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Table 2. Observed vs. expected rates of birth for every season in 340 patients with Crohn's disease

	Observed number	Expected number
Season		
Spring	87	85.7
Summer	99	85.7
Autumn	70	84.8
Winter	84	83.8
χ^2(11 d.f.)	4.66	
P value	0.198	

ically predisposed individuals. Exposure to external seasonal factors during the maturation of the immune system is suspected to be an inducing factor for IBD. Perinatal exposure to infections has been proposed as such an environmental factor. Many childhood infections show seasonal variation. Other environmental factors, which are potential trigger factors for IBD, including the use of cigarettes, non-steroidal anti-inflammatory drugs, antibiotics, and contraceptives, have shown seasonal variation since seasonal changes in immune response were first reported (10,11).

In conclusion, in this study, the first performed in Italy, a significant association between the occurrence of CD and birth in the month of July was found. On the other hand, no different seasonal birth patterns were observed. We think that the numerous potential links between the clustering of birth dates and seasonal variations of both immune function and environmental factors should be evaluated by large population-based studies.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Development of Pouchitis With Combination Therapy With Peg-Interferon α-2b and Ribavirin for Chronic Hepatitis C in a Patient With Ulcerative Colitis Who Underwent Pouch Surgery

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To the Editor: A 41-year-old woman was diagnosed with ulcerative colitis (UC), total colitis type, in 2002. She was also infected with hepatitis C virus, genotype 1b, viral load 827 KIU/ml. In April 2004, she had a recurrence of UC that proved refractory to medication, and underwent colectomy with temporary ileostomy in May 2004. Ileal pouch anal canal anastomosis was performed in February 2005. In August 2005, endoscopic examination revealed only some aphthous lesions in the ileal pouch. At that time, she had four defecations per day, but was otherwise asymptomatic. The modified Pouchitis Disease Activity Index (PDAI) score (1) was two points. She had a good clinical course after surgery. Combination therapy for chronic hepatitis C with Peg-interferon (Peg-IFN) α-2b (1.5 μg/kg per week) and ribavirin (600 mg/day) for 24 weeks was started in June 2006.

The frequency of watery diarrhea had gradually increased over 3 months after the initiation of Peg-IFN and ribavirin therapy. However, no abdominal pain, fever, or bloody diarrhea was noted. Bloody diarrhea appeared 6 weeks later. After completion of combination therapy in mid-November 2006, endoscopy revealed erosions, friable mucosa, and purulent mucus in the pouch (**Figure 1a**). The modified PDAI score at that time was 9 points, confirming the diagnosis of pouchitis. Treatment with metronidazole (500 mg/day) was begun, and her symptoms improved rapidly. Endoscopy after treatment with metronidazole revealed only several tiny aphthae in the pouch (**Figure 1b**).

Antiviral treatment appears to have induced pouchitis in the present case, as the patient had been doing well with no pouchitis until antiviral treatment was initiated. Several case reports of development and exacerbation of UC after IFN-α treatment (2,3) also suggest that antiviral treatment can cause pouchitis.

The etiology of pouchitis remains unknown. We previously detected specific

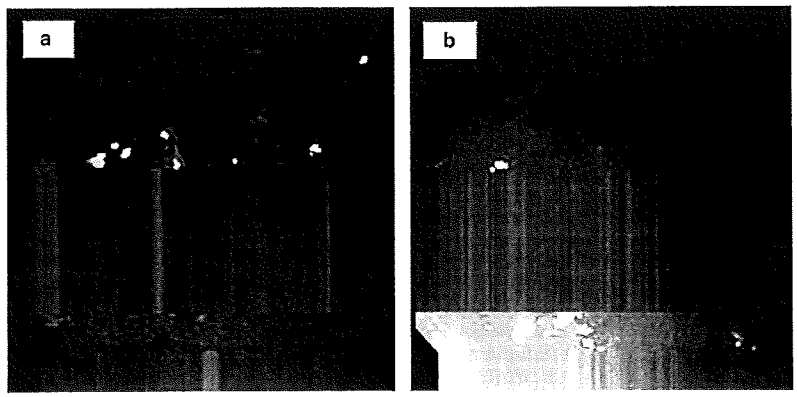

Figure 1. Endoscopic findings for the pouch mucosa. (a) Pouchitis induced by combination therapy before treatment with metronidazole. Erosions, friable mucosa, and purulent mucus were noted in the pouch. (b) No erosions were found after treatment with metronidazole.

proliferation of immature plasma cells in the inflamed pouch mucosa of patients with UC (4). In the present case, enhancement of humoral immunity by IFN-α administration could have caused dysregulation of differentiation of plasma cells that led to the development of severe pouchitis.

CONFLICT OF INTEREST

Guarantor of the article: Kenichi Morimoto, MD.

Specific author contributions: Kenichi Morimoto: planned the study; collected and interpreted data, and drafted the manuscript; Hirokazu Yamagami, Shuhei Hosomi, Mizuki Ohira, Takehisa Suekane, Noriko Kamata, Mitsue Sogawa, and Kenji Watanabe: collected and interpreted data; Kazunari Tominaga, Toshio Watanabe, Yasuhiro Fujiwara, and Akihiro Tamori: interpreted data; Nobuhide Oshitani: collected and interpreted data; Tetsuo Arakawa: interpreted data.

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Acute Hemorrhage With Retroperitoneal Hematoma After Endoscopic Ultrasound-Fine Guided-Needle Aspiration of an Intraductal Papillary Mucinous Neoplasm of the Pancreas

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To the Editor: Endoscopic ultrasound (EUS)-guided fine-needle aspiration (FNA) is effective for tissue diagnosis in suspected pancreatic cancer (1) and for collecting fluid from cystic tumors (2). Major complications after EUS-FNA of solid masses are rare, but cystic tumors seem to have a higher risk of infection and bleeding. In a series of 50 patients undergoing EUS-FNA of pancreatic cystic lesions, Varadarajulu and Eloubeidi (3) found acute intracystic hemorrhage at the site of cyst aspiration in three cases (frequency 6%: 95% confidence interval 1.3–16.6). There was no pancreatitis or infectious complications. Clinical history and laboratory parameters did not predict which patients were at risk for intracystic hemorrhage (3).

A 66-year-old woman presented with multiple pancreatic cysts found by chance during a transabdominal ultrasound. Computed tomography and magnetic resonance imaging confirmed an intraductal papillary mucinous neoplasm of the main pancreatic duct and side branches involving the whole pancreas. The largest cyst, measuring 3 cm, was in the tail. EUS-FNA of this cyst was done from the stomach with a 22-gauge fine needle (**Figure 1**); 10 ml of clear mucous were aspirated and the cyst collapsed completely. After removal of the needle, intracystic hemorrhage occurred at the site of aspiration, manifesting as a fine hyperechoic flow that progressed gradually to involve the entire cyst, then leaked into the retroperitoneal space along the needle path as an expanding echo-rich region (**Figure 2**). Color and power Doppler before the puncture had

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Frequent Detection of Hepatitis B Virus DNA in Hepatocellular Carcinoma of Patients With Sustained Virologic Response for Hepatitis C Virus

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Hepatocellular carcinoma (HCC) develops several years after the eradication of hepatitis C virus (HCV) by interferon therapy. Risk factors for the development of HCC are only partly understood. To elucidate the role of occult hepatitis B virus (HBV) infection in hepatocarcinogenesis in patients with sustained virologic response, the prevalences of HBV-related makers were examined. Study group comprised 16 patients with sustained virologic response (group A) and 50 with HCV (group B). Anti-HBc and anti-HBs in serum were examined by enzyme-linked immunoassay. HBV DNA in liver was examined by nested polymerase chain reaction, using primers specific for genes encoding for HBx, HBsAg, HBcAg, and HBV cccDNA. Sequence of the amplified HBV DNA for 'a' determinant of HBsAg was determined in HCC. Anti-HBc was positive in 10 of 16 in group A and 25 of 50 in group B. HBV DNA in liver was detected in 12 of 16 in group A and 21 of 50 in group B ($P=0.044$). In group A, HBV DNA in liver was detected frequently in patients without cirrhosis and in those with a longer period from the time of HCV eradication to the development of HCC. Mutation in 'a' determinant of HBsAg was found in three HCC of group A. Occult HBV infection may be one of the most important risk factors in hepatocarcinogenesis of Japanese patients with sustained virologic response. ***J. Med. Virol. 81:1009–1014, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: anti-HBc; anti-HBs; interferon; occult HBV infection

INTRODUCTION

In Japan, a country endemic for hepatitis B virus (HBV) and hepatitis C virus (HCV), more than 75% of cases of hepatocellular carcinoma (HCC) are attributable to HCV-related chronic liver disease, and nearly 15% are attributable to HBV-related liver disease [Ikai et al., 2007]. Several reports have focused on the clinical role of past HBV infection as indicated by the presence of anti-HBc and the absence of HBsAg in patients with HCV. Marusawa et al. [1999] reported that the prevalence of anti-HBc was high in patients with chronic liver disease, especially in HCC associated with HCV. A retrospective study of 412 patients with HCV showed that the risk of hepatocarcinogenesis increased twofold in patients with anti-HBc [Chiba et al., 1996]. Prospective studies have also demonstrated that the presence of anti-HBc is an important risk factor for HCC in patients with HCV [Tanaka et al., 2006; Ikeda et al., 2007]. Conversely, some studies failed to support a role of previous HBV infection in hepatocarcinogenesis [Hiraoka et al., 2003; Stroffolini et al., 2008].

Another important clinical feature is occult HBV infection, defined as the presence of detectable levels of HBV DNA in liver despite the absence of serum hepatitis B surface antigen (HBsAg). The HBV genome is detectable frequently in liver tumors from HBsAg-negative patients with HCV-related liver disease, suggesting that occult HBV infection may contribute to the progression of liver damage and the development of HCC in HCV-positive patients [Cacciola et al., 1999; Tamori et al., 1999, 2003; Squadrito et al., 2006; Pollicino et al., 2004].

Interferon (IFN) has potent antiviral activity against HCV. Complete eradication of HCV by antiviral therapy is associated with a considerable reduction in the

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incidence of HCC [Yoshida et al., 1999; Tanaka et al., 2000]. Nevertheless, recent studies have shown that HCC develops in 2.5–4.2% of patients who have a sustained virologic response [Toyoda et al., 2000; Ikeda et al., 2005; Kobayashi et al., 2007]. These patients may have had advanced liver fibrosis at the time of HCV eradication, and subclinical tumors might already be present in the liver at the end of IFN therapy [Makiyama et al., 2004]. In some patients with sustained virologic response, however, HCC develops from liver without fibrosis several years after the eradication of HCV by IFN. The etiology of such cases of HCC remains obscure. Delineation of important features of HCC that develop after the elimination of HCV as compared with those established during sustained HCV infection may contribute to a better understanding of the mechanisms involved.

In the present study, resected liver specimens were examined to evaluate the role of previous and occult HBV infection in the development of cancer after the clearance of HCV by IFN treatment. The present results might contribute to a clearer definition of patients at high risk for HCC after the eradication of HCV.

MATERIALS AND METHODS

Patients

Sixteen consecutive patients who underwent surgical resection of HCC in Osaka City University Hospital after eradication of HCV by interferon monotherapy from June 1998 through July 2007 (group A) were studied (Table I). All patients with sustained virologic response were male in the present study. As a sex-matched control, 50 consecutive HCV-RNA-positive men with HCC during the same period were studied (group B). Serological markers showed that anti-HCV was positive and HBsAg was negative in all patients. One portion of each tissue sample from the 66 patients with HCC was frozen in liquid nitrogen immediately after resection and stored at −80°C until analysis. Total DNA was extracted from these portions by conventional methods as described previously [Tamori et al., 2003]. None of the patients had a history of exposure to aflatoxin B1, insulin administration, hereditary hemochromatosis, autoimmune hepatitis, or primary biliary cirrhosis. The activity of hepatitis and the stage of fibrosis were determined according to a modified version of Desmet's classification in liver tissue specimens obtained before IFN therapy and in noncancerous liver tissue obtained intraoperatively.

Anti-HBc and Anti-HBs in Serum

Antibodies to HBsAg (anti-HBs) and anti-HBc in patient sera were tested by enzyme immunoassay and/or radioimmunoassay, using commercially available kits (Dainabott, Tokyo, Japan).

Detection of HBV DNA in Serum and Liver

DNA was extracted from 100 μl of serum or 10 mg of liver tissue by phenol/chloroform extraction, as described previously [Tamori et al., 2003]. HBV DNA in serum or in liver was amplified with specific primers for genes encoding for HBx, HBsAg, and HBcAg (sequences of the primers shown in Table II). Amplification was done in a thermal cycler for 35 cycles: 95°C for 30 sec, 55°C for 60 sec, and 72°C for 60 sec in 40 μl of a reaction buffer containing 30 pmol of the two appropriate primers, four deoxynucleotides each at a concentration of 100 μM, polymerase chain reaction (PCR) buffer, and 2.5 units of Gold Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). The first PCR product (2 μl) was used in a second PCR. To examine HBV covalently closed circular DNA (cccDNA) in liver, extracted DNA

TABLE I. Clinical Characteristics of HCC-Patients With Sustained Virological Response and With HCV

	Group A: sustained virologic response	Group B: HCV	*P*-value
Patients, n	16	50	
Age	66.1 (55–79)	65.7 (55–76)	0.465
HBsAg (+/−)	0/16	0/50	
Anti-HBs (+/−)	6/10	9/41	0.201
Anti-HBc (+/−)	10/6	25/25	0.559
Anti-HCV (+/−)	16/0	50/0	
HCV RNA (+/−)	0/16	50/0	
Alcohol			
>30 g/day	0	6	
<30 g/day	6	20	
None	10	24	0.47
Diabetes melitus (+/−)	2/14	17/33	0.182
Hypertention (+/−)	7/9	18/32	0.795
BMI	24.1 (17.4–28.1)	23.0 (17.9–29.8)	0.188
Hepatic cirrhosis (+/−)	4/12	24/26	0.184
Tumor grades			
Well-d	0	5	
Moderately d	6	25	
Poorly d	10	20	0.199

BMI, body mass index, Well-d, well-differentiated HCC, Moderately d: moderately differentiated HCC, Poorly d: poorly differentiated HCC.

TABLE II. Primers Used for Amplification of HBV DNA

Forward primers		Reverse primers	
HBx-1: 1220–1239	5′-CTCTCTCGGAAATACACCTC	HBx-2: 1818–1799	5′-GTAACTCCACAGAAGCTCCA
HBx-3: 264-1293	5′-TGCCAACTGGATCCTGCGCGG-GACGTCCTT	HBx-4: 1742–1723	5′-GGCTTGAACAGTAGGACATG
HBs-1: 391–408	5′-AAGACCTGCACGATTCCT	HBs-2: 672–654	5′-TAGAGGTAAAAAGGGACTC
HBs-3: 499–517	5′-TTCGCAAGATTCCTATGGG	HBs-4: 634–617	5′-GCCCCCAATACCACATCA
HBc-1: 1690–1708	5′-AACTTTTTCACCTCTGCCT	HBc-2: 1945–1929	5′-GCTTGCCTGAGTGCTGT
HBc-3: 1731–1749	5′-ACTGTTCAAGCCTCCAAGC	HBc-4: 1848–1829	5′-AAGGAAAGAAGTCAGAAGGC
P23: 1443–1462	5′-CTGAATCCCGCGGACGACCC	P24: 1891–1871	5′-ACCCAAGGCACAGCTTGGAGG
P25: 1553–1573	5′-GTCTGTGCCTTCTCATCTGCC	P26: 1846–1823	5′-AGATGATTAGGCAGAGGT-GAAAAA
HBs'a'-1: 52–71	5′-CTAGGACCCCTGCTCGTGTT	HBs'a'-2: 545–526	5′-AGCCAGGAGAAACGGACTGA
HBs'a'-3: 69–89	5′-GTTACAGGCGGGGTTTTTCTT		

was amplified with primers P23, 24, 25, and p26 (Table II). The amplification procedure and primers have been described previously [Tamori et al., 2005]. The nested PCR analysis was sufficiently sensitive to detect more than 10 copies of HBV DNA [Yotsuyanagi et al. 2000]. Occult HBV infection was diagnosed when two or more regions of HBV DNA were amplified, as reported previously [Pollicino et al., 2004].

Sequence of 'a' Determinant of HBsAg and HBV Genotyping

HBV DNA in liver was amplified with specific primers for HBS 'a' determinant (sequences of the primers shown in Table II). PCR (an initial incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min) was performed in a final volume of 50 μl with a GeneAmp PCR system 9600 (Perkin-Elmer Life Sciences Japan, Tokyo, Japan). Aberrant PCR products were purified with a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and sequenced with an Applied Biosystems DNA sequencer (Perkin-Elmer) and a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Tokyo, Japan). HBV genotyping in liver was performed by PCR using type-specific primers [Naito et al., 2001].

Statistical Analysis

Statistical analysis was performed with the Statview SE+Graphics program, version 5.0 (SAS Institute, Cary, NC). The Mann–Whitney *U*-test was used to compare two continuous variables, and the χ^2 test was used to compare two categorical variables. All tests was two-sided and P value <0.05 was considered to be statistically significant.

Ethical Considerations

This study protocol complied with the ethical guidelines of the Declaration of Helsinki (1975) and was approved by the Ethics Committee of Osaka City University Graduate School of Medicine.

RESULTS

Histological Findings in Patients With HCC

In group A, 4 patients (25%) had hepatic cirrhosis. In group B, 24 patients (48%) had hepatic cirrhosis. This difference was not significant. Table I shows the histological tumor grade in each group. There was no significant difference between the two groups.

In patients with sustained virologic response, the period from the end of IFN treatment to the diagnosis for HCC ranged from 13 to 177 months. Histological examinations before and after IFN treatment, performed in 11 of the 16 patients in group A, showed that the staging of hepatic fibrosis improved in 5 patients and the grade of hepatic activity improved in 10 patients (Table III). In noncancerous liver, there was no fat accumulation.

Anti-HBc, Anti-HBs, and HBV DNA in Serum

Anti-HBc was positive in 10 (62.5%) of 16 patients in group A and 25 (50%) of 50 patients in group B ($P=0.56$). Anti-HBs was detected in 6 (37.5%) of 16 patients in group A and 9 (18%) of 50 patients in group B ($P=0.2$). HBV DNA was not detected in serum from either patients in group A or those in group B.

HBV DNA in the Resected Liver

HBV DNA in the resected liver was found in 12 (75%) of 16 patients in group A and 21 (42%) of 50 in group B. The rate of HBV DNA detection was significantly higher in patients with sustained virologic response than in patients with HCV ($P=0.044$).

In group A, HBV DNA was detected in 7 resected livers from 10 patients with anti-HBc and in 5 resected livers from 6 patients without anti-HBc.

Among patients in group A, HBV DNA was detected in 11 (92%) of 12 patients without cirrhosis and 1 (25%) of 4 patients with cirrhosis ($P=0.046$). Among patients in group B, HBV DNA was detected in 11 (42%) of 26 patients without cirrhosis and in 10 (42%) of 24 patients with cirrhosis (Table IV).

TABLE III. Clinicopathological Data in HCC-Patients With Sustained Virologic Response

Case	Pre-IFN therapy				Period from HCV eradication to diagnosis of HCC	At operation	
	Genotype	Viral load	F factor	A factor		F factor	A factor
56	1b	1 Meq	2	2	45 months	2	1
101	2a	Unknown	3	2	19 months	4	2
149	2a	1.1 Meq	4	3	20 months	4	2
196	2b	Unknown	2	2	41 months	1	2
198	2a	0.4 Meq	2	2	103 months	1	1
200	2a	1.1 Meq	2	2	13 months	2	1
221	2a	0.9 Meq	2	3	80 months	2	2
268	Unknown	Unknown	Unknown	Unknown	144 months	1	1
269	2a	0.4 Meq	2	3	156 months	0	0
271	1b	Unknown	4	1	156 months	3	1
325	1b	300 KIU	3	2	15 months	2	1
327	2b	160 KIU	3	3	36 months	4	2
328	1b	Unknown	Unknown	Unknown	14 months	4	2
336	1b	100 KIU	2	2	152 months	2	0
340	1b	0.9 Meq	2	3	177 months	2	1
347	Unknown	Unknown	Unknown	Unknown	60 months	3	1

F factor is hepatic fibrosis score in the liver. A factor is hepatitis activity in the liver.

HBV cccDNA was detected in 5 (31%) of 16 patients in group A and in 7 (14%) of 50 patients in group B ($P = 0.24$).

Average of the period from the end of IFN treatment to the diagnosis of HCC was 94 months in patients with occult HBV infection and 22 months in those without occult HBV infection. In group A, HBV DNA was detected in all patients in whom HCC developed more than 40 months after the disappearance of HCV (Table IV).

Mutation of the 'a' Determinant of HBsAg and HBV Genotype in Patients With Sustained Virologic Response

In 6 of 12 patients with occult HBV infection, the 'a' determinant area of HBsAg was amplified. Sequencing of the PCR product showed that codon 126 ATT (Ile) had mutated to GTT (Val) in two cases. One of them had two mutations, which were TCG to TTG at codon 54 and GCT to GGT at codon 157. In the other case, TTC had mutated to TCT at codon 93. No other mutations in the 'a' determinant area were found. HBV genotyping was performed in 12 patients with occult HBV infection. Eight patients were infected with HBV genotype C. In the four other patients, the HBV genotype could not be determined.

DISCUSSION

Previous studies have reported that 43–59% of patients with HCC related to HCV are positive for anti-HBc [Marusawa et al., 1999; Ikeda et al., 2007; Stroffolini et al., 2008]. In the present study, anti-HBc and anti-HBs were detected respectively in 50% and 18% of 50 patients with HCV-HCC. As compared with patients who had HCV-HCC, anti-HBc and anti-HBs were detected more frequently in HCC-patients with

TABLE IV. Clinical Characteristics of HCC-Patients With Sustained Virologic Response and With HCV; Stratified According to Occult HBV Infection

	Sustained virologic response		HCV	
	With occult HBV	Without occult HBV	With occult HBV	Without occult HBV
N	12	4	21	29
Age	66.1	66.3	63.8	65.3
Cirrhosis/non-cirrhosis	1/11*	3/1*	10/11	14/15
Period from HCV eradication to diagnosis of HCC (months)	94** (14–177)	22** (13–36)	—	—
Alcohol				
>30 g/day	0	0	4	2
<30 g/day	5	1	5	15
None	7	3	12	12
Diabetes melitus (+/−)	2/10	0/4	5/16	12/17
Hypertension (+/−)	5/7	2/2	8/13	10/19
BMI	23.9	24.4	23.6	22.5

*$P = 0.046$.
**$P = 0.036$.

sustained virologic response. Recent studies show a higher prevalence of anti-HBc in HCC-patients with sustained virologic response [Ikeda et al., 2007; Stroffolini et al., 2008]. The results in the present study were consistent with these reports. The prevalence of anti-HBc is higher among the elderly population in Japan. In the present study, mean ages and age distributions did not significantly differ between the patients with sustained virologic response and the patients with HCV. Taken together, anti-HBc might be one of the specific characteristics of HCC-patients with sustained virologic response.

Occult HBV infection was diagnosed when HBsAg in serum was negative and HBV DNA in serum or liver was positive, irrespective of test results for serum anti-HBc. Previous studies showed a high prevalence of occult HBV infection in patients with HCV and progressive liver disease, particularly HCC [Cacciola et al., 1999; Tamori et al., 1999]. Another report suggested that occult HBV infection is an independent risk factor for carcinogenesis in patients with HCV [Pollicino et al., 2004]. It is controversial whether very small amounts of HBV have carcinogenic potential in patients with HCV. HBV DNA integration into the human genome, one of the most important mechanisms of HBV-related hepatocarcinogenesis [Bréchot, 2004], was suggested to accelerate tumor progression in HBsAg-negative patients with HCV infection [Tamori et al., 2003]. However, a recent study showed that the incidence of HCC in HBsAg-negative patients with HBV DNA integration was not higher than that in patients without such integration [Toyoda et al., 2008]. The role of HBV DNA integration in patients with HCV infection remains to be clarified. On the other hand, there are few reports about occult HBV infection in patients with HCC in whom HCV was eradicated by IFN treatment. The present study clearly showed that the detection rate for HBV DNA was significantly higher in HCC-patients with sustained virologic response than in HCC-patients with HCV. Stroffolini et al. [2008] speculated that the disappearance of HCV might up-regulate HBV replication later on. In the present study, HBV cccDNA essential for HBV replication was detected in the liver from patients with occult HBV infection. HBV cccDNA was detected in small amounts, but not significantly more frequently in patients with sustained virologic response than in patients with HCV. It was suggested that HBV is re-amplified or accumulated in the liver of HCC-patients with sustained virologic response. Although HBV DNA integration in HCC of patients with sustained virologic response has been confirmed [Tamori et al., 2005], further studies are needed to elucidate the precise role of occult HBV infection in patients with sustained virologic response.

In the present study, eight patients with occult HBV infection were infected with HBV genotype C. A previous study reported that 610 (84.7%) of 720 Japanese patients with chronic HBV infection had genotype C [Orito et al., 2001]. In Japan, genotype C is predominant in both patients with occult HBV infection and patients with HBsAg. It is speculated that occult HBV infection occurred after the disappearance of HBsAg in patients with HBV infection.

Mutation in 'a' determinant of HBsAg, which contributes to defective HBsAg expression [Chen and Oon, 1999], was detected in only three of 12 HCC with occult HBV infection. HBV DNA was not detected in serum from any patient in the present study. It appears that the 'a' determinant mutation does not have a major role in the pathogenesis of occult HBV infection in patients with sustained virologic response. Molecular analysis of the full-length HBV genome in patients with occult HBV infection has shown that viral factors are not responsible for the unique HBV status [Pollicino et al., 2007]. Available evidence suggests that small amounts of HBV, detected only in liver, exist without inducing hepatic injury or that HBV DNA is integrated into hepatocyte nuclei.

Previous studies in Japanese patients indicated that male sex, higher age, and advanced liver fibrosis before IFN therapy are risk factors for HCC in patients with sustained virologic response [Toyoda et al., 2000; Makiyama et al., 2004]. It has been suggested that subclinical tumors are most likely present in liver with severe fibrosis before HCV eradication. In the present study, however, histological findings before IFN therapy showed mild fibrosis (stage 2) in eight HCC-patients with sustained virologic response. In five patients, HCC was diagnosed more than 10 years after the eradication of HCV by IFN. It is doubtful whether neoplastic cells induced by HCV had existed in liver with milder fibrosis and proliferated slowly over such a long period. On the other hand, HBV DNA was detected frequently in HCC-patients without cirrhosis. In addition, HBV DNA was detected in HCC-patients with sustained virologic response that developed more than 40 months after HCV eradication. These results suggest that occult HBV infection is related to carcinogenesis in non-cirrhotic patients with sustained virologic response.

Recently, nonalcoholic steatohepatitis (NASH) is one of the causes of underlying non-viral hepatic cirrhosis and cryptogenic HCC. At the onset of HCC in patients with steatohepatitis, the noncancerous liver is often cirrhotic. In the present study, the noncancerous liver was not cirrhotic in 12 of 16 HCC-patients with sustained virologic response. The frequency of obesity, diabetes mellitus, and alcohol intake, factors related to steatohepatitis, in HCC patients with sustained virologic response were similar to those in HCC-patients with HCV. These findings suggest that steatohepatitis was not a major risk factor for HCC in patients with sustained virologic response.

In the present retrospective study, HBV DNA in liver was detected frequently in HCC of patients with sustained virologic response compared to in HCV-HCC. On the other hand, a follow up study for 15 patients with sustained virologic response showed that HBV DNA in liver was not detected in two patients in which HCC developed [Tsuda et al., 2004]. However, the number of examined patients in this report was small. A

large scale of prospective study is necessary to define the role of occult HBV infection on hepatocarcinogenesis in patients with sustained virologic response.

In conclusion, the above findings provide compelling evidence that continuous existence of HBV DNA in liver may be one of the risk factors for the development of HCC in patients with sustained virologic response in Japan.

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Short Communication

Optimal duration of additional therapy after biochemical and virological responses to lamivudine in patients with HBeAg-negative chronic hepatitis B: a randomized trial

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Aim: The endpoint of treatment with nucleoside analogs remains unclear for patients with hepatitis B e antigen (HBeAg)-negative chronic hepatitis B. We report the results of a randomized trial to determine the optimal duration of additional therapy after response to lamivudine in HBeAg-negative patients.

Methods: Twenty-two patients with HBeAg-negative chronic hepatitis B who exhibited biochemical and virological responses to lamivudine were enrolled. When patients responded to treatment, they were randomly assigned to receive 12 more months of therapy (Group A, 11 patients) or 24 more months of therapy (Group B, 11 patients).

Results: The baseline characteristics of the patients were similar in the two groups. Biochemical and virological responses were obtained in all patients within 6 months. Drug resistance developed in one patient in Group A during month 7 of additional therapy, and in five patients in Group B from months 13–23 of additional therapy. Ten patients in Group A and six in Group B completed the protocol and were included in analysis. Eight of the 10 patients in Group A experienced relapse between months 2 and 14 after the discontinuation of therapy, while three of the six patients in Group B experienced relapse between months 2 and 24. There was no difference in cumulative relapse rate between the groups ($P = 0.275$).

Conclusion: Additional therapy with lamivudine for longer than 12 months after biochemical and virological responses in patients with HBeAg-negative chronic hepatitis B could increase the risk of drug resistance, but did not reduce the rate of relapse.

Key words: HBeAg-negative chronic hepatitis B, hepatitis B virus, lamivudine, YMDD variant

INTRODUCTION

INFECTION WITH HEPATITIS B virus (HBV) affects more than 350 million people worldwide.[1,2] Seroconversion from hepatitis B e antigen (HBeAg) to anti-HBe usually represents a transition from chronic hepatitis to an inactive carrier state with normal alanine aminotransferase (ALT) and decreased HBV-DNA levels. However, in a portion of patients with HBV variants with mutations in the precore or core promoter regions that abolish or downregulate HBeAg synthesis,[3,4] serum ALT and HBV-DNA levels sometimes remain persistently elevated even after HBeAg seroconversion. Sustained spontaneous remission is uncommon in such patients with HBeAg-negative chronic hepatitis B.

Currently available antiviral therapy for chronic hepatitis B includes interferon[5,6] and nucleos(t)ide analogs, such as lamivudine,[7–10] adefovir dipivoxil[11,12] and entecavir.[13,14] Although interferon-induced remission of chronic hepatitis B is durable, it is achieved in only a minority of patients. In contrast, treatment with nucleos(t)ide analogs induces biochemical and virological responses in a majority of patients, but viral relapse and

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exacerbations of hepatitis are common after discontinuation of treatment. One possible approach to reducing the risk of relapse is extending the duration of treatment.

However, a major concern with the long-term use of nucleos(t)ide analogs is the selection of antiviral-resistant mutations in the reverse transcriptase (rt) domain of the polymerase gene. For instance, mutations at amino acid rt204 in the tyrosine–methionine–aspartate–aspartate (YMDD) motif are associated with resistance to some nucleoside analogs.[15–17] The decision of how long to continue treatment after response to therapy should be based on a careful assessment of the balance between the likelihood of sustained remission and the risk of development of drug resistance.

Lamivudine was the first approved oral nucleoside analog for the treatment of chronic hepatitis B. The recently updated guidelines recommend that, in HBeAg-positive patients, lamivudine treatment be continued for an additional 6–12 months after seroconversion to anti-HBe.[18,19] However, the ideal endpoint of treatment remains unclear for HBeAg-negative patients. One reason for this is that the endpoint is difficult to assess, since normal ALT and undetectable HBV–DNA levels are the only practical criteria for determination of it, rather than HBeAg seroconversion. Another reason is that longer term treatment is needed for HBeAg-negative patients because of the high rates of post-treatment relapse.[20]

We report here the results of a randomized trial to determine the optimal duration of additional therapy after response to lamivudine in patients with HBeAg-negative chronic hepatitis B. Patients with HBeAg-negative chronic hepatitis B who had biochemical and virological responses to lamivudine were randomly assigned to receive 12 or 24 more months of therapy.

METHODS

Patients

BETWEEN SEPTEMBER 2000 and May 2004, 22 patients (16 men and 6 women; mean age, 49 ± 11 years) with HBeAg-negative chronic hepatitis B who exhibited biochemical and virological responses to lamivudine therapy were enrolled in this study. The inclusion criteria were as follows: (1) persistent elevation of serum ALT levels for at least 6 months before the start of therapy; (2) presence of hepatitis B surface antigen; (3) absence of HBeAg and presence of anti-HBe; (4) presence of HBV–DNA > 10^5 copies/ml; (5) no use of immunomodulatory drugs, including interferon, within one year before the start of therapy; (6) no previous use of nucleos(t)ide analogs; (7) absence of other likely causes of chronic liver disease; and (8) no clinical signs of decompensated cirrhosis or hepatocellular carcinoma. The procedures of the study accorded with the Helsinki Declaration of 1975 (2000 revision) and were approved by the Ethics Committee of the Osaka City University Medical School.

Study protocol

Lamivudine was given orally in a dose of 100 mg once daily. After lamivudine therapy was started, serum ALT and HBV–DNA levels were tested every 1–2 months. When patients exhibited biochemical and virological responses, they were randomly assigned to one of two groups: (1) 11 were assigned to receive 12 more months of therapy (Group A); and (2) 11 were assigned to receive 24 more months of therapy (Group B). Biochemical response was defined as a decrease in serum ALT levels to within the reference range. Virological response was defined as an undetectable HBV–DNA level on polymerase chain reaction (PCR) testing. The endpoint for analysis was relapse, defined as increase in serum ALT to >2× the upper limit of normal and increase in HBV–DNA to >10^5 copies/ml.

Assays

Hepatitis B surface antigen, HBeAg and anti-HBe were detected by chemiluminescence enzyme immunoassay. Genotypes of HBV were identified by enzyme-linked immunosorbent assay (Institute of Immunology, Tokyo, Japan).[21] The mutations at nucleotide (nt) 1896 in the precore region, and at nt 1762 and nt 1764 in the basal core promoter region of HBV–DNA were detected by enzyme-linked minisequence assay (Genome Science Laboratory, Tokyo, Japan). HBV–DNA was measured by transcription-mediated amplification assay (Chugai Diagnostics, Tokyo, Japan).[22] The range of detection of the assay was between 3.7 and 8.7 $\log_{10}$ copies/ml of HBV–DNA. If HBV–DNA was not detected by this method, a PCR-based Amplicor Monitor test (Roche Molecular Systems, Pleasanton, CA, USA)[23] was utilized. The range of detection of the assay was between 2.6 and 7.6 $\log_{10}$ copies/ml. When virological breakthrough – defined as one $\log_{10}$ increase in HBV–DNA during continuous treatment – was observed, the mutations in the YMDD motif of the polymerase gene were examined by a line probe assay (INNO-LiPA HBV DR; Innogenetics NV, Ghent, Belgium).[24]

Table 1 Baseline characteristics of enrolled patients with HBeAg-negative chronic hepatitis B

	Group A ($n = 11$)	Group B ($n = 11$)	*P*-value
Age (year)	52 ± 9	47 ± 12	0.412
Sex (male/female)	9/2	6/5	0.311
ALT (IU/l)	85 (40–545)	203 (53–612)	0.412
HBV genotype (A/B/C)	0/1/10	1/1/9	0.591
Precore (wild/mixed/mutant)	2/1/8	1/4/6	0.298
Core promoter (wild/mixed/mutant)	3/1/7	2/0/9	0.484
HBV-DNA ($\log_{10}$ copies/ml)	6.1 (4.9–7.6)	7.1 (3.7–8.1)	0.431
Grade of inflammation (mild/moderate/severe)	4/3/1	4/1/0	0.385
Stage of fibrosis (mild/moderate/severe/cirrhosis)	1/1/4/2	1/0/3/1	0.565

Values are means ± SD for normally distributed variables, and medians (range) for non-normally distributed variables.
ALT, alanine aminotransferase; HBV, hepatitis B virus.

Histopathology

After informed consent had been obtained, liver biopsy was undertaken within 6 months before the start of therapy. Histopathologic findings were assessed by grading of inflammatory activity and staging of fibrosis using the classification of Desmet *et al.*[25]

Statistical analysis

Statistical analysis was performed with the Statview SE+Graphics program, version 5.0 (SAS Institute, Cary, NC, USA). Distributions of continuous variables were analyzed by the Mann-Whitney *U*-test. Differences in proportions were tested by Fisher's exact test. Cumulative incidences were plotted using the Kaplan–Meier method, and the significance of differences was determined using the log–rank test. A two-tailed *P*-value less than 0.05 was considered significant.

RESULTS

Baseline characteristics of patients

THE BASELINE CHARACTERISTICS of the 22 patients with HBeAg-negative chronic hepatitis B included in this study are shown in Table 1. Liver biopsy was not performed in eight of the 22 patients, because informed consent could not be obtained. There were no significant differences between groups A and B with respect to mean age, sex ratio, serum ALT activity, proportions of HBV genotypes, serum HBV-DNA level, or histological findings for the liver.

Flow of participants though the trial

The flow of participants though the trial is shown in Figure 1. Biochemical and virological responses were obtained from all patients within 6 months. Drug-resistant YMDD variants emerged in one patient in Group A during month 7 of additional therapy and in five patients in Group B from months 13–23 of additional therapy. These patients were treated with adefovir dipivoxil in addition to lamivudine, and excluded from analysis. Ten patients in Group A and six in Group B

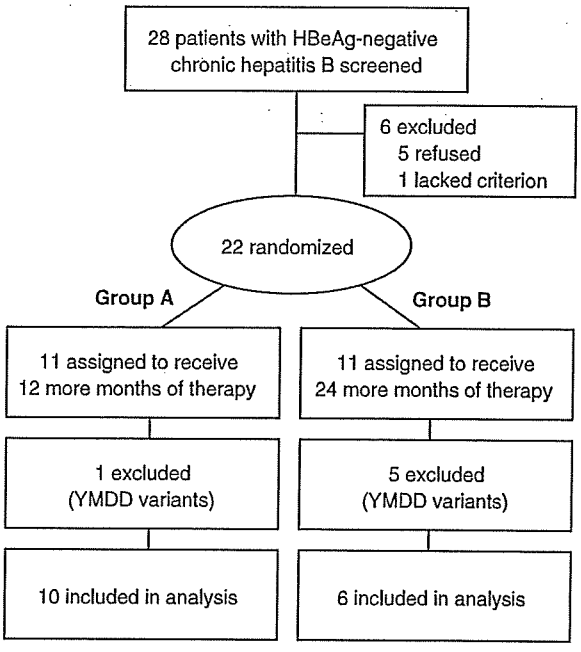

Figure 1 Flow of participants though the trial. Ten patients in Group A and six in Group B completed the study according to the protocol, and were included in analysis.
YMDD, tyrosine-methionine-aspartate-aspartate.

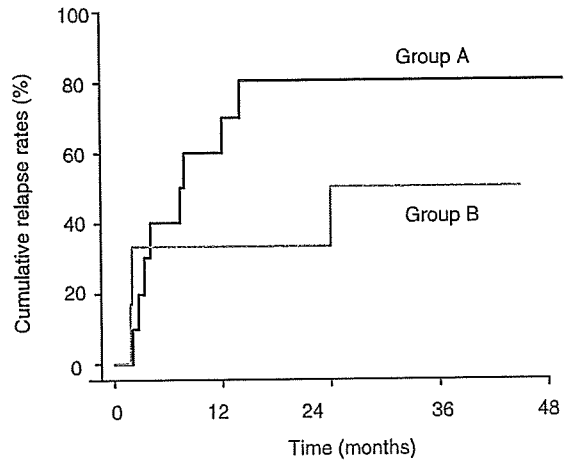

Figure 2 Cumulative rates of post-treatment relapse by duration of additional therapy after biochemical and virological responses to lamivudine. There was no significant difference in cumulative post-treatment relapse rates between the groups according to the duration of therapy ($P = 0.275$). Log–rank test.

completed the study according to the protocol, and were included in analysis.

Cumulative rates of post-treatment relapse

The cumulative rates of post-treatment relapse are shown by duration of additional therapy in Figure 2. Eight of the 10 patients in Group A experienced relapse between months 2 and 14 after discontinuation of therapy, while three of the six patients in Group B experienced relapse between months 2 and 24. There was no difference in cumulative relapse rate between the groups ($P = 0.275$). Reinstitution of lamivudine resulted in prompt responses in the patients with relapse, since drug resistance had not developed.

DISCUSSION

IN PREVIOUS LARGE multicenter trials, lamivudine treatment resulted in biochemical, virological and histological improvement,[7-9] as well as reducing the incidence of hepatic decompensation and hepatocellular carcinoma.[26] Currently, limited findings are available on the long-term efficacy of newer analogs in preventing the progression of liver disease to cirrhosis and hepatocellular carcinoma.

In HBeAg-positive chronic hepatitis B, a study in non-Asian countries found that 30 of 39 (77%) patients who experienced HBeAg seroconversion during 12-month lamivudine treatment had a durable response during a median follow-up of 37 months.[27] However, studies from Korea reported lower rates of durability (50–60%).[28,29] This discrepancy may be due, at least in part, to differences in viral genotypes among study populations.[30,31] In Asian countries, the most prevalent type is genotype C, which is associated with more progressive liver disease and a low likelihood of a sustained response to antivirals. In HBeAg-negative chronic hepatitis B, durable response during 12-month follow-up after the end of 12-month lamivudine treatment was found in only 2 of 15 (13%) patients.[20]

How long treatment with nucleos(t)ide analogs should be continued after response to treatment has yet to be tested in a randomized trial. Previous open trials suggested that long-term therapy with lamivudine increases rates of durable response. Fung *et al.*[32] reported that rates of durable virological response were increased to 50%, 70% and 50% at 6, 12, and 18 months after discontinuation of treatment, respectively, in HBe-negative patients who had completed 2 years of treatment and had persistently undetectable HBV-DNA on PCR testing during the second year. Ryu *et al.*[33] reported similar results for HBe-positive patients. However, virological breakthrough was found in 12–25% of the patients included in these studies during the 12–24 months of additional therapy.

The incidence of resistance to lamivudine increases with the duration of treatment: 24% at 1 year, increasing to 70% at 4 years.[8] Patients with detectable HBeAg and high HBV-DNA levels at baseline are at high risk for the emergence of drug-resistant variants.[34,35] In addition, patients who had a slow decrease in viral loads after the initiation of treatment are more likely to develop drug resistance. However, Kurashige *et al.*[36] reported that the cumulative rates of resistance to lamivudine were 4% at 1 year and 25% at 2 years of therapy, even when the initial viral response was achieved at 6 months. The benefits of extended therapy must thus be balanced against the risk of drug-resistant mutation.

Our randomized trial showed that additional therapy with lamivudine for longer than 12 months after biochemical and virological responses in patients with HBeAg-negative chronic hepatitis B could increase the risk of drug resistance, but did not reduce the rate of relapse. One limitation of this study was the small number of patients included. The cumulative rate of post-treatment relapse was lower in patients assigned to

Group B than in patients assigned to Group A (Fig. 2). However, this difference was not statistically significant, and our analysis excluded patients in whom drug resistance developed during additional therapy. Overall, sustained remission after discontinuation of lamivudine therapy was observed at similar rates in the two groups (2 of 11 in Group A and 3 of 11 in Group B). Even larger studies would be unable to demonstrate the benefit of additional therapy for longer than 12 months.

Among the nucleos(t)ide analogs approved for use in treating hepatitis B, lamivudine is associated with the highest and entecavir with the lowest rate of drug resistance (<1% at year 4) in nucleos(t)ide-naïve patients.[37] Entecavir is the nucleoside analog most potent against HBV, and has exhibited superiority to lamivudine in randomized controlled trials.[13,14] The use of new analogs with potent antiviral effects and low rates of resistance could make it possible to extend the duration of therapy, which might reduce the risk of post-treatment relapse. Further studies are needed to determine the optimal duration of additional therapy after response to new analogs.

In lamivudine-refractory patients with YMDD variants, resistance to entecavir occurred more frequently (15% at year 4) due to an additional substitution (at rt169, rt184, rt202, or rt250) in the polymerase gene.[37] Drug-resistant mutations limit subsequent treatment options because of cross-resistance. Based on the results of this study, we recommend that lamivudine should be switched to entecavir before the emergence of YMDD variants, or be discontinued 12 months after response to therapy. Entecavir is the treatment of choice when relapse occurs.

In conclusion, this randomized trial showed that additional therapy with lamivudine for longer than 12 months after biochemical and virological response in patients with HBeAg-negative chronic hepatitis B can increase the risk of drug resistance, but did not reduce the rate of relapse. The optimal duration of therapy, with which both the risk of development of drug resistance during additional therapy and the risk of relapse after discontinuation of therapy are reduced, was not established, because of the high incidence of drug resistance and insufficient antiviral potency when lamivudine was used.

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Platelet-associated IgG for the diagnosis of immune thrombocytopaenic purpura during peginterferon α and ribavirin treatment for chronic hepatitis C

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To the Editor:

Mild-to-moderate thrombocytopaenia is a common adverse event of treatment with conventional or pegylated interferon α, attributed primarily to bone marrow suppression, in patients with chronic hepatitis C. Nevertheless, severe, life-threatening immune thrombocytopaenic purpura (ITP) has rarely been associated with interferon treatment (1–7). The pathogenesis of ITP is incompletely understood, but immunoglobulin G (IgG)-type antibodies against platelet membrane glycoproteins (IIb/IIIa, Ib/IX, etc.) are involved (8). We describe a case of ITP induced by peginterferon treatment for chronic hepatitis C. Detection of platelet-associated IgG was helpful for the diagnosis.

A 69-year-old woman with chronic hepatitis C genotype 1b infection started to receive peginterferon α2b 80 μg/week and ribavirin 600 mg/day in October 2006 (Fig. 1). At the start of therapy, she was well, with a height of 155 cm and a weight of 58 kg. The laboratory values were as follows: aspartate aminotransferase 44 IU/L, alanine aminotransferase 58 IU/L, γ-glutamyltransferase 32 IU/L, bilirubin 0.9 mg/dl, albumin 4.1 g/dl, hepatitis C virus (HCV) RNA 43 kIU/ml, haemoglobin concentration 14.4 g/dl, white blood cell count 5400/mm^3 and platelet count 139 000/mm^3. A liver biopsy specimen showed moderate inflammation and mild fibrosis. After the start of therapy, the serum HCV RNA level rapidly decreased and became negative on polymerase chain reaction at the fourth week.

The platelet count gradually declined to 86 000/mm^3 by the 13th week of therapy and then rapidly declined to 14 000/mm^3 at the 16th week. She had petechiae on the upper extremities. Peginterferon and ribavirin were withdrawn. Coagulation test results were normal. A direct Coombs' test result was negative. Antinuclear and anticardiolipin antibodies were negative. Cryoglobulins were not detected. Serum was negative for antiplatelet antibody by mixed passive haemagglutination. However, the platelet-associated IgG level on the platelet surface had increased to 372 (reference range, 9.0–25.0) ng/10^7 cells as measured by an enzyme-linked immunoassay. Bone marrow

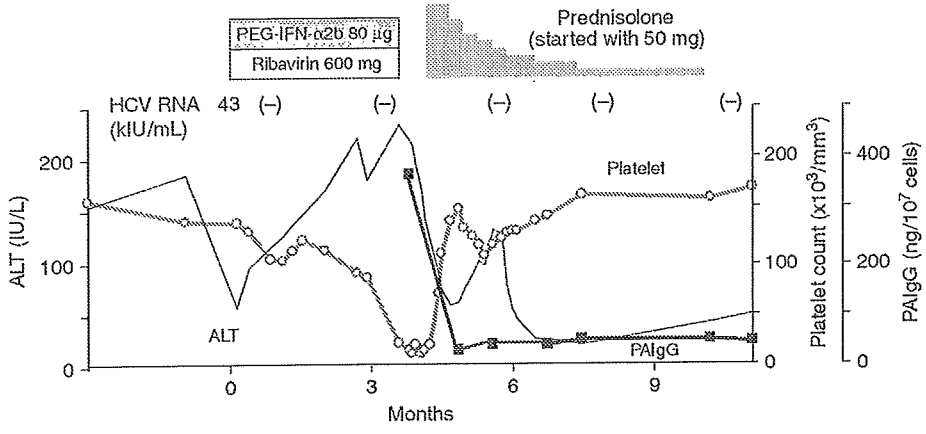

Fig. 1. Clinical course of a 69-year-old woman with chronic hepatitis C in whom immune thrombocytopaenic purpura developed during treatment with peginterferon α and ribavirin. ALT, alanine aminotransferase; PAIgG, platelet-associated immunoglobulin G; PEG-IFN, peginterferon.

aspiration demonstrated increased numbers of megakaryocytes, compatible with a diagnosis of ITP. The results of a ^{13}C-urea breath test were negative for *Helicobacter pylori* infection. Corticosteroid therapy was started with 50 mg oral prednisolone. The platelet count returned to 141 000/mm^3 in 14 days, and remained normal while tapering the dose of prednisolone. The platelet-associated IgG titre decreased in response to corticosteroid therapy. HCV RNA continued to remain negative after the withdrawal of peginterferon.

Immune thrombocytopaenic purpura is an autoimmune disorder characterized by peripheral consumption of platelets and clinical manifestations of a haemorrhagic diathesis (8). ITP is a diagnosis of exclusion, often difficult to establish. In the literature, interferon-induced ITP has developed after 4 week to 12 months of therapy (2, 4), or even 6 months after the completion of therapy (6). The ages and baseline platelet counts of patients have varied widely, ranging from 27 (6) to 73 (5) years and from 80 000 (3) to 260 000 (4)/mm^3 respectively. As demonstrated in our patient, the detection of circulating antiplatelet antibodies unbound to platelets is not sensitive enough for diagnosis. Such autoantibodies can develop in patients immunized by pregnancy, allogenic transfusions or organ transplantation and are thus not specific for ITP. In contrast, a direct assay of platelet-associated IgG (bound to platelets) is more useful for the diagnosis of ITP, with a sensitivity of 49–66% and a specificity of 78–92% (8). In our patient, platelet-associated IgG was also helpful for monitoring the response to corticosteroid therapy.

In summary, we have described a patient with chronic hepatitis C in whom ITP developed during treatment with peginterferon α and ribavirin. Albeit rare, ITP can occur any time during interferon treatment. Physicians treating patients with chronic hepatitis C should be aware that platelet-associated IgG is helpful for promptly diagnosing this potentially fatal complication.

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Hepatocellular apoptosis in polycystic liver disease

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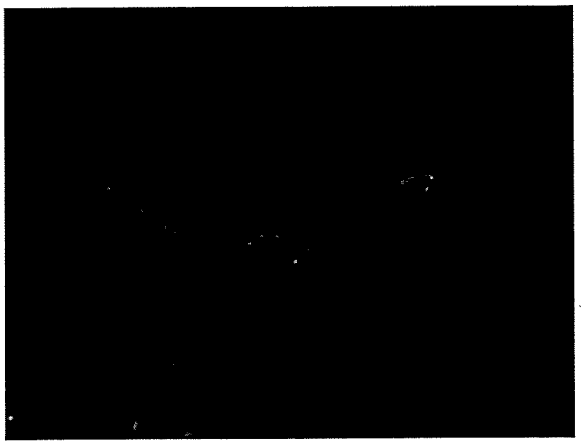
To the Editor:

Polycystic liver disease (PCLD) is asymptomatic in 80% of patients and often diagnosed incidentally. After diagnosis, surgical therapy is the mainstay of treatment tailored to the extent of disease for symptomatic patients. In the past several decades, there have been great advances in the knowledge of the pathogenesis, genetics and effective treatment for PCLD. These lesions in PCLD have been attributed to bile duct overgrowth after the arrest of embryogenesis and failure of the intralobar bile ducts to involute. This involutional failure results in cystic dilations that are known as biliary microhamartomas or von Meyenburg complexes (VMC) (1). However, we focused on the role of hepatocellular apoptosis in PCLD.

Figure. Direct immunofluorescence revealing perivascular deposits of C3 within the affected vessels.



Original magnification, ×60.

hours at 4 °C, and cryoprecipitates were separated by centrifuge for 5 minutes. The cryoprecipitates were washed 3 times with a small volume of ice-cold, phosphate-buffered saline (pH, 7.2). Then the cryoprecipitates were dissolved in warmed (37 °C), phosphate-buffered saline for 2 hours, and the IgG anti-PS/PT antibody level was measured (at 37 °C). We detected that IgG anti-PS/PT antibody level in the cryoprecipitates was higher (120 U/mL) than that in cryoglobulin-free sera (20 U/mL) under the same dilution conditions.

The patient was treated with entecavir monotherapy, and within 6 months, HBV DNA levels decreased below the limit of detection; his purpuric rash with paresthesia and numbness disappeared; and his serum cryoglobulins, anti-PS/PT antibodies, and lupus anticoagulant also became negative.

Discussion: The mechanism of association between hepatitis infection and vasculitis is unclear. Phosphatidylserine is a constituent of cell membranes that are exposed during apoptosis or other forms of cell damage, and some reports have suggested that it is initially present in the particles produced by infected hepatocytes during HBV infection (2). Also, prothrombin binds to the surface of apoptotic cells (3). On the basis of our findings of higher anti-PS/PT antibody titers in cryoprecipitates than in cryoglobulin-free sera and of C3 deposition by direct immunofluorescence testing within the cryoglobulinemic vasculitis lesions, we propose that prothrombin induced by destroyed hepatocytes binds to phosphatidylserine produced by HBV, triggering production of anti-PS/PT antibody and other cryoglobulins; endothelial cell damage, an inflammatory cytokine cascade, and complement activation; and vasculitis. Demonstration of remission of cryoglobulinemic vasculitis by treatment with entecavir suggests that the effect of entecavir against HBV might prevent this cascade of events.

Conclusion: Entecavir treatment seemed to lead to prompt suppression of HBV replication and prompt resolution of cryoglobulinemic vasculitis.

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Entecavir to Treat Hepatitis B–Associated Cryoglobulinemic Vasculitis

Background: Hepatitis C virus infection is associated with mixed cryoglobulinemia (1). The association of hepatitis B virus (HBV) with cryoglobulinemia is less certain, and treatment is nonspecific.

Objective: To describe a patient with HBV-associated cryoglobulinemic vasculitis that resolved with entecavir.

Case Report: A 57-year-old Taiwanese woman was referred to us because of a purpuric rash on her legs (Figure). Skin biopsy specimens showed leukocytoclastic vasculitis in the upper dermis. Her aspartate aminotransferase level was 143 U/L, alanine aminotransferase level was 119 U/L, γ-glutamyltransferase level was 27 U/L, total bilirubin level was 29.1 μmol/L (1.7 mg/dL), albumin level was 3.2 g/dL, creatinine level was 38.13 μmol/L (0.50 mg/dL), leukocyte count was 2.6×10^9 cells/L, hemoglobin concentration was 10.5 g/dL, and platelet count was 63×10^9 cells/L. C-reactive protein and antinuclear and antineutrophil cytoplasmic antibodies were negative. Cryoglobulins were detected by cold precipitation. Test results for antibodies to hepatitis C virus and viral RNA and anti–hepatitis B envelope antigen were negative, and results for hepatitis B surface antigen and hepatitis B envelope antigen were positive. Her HBV DNA level was 6.4 $\log_{10}$ copies/mL. The genotype of the HBV was type B. A precore stop codon mutation was found at nucleotide 1896, but no mutations were found at nucleotide 1762 or nucleotide 1764 in the basal core promoter. Abdominal ultrasonography showed no evidence of cirrhosis or hepatocellular carcinoma. A liver biopsy specimen showed moderate inflammation and severe fibrosis.

On the basis of these findings, HBV-associated cryoglobulinemic vasculitis was diagnosed, and entecavir treatment was started at a dose of 0.5 mg/d. The serum HBV DNA level decreased immediately after the start of therapy and became undetectable by polymerase chain reaction testing (<2.6 $\log_{10}$ copies/mL) by week 6. The aminotransferase activity fell to the normal range by week 12. Cryo-

Figure. Purpuric rash on the patient's legs.

globulins became undetectable by week 20, and skin lesions resolved gradually.

Discussion: Cryoglobulins are abnormal immunoglobulins that undergo reversible precipitation at low temperatures, are deposited in microvessels, and evoke vasculitis. Secondary cryoglobulinemia is best managed by treating the underlying disease. Some cases of chronic hepatitis B complicated by cryoglobulinemic vasculitis have responded to lamivudine or adefovir dipivoxil (2–4), suggesting an association between HBV infection and cryoglobulinemia. Of particular interest, Çakir and colleagues (4) described a patient whose cryoglobulinemic vasculitis responded to lamivudine, recurred owing to the emergence of lamivudine-resistant HBV, and then resolved after rescue therapy with adefovir. The suppression of HBV by antiviral agents may have resulted in decreased numbers of viral antigens that can form cryoglobulins.

Entecavir is the most potent currently available nucleoside or nucleotide analogue against HBV and has been shown to be superior to lamivudine in randomized, controlled trials (5). In addition, it is associated with the lowest rate of drug resistance. New nucleoside analogues with high antiviral potency and low resistance rates would be useful not only for treating hepatitis, but also for managing extrahepatic manifestations.

Interferon-α is an alternative treatment for chronic HBV infection, but it is not preferred to nucleoside or nucleotide analogues for the management of extrahepatic manifestations. One reason is that interferon-α produces rapid viral suppression in only some patients. Another is that interferon-α has immunomodulatory activity. The pathogenesis of extrahepatic manifestations is not completely understood, but immune-mediated mechanisms are most likely involved. The use of corticosteroids or immunosuppressive agents alone is not recommended because of possible flare-ups of viral replication.

Conclusion: Entecavir may be a first-line treatment for extrahepatic manifestations of chronic HBV infection.

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Sildenafil-Induced Severe Cholestatic Hepatotoxicity

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To the Editor: Sildenafil citrate (Viagra) is a potent, orally active, cyclic guanosine monophosphate-specific phosphodiesterase type 5 inhibitor, used globally for the treatment of penile erectile dysfunction. The most common adverse effects are headache, flushing, dyspepsia, and cardiovascular events. Liver toxicity attributed to sildenafil appears to be very rare. In the English-language literature, only one case of mild hepatotoxicity induced by sildenafil has been reported earlier (1). We describe a case of severe cholestatic hepatotoxicity induced by sildenafil.

A previously healthy 58-year-old man was referred to us because of jaundice, pruritus, and malaise. The laboratory values were as follows: aspartate aminotransferase 42 IU/l, alanine aminotransferase 64 IU/l, alkaline phosphatase 476 IU/l, total bilirubin 8.5 mg/dl, direct bilirubin 6.3 mg/dl, albumin 3.6 g/dl, white blood cell count 4,300/mm^3 (with 2.8% eosinophils), hemoglobin concentration 13.3 g/dl, platelet count 239,000/mm^3, and prothrombin time 95%. Tests for immunoglobulin M anti-hepatitis A virus, hepatitis B surface antigen, immunoglobulin M anti-hepatitis B core antibodies, anti-hepatitis C virus antibodies, immunoglobulin M anti-viral capsid antigen of Epstein-Barr virus, and immunoglobulin M anti-cytomegalovirus were all negative. C-reactive protein and anti-nuclear, anti-mitochondrial, and anti-neutrophil cytoplasmic antibodies were also negative. No history of recent drug use or excessive alcohol intake was reported. Abdominal ultrasound, computed tomography, and magnetic resonance cholangiopancreatography showed no signs of bile duct obstruction. Macroscopically, the liver was green, enlarged, and had a smooth surface without nodularity on laparoscopic examination (**Figure 1**). A liver biopsy specimen revealed features of intrahepatic cholestasis; marked bile stasis was seen in canaliculi around the pericentral area, and cellular necroinflammation in the portal area was minimal (**Figure 2**).

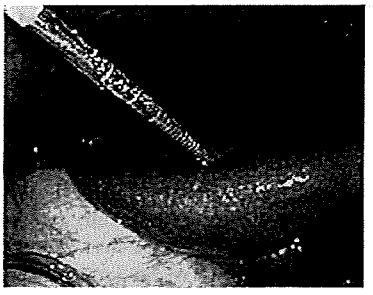

Figure 1. Laparoscopic image showing a green and enlarged liver without nodularity.

On carefully obtaining his history again, the patient admitted that he had taken sildenafil 50 mg 1 month before symptom onset. No other medications, including nonsteroidal anti-inflammatory drugs, were used. On the basis of criteria for drug-induced liver disorders (2) and the Naranjo adverse drug reaction probability scale, (3) we diagnosed probable sildenafil-induced cholestatic hepatotoxicity. The laboratory data steadily improved thereafter without any medical treatment and returned to normal 4 months after symptom onset.

Daghfous *et al.* (1) reported a case of acute hepatotoxicity attributed to sildenafil. However, the causal relation between sildenafil use and subsequent liver damage was uncertain. The alanine aminotransferase level increased to only 1.2 times the upper limit of normal, and the biluribin and alkaline phosphatase levels were normal. A liver biopsy was not undertaken. Liver injury did not recur after rechallenge with sildenafil.

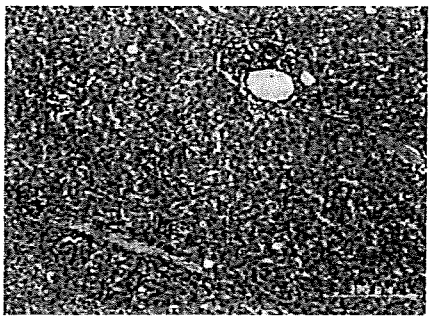

Figure 2. Liver biopsy specimen showing intrahepatic cholestasis (hematoxylin and eosin; original magnification, ×200).