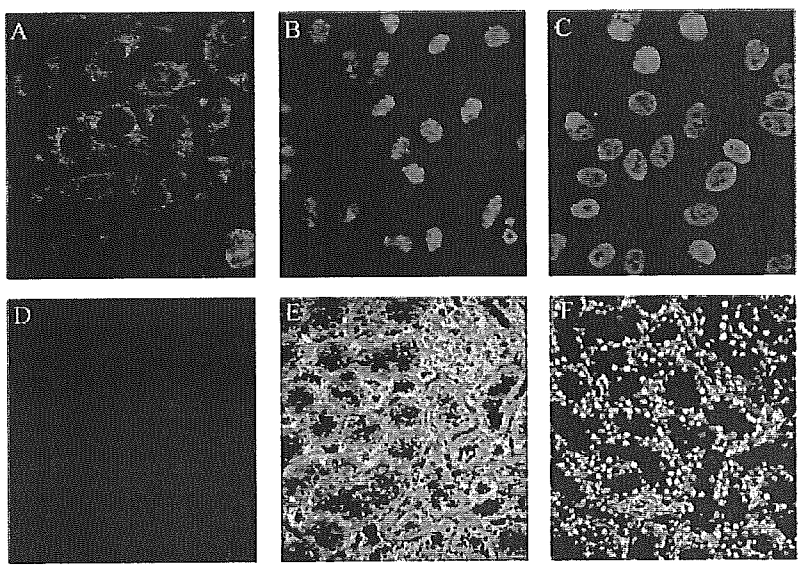

Fig. 4. Presence of autoantibodies in the sera of mice injected with polyinosinic polycytidylic acid (poly I:C). HEp-2 cells were incubated with the sera from mice injected with poly I:C. The presence of anticytoplasmic antibodies (A), antinuclear antibodies (B) and coexistence of both antibodies (C) was evaluated by an immunofluorescence method using Alexa Fluor 555 conjugated polyclonal anti mouse immunoglobulin antibody. Frozen sections of rat kidney were incubated with the sera from mice injected with poly I:C or untreated mice (negative control). Color development was done by Alexa Fluor 488. Autoantibody was not detected in the sera from untreated mice (D). The existence of anticytoplasmic antibodies (E) and coexistence of both antibodies (F) in the sera of poly I:C injected mice are shown. A representative staining pattern of autoantibodies from the sera of female C57BL/6 mice injected with poly I:C is shown.

Table 1. Prevalence of autoantibodies in female C57BL/6 mice administered poly I:C

Injection periods (weeks)	4	8	12	16	20	24	28
Anticytoplasmic type	41.7	60.0	66.7	64.0	90.0*	100.0†	83.3
Antinuclear type	33.3	30.0	40.0	60.0	50.0	75.0	50.0

Female C57BL/6 mice were injected with poly I:C and sera were collected at the indicated time. Each groups contained at least five mice. Autoantibodies in these sera were measured by an immunofluorescence method using HEp-2 cells and were defined as anticytoplasmic type and antinuclear type based on the manufacturer's instructions. The data are shown as autoantibody positivity among total mice used in the study. poly I:C, polyinosinic polycytidylic acid. *$P = 0.031$ compared with that of 4 weeks, †$P = 0.015$ compared with that of 4 weeks.

Table 2. Incidence of extrahepatic lesions in mice injected with poly I:C

Injection periods (weeks)	4	8	12	16	20	24	28
Salivatitis	0	26.7	40.0	70.0	62.5	83.3	90.9
Pancreatitis	16.7	26.7	64.0*	100.0†	87.5	83.5	54.5
Interstitial nephritis	0	0	12.0	30.0	37.5	33.3	45.5

Female C57BL/6 mice were injected with poly I:C and different tissues were collected at different times after the start of poly I:C administration. Data show the incidence of extrahepatic lesions at different times after the start of poly I:C administration among the total mice in the groups, which consisted of at least five mice. poly I:C, polyinosinic polycytidylic acid *$P = 0.048$, compared with that of 8 weeks. †$P = 0.036$, compared with that of 12 weeks.

The AMA in the sera was also confirmed by immunoblotting. Figure 5B showed the representative patterns of the immunoblotting. Sera 1 recognized 74 kDa protein (PDC-E2) and sera 2 recognized 41 kDa protein (PDC-E1).

Extrahepatic lesions in female C57BL/6 mice injected with poly I:C

PBC is associated with an autoimmune type of lesion in various extrahepatic organs. The relative frequencies of salivatitis, pancreatitis, and interstitial nephritis in these mice at every 4 weeks of injection have been summarized in Table 2.

Nature of infiltrating cells at the liver because of poly I:C injections

Poly I:C injection led to the accumulation of mononuclear cells in the liver tissues. At the same time, high levels of IFN-α were detected in the sera after poly I:C injection. Thus, it was important to analyze the types of cells that infiltrated the liver because of the poly I:C injection. As shown in Fig. 6, T and B lymphocytes constituted the main bulk of the infiltrating cells of the liver (A). Among the T lymphocytes, both $CD4^+$ and $CD8\alpha^+$ T cells were localized in the liver (B). $CD8\alpha^+$ T cells were localized near the bile ducts as detected by using Fuchsin as chromogen (data not shown). In addition, $CD11b^+$ macrophages and $CD11c^+$ dendritic cells were detected in the liver tissues (C).

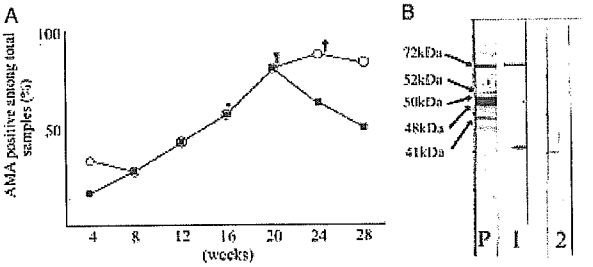

Fig. 5. (A) Antimitochondrial antibody (AMA) expression in the sera of female C57BL/6 mice injected with polyinosinic polycytidylic acid (poly I:C). AMA was measured by enzyme-linked immunosorbent assay (ELISA). Sera negative for autoantibodies by the immunofluorescence method were used as the negative controls. Monoclonal goat anti-mouse IgM or IgG was used as the secondary antibody. AMA positivity was ascertained from the optical density (OD) values of the AMA-negative sera. Sera showing OD values greater than the mean+3 standard deviations from the negative controls were regarded as AMA positive. Open circle indicate IgG, closed square indicate IgM. *$P = 0.01$ (IgG), compared with 4 weeks, ¶$P = 0.046$ (IgG) or 0.003 (IgM), compared with 4 weeks, 0.016 compared with 8weeks, †$P = 0.03$, compared with 4 weeks, 0.009 compared with 8 weeks. (B) The presence of AMA in the sera was confirmed by immunoblotting. Mitochondrial antigen resolved by SDS-PAGE contained pyruvate dehydrogenase complex (PDC)-E2 (74 kDa), protein-X (52k Da), branched chain 2-oxo dehydrogenase complex (BCOADC)-E2 (50 kDa), 2-oxoglutarate dehydrogenase complex (OGDC)-E2 (48 kDa) and PDC-E1 (41 kDa). Horseradish peroxidase-conjugated anti mouse immunoglobulin was used as the secondary antibody and diaminobenzidine was used as chromogen. Immunoblotting profile of a known AMA-positive human serum was shown in lane P as positive control. Sera positive for AMA by ELISA method (taken from poly I:C-treated mice) were applied to Lanes 1 and 2.

Discussion

A proper animal model of disease is essential when the cellular and molecular events underlying the pathogenesis of that disease could not be adequately understood from patient examination. Moreover, animal models are important to study the effects of newly developed drugs and therapeutic regimens because most of these approaches