TABLE I. Demographic and Clinical Differences Among Patients With Acute Hepatitis Who Were Infected With HBV of Distinct Genotypes

Features	Genotypes of HBV				Differences (A vs. C)	
	A (n = 27)	B (n = 8)	C (n = 109)	B/C (n = 1)	Univariate (*P*-value)	Multivariate logistic regression (*P*-value)
Areas					<0.001	0.03
Metropolitan (n = 69)	21 (30%)	5 (7%)	43 (63%)	0		
Others (n = 76)	6 (8%)	3 (4%)	66 (87%)	1 (1%)		
Age (years)	29.3 ± 8.0	35.7 ± 10.1	36.6 ± 13.6	51	0.016	0.152
Male	25 (93%)	7 (88%)	69 (57%)	1 (100%)	0.003	0.018
Transmission routes						
Heterosexual	15 (56%)	3 (37%)	52 (48%)	0	0.197	
Homosexual	5 (19%)	1 (13%)	2 (2%)	0	<0.001	0.133
IV drugs	0	0	8 (7%)	0	0.280	
Unknown	7 (25%)	4 (50%)	47 (43%)	1 (100%)	0.102	
Fulminant hepatic failure	0	1 (13%)	5 (5%)	0	0.582	
ALT (IU/L)[a]	2069 ± 1075	2952 ± 1106	2889 ± 1867	646	0.030	0.084
Bilirubin (mg/dl)[a]	10.7 ± 14.1	10.3 ± 4.9	7.8 ± 6.7	4.8	0.533	
ALP (IU/L)[a]	476 ± 161	501 ± 94	432 ± 116	No data	0.542	
HBeAg	24/26 (92%)	4/8 (50%)	57/93 (61%)	1/1 (100%)	0.357	
Precore and BCP mutations						
Precore (1896A)	0/27	1/8 (13%)	20/102 (20%)	No data	0.250	
BCP (1762T/1764A)	0/27	1/6 (17%)	14/75 (19%)	No data	0.357	
Precore or BCP	0/27	2/8 (25%)	27/102 (26%)	No data	0.096	

[a]Maximum data are shown for alanine aminotransferase (ALT), bilirubin and alkaline phosphatase (ALP).

different genotypes are compared in Table I. Patients with genotype A were younger than those with genotype C (29.3 ± 8.0 vs. 36.6 ± 13.6 years, $P = 0.016$). The proportion of male patients was higher in genotype A than C infection (93% vs. 57%, $P = 0.003$). The main route of transmission identified in the patients with acute hepatitis B was extramarital heterosexual contacts. Homosexual activity was more frequent in patients with genotype A than C (5/27 (19%) vs. 2/109 (1.8%), $P < 0.001$).

The maximum ALT levels were lower in patients with genotype A than B or C infection (2069 ± 1075, 2952 ± 1106 and 2889 ± 1867 IU/L, respectively: A vs. B, $P = 0.02$; A vs. C, $P = 0.03$). The maximum bilirubin and alkaline phosphatase levels were no different among patients infected with HBV of different genotypes. Fulminant hepatic failure developed in one (13%) patient with genotype B and five (5%) with genotype C; no patients with genotype A came down with it. Evolution into chronic infection occurred in two patients (one with genotype A and one with genotype C). The remaining 137 (96%) patients ran a non-fulminant and self-limited disease.

HBeAg was found in 24 of the 26 (92%) patients with genotype A, 4 of the 8 (50%) with genotype B and 57 of the 93 (61%) with genotype C; it was no different between genotype A than genotype C infection ($P = 0.357$). Of the six patients with fulminant hepatic failure, only one (17%) had HBeAg.

With logistic multivariate regression analysis, the variables for differences between genotypes A and C were sex (odds ratio (OR), 6.45; 95% confidence interval (CI), 1.378–30.213; $P = 0.0018$) and area (OR, 0.25; 95% CI, 0.076–0.830; $P = 0.0024$).

Routes of transmission were compared between genotypes A and C in patients with acute hepatitis B from metropolitan areas. Although the mean age was no different, frequently the proportion of male patients was higher in genotype A than C infection (20/21 (95%) vs. 28/43 (65%), $P = 0.012$). Homosexual patients had more frequently genotype A than C infection (5/21 (24%) vs. 1/44 (2%), $P = 0.012$). Additionally heterosexuals with multiple unspecific partners had in genotype A more frequently than C infection (7/12 (58%) vs. 6/26 (23%), $P = 0.035$, respectively). However, with logistic multivariate regression analysis, none of these variables differed between genotype A and C infections.

Figure 1 compares serum HBV DNA levels on admission among patients infected with different genotypes. HBV DNA levels were higher in patients with genotype A than C (5.90 ± 1.45 vs. 5.13 ± 1.36 LGE/ml, $P = 0.002$).

Among the 145 patients whose HBV genotypes could be determined, 54 (A: 15, B: 4, and C: 35) were followed for HBsAg in serum every 2–4 weeks until it disappeared. The time between the first and last detection of HBsAg was defined as the duration of HBsAg, and compared between patients infected with HBV of genotypes A and C (Fig. 2a). The duration of HBsAg was longer in patients with genotype A than C infection (1.95 ± 1.09 (n = 15) vs. 1.28 ± 1.42 months (n = 35), $P = 0.02$). When patients with fulminant hepatic failure were excluded, the mean duration of HBsAg in patients with genotype C became longer, but it was still shorter

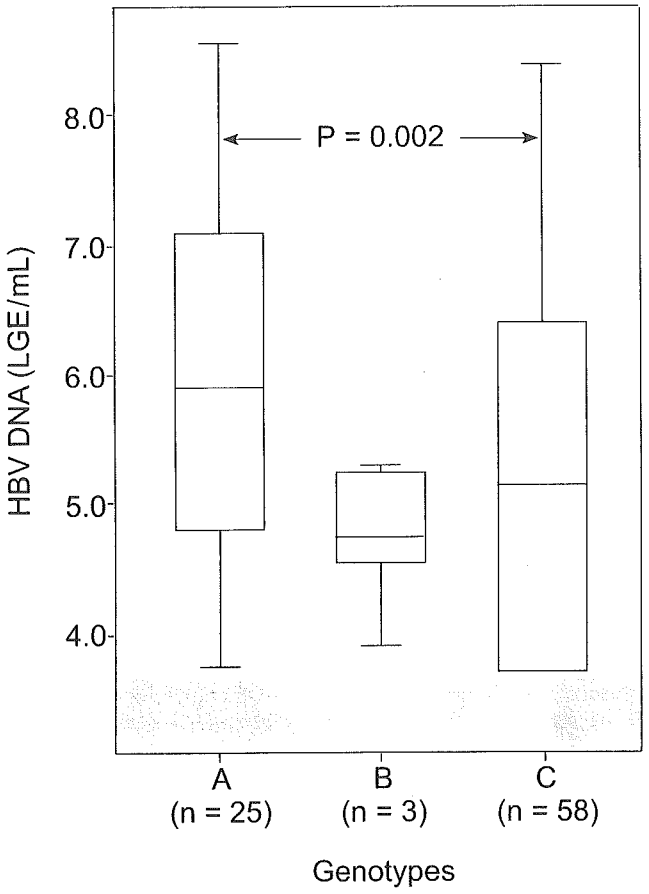

Fig. 1. HBV-DNA levels in patients with acute hepatitis B with genotypes A, B, or C at the presentation. Box plots are given with horizontal lines for the medians, upper and lower edges indicating the 25th and 75th centiles, respectively, and bars represent the extremes without including outliers. Shaded areas are outside the range of detection by the TMA method.

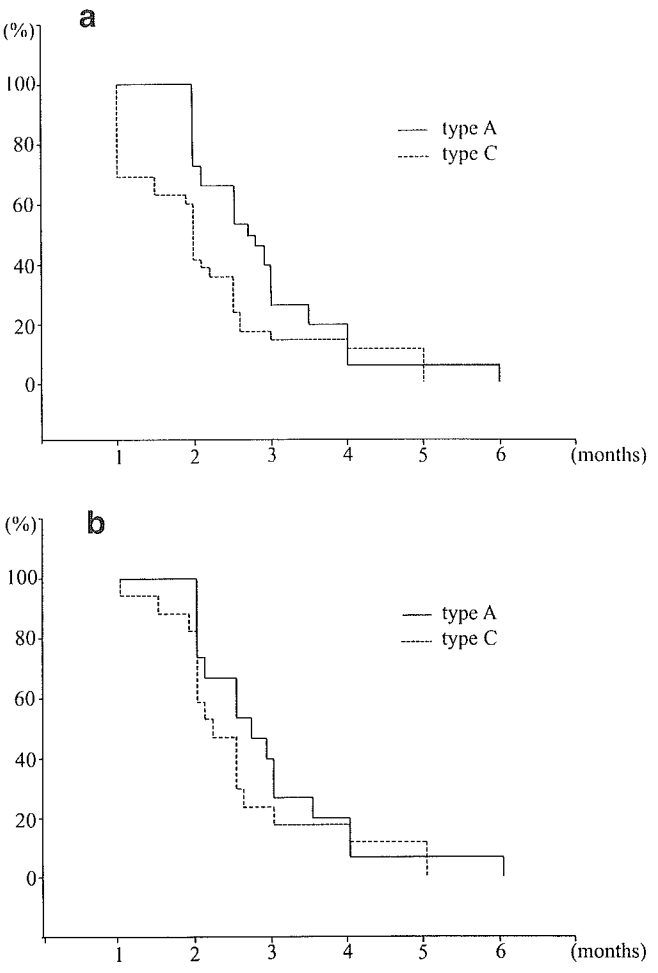

Fig. 2. The duration of HBsAg in patients with acute hepatitis B with genotypes A or C. The results are shown for (**a**) all patients, and (**b**) patients with the wild-type sequences both in precore and BCP regions of HBV.

than that in those with genotype A (1.95 ± 1.09 (n = 15) vs. 1.41 ± 1.42 (n = 31) months, $P = 0.03$).

Subtypes of Genotypes A and B

Among the 27 HBV/A isolates, 9 were selected at random and the entire S region was amplified and sequenced for them. Seven of them were classified into genotype A and the remaining 2 into subgroup A′. The sequence divergence within the seven genotype A isolates ranged from 0.12% to 2.01% in pair-wise comparison, while that between two subgroup A′ and seven genotype A isolates spanned from 5.70% to 6.53%.

A phylogenetic tree was constructed on the entire *S*-gene sequences from these nine sequences along with those from 31 HBV isolates retrieved from the database (Fig. 3). The seven (78%) HBV isolates classified into genotype A clustered with reported HBV/A isolates, while the remaining two isolates classified into subgroup A′ (cases 3 and 4) joined the branch of subgroup A′.

Six of the eight HBV/B isolates were available for analysis of subtypes. Two (both from the metropolitan area) were classified as Ba and the remaining four, including two from Tokyo and two from the other areas, as Bj. One of the four patients infected with subtype Bj developed fulminant hepatic failure, while the remaining three with subtype Bj as well as the two with subtype Ba ran a non-fulminant course.

Point Mutations in the Precore and Basic Core Promoter Regions of HBV

All the 27 HBV isolates of genotype A in which mutations were sought had the wild-type sequences both in the precore and BCP regions. In contrast, of the 102 genotype C isolates whose precore and BCP sequences were examined, 27 (26%) had mutations in the precore or BCP regions ($P = 0.096$). Furthermore, of the four genotype C isolates from patients with fulminant hepatic failure whose genetic mutations could be determined, three had mutations in the BCP region (T1762/A1764) and two had a mutation in the precore region (A1896). Only one isolate had the wild-type sequences both in the precore and BCP regions. Of

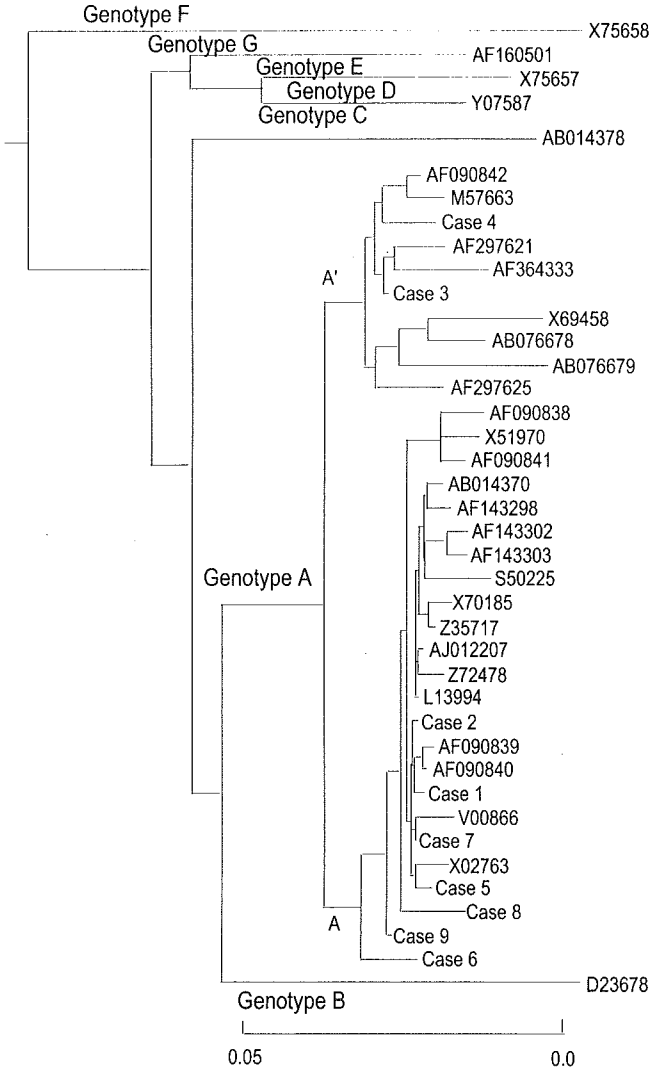

Fig. 3. A phylogenetic tree constructed on HBV DNA sequences spanning the major *S*-gene of all known HBV genomes, including the nine of genotype A. The horizontal bar indicates the number of nucleotide substitutions per site. Accession numbers are shown for the isolates, which have been deposited in the DDBJ/EMBL/GenBank databases. HBV sequences in cases 1–9 were determined in the present study. The HBV/A sequences from cases 1, 2, and 5–9 clustered with the European-American genotype A, while those from cases 3 and 4 clustered with genotype A' that is the African subgroup of genotype A.

the eight genotype B isolates, two (25%) had mutations in the precore or BCP region (Table I).

To examine further differences between genotype A and C infections, patients infected with HBV strains with the wild-type sequences both in precore and BCP regions were compared. The maximum ALT levels were still lower in patients with genotype A than C infection (2069 ± 1075 and 2594 ± 1015 IU/L, respectively, $P = 0.02$), but the maximum bilirubin and alkaline phosphatase levels were no different amongst patients infected with HBV of distinct genotypes. There were no differences in the duration of serum HBsAg between patients with genotype A and C infections (1.95 ± 1.09 vs. 1.58 ± 1.24 months, $P = 0.35$) (Fig. 2b).

DISCUSSION

The salient finding in this study is that infection with HBV genotype A is frequent in patients with acute hepatitis in Japan, lending support to previous studies [Kobayashi et al., 2002; Ogawa et al., 2002]. Substantial portion of patients with acute hepatitis were infected with genotype A, which is detected rarely among patients with chronic hepatitis in Japan [Orito et al., 2001a; Kobayashi et al., 2002]. Genotype A prevails in North-Western Europe, United States, Central Africa, and India [Kao, 2002; Miyakawa and Mizokami, 2003]. This genotype may be prevalent in countries elsewhere, since the distribution of HBV genotypes has not been examined in many districts of the world. Phylogenetic analysis has shown that seven (78%) HBV/A strains of the nine patients examined with acute hepatitis B were of the European-American type. Although the HBV/A sequences from four, (cases 1, 2, 5, and 7) clustered with those reported previously, those from three (cases 6, 8, and 9) were separated genetically (Fig. 3), which suggests their distinct geographic origin.

Notably, the genotype distribution differed between patients with acute hepatitis B from metropolitan areas and the others including many large cities. As genotype A is seen rarely in patients with chronic hepatitis [Orito et al., 2001a; Kobayashi et al., 2002], it is suspected that genotype A in metropolitan areas has a distinct geographic origin. Many patients with genotype A infection in these areas had a history of extramarital sexual contacts with plural unspecified partners. Such sexual behavior may increase the risk of infection with genotype A. In support of this view, most homosexual people in Tokyo who have human immunodeficiency virus type I are coinfected with HBV genotype A [Koibuchi et al., 2001]. Taken together, homosexual activity would increase the risk of genotype A infection in metropolitan areas. Further molecular analysis on HBV isolates from transmitters and recipients will verify this hypothesis. With respect to genotype B, both Ba, and Bj subtypes [Sugauchi et al., 2002b] were detected. Although the number of studied patients was small, patients with subtype Ba were found in the Tokyo metropolitan area exclusively. Whether subtype Ba intrinsic to the metropolitan area has a peculiar geographic origin is currently unknown and awaits further analyses.

Another point made in this study is that HBV genotypes influence clinical features and the outcome of acute hepatitis B. It has been shown that the proportion of patients who develop chronic HBV infection is close to 10% in European and American countries [Sherlock S, 1997] but rare in Japan [Kobayashi et al., 2002]. Recent studies suggest that chances for evolution into chronicity may differ among patients acutely infected with HBV of distinct genotypes [Mayerat et al., 1999; Ogawa et al., 2002]. Our study has shown that patients with genotype A had higher HBV DNA and lower ALT levels, as well as a longer duration of HBsAg in serum. Development of chronic hepatitis was seen in one of the 27 (4%) patients with genotype A as against one of the 109 (1%)

with genotype C infection. Although the number of patients studied was not large enough for statistical evaluation, the transition to chronic infection may be more frequent in infection with genotype A than the other genotypes, insofar as higher viral loads can predict chronic infection [Fong et al., 1994]. Further studies on more patients are required to evaluate whether or not viral persistence occurs more often after HBV infection with genotype A than the other genotypes.

Patients with fulminant hepatic failure in the present study were infected with either genotypes B or C; no patient with genotype A developed hepatic failure. As mutations at nt 1896 in the precore and nt 1762/1764 in the BCP regions, which are found frequently in patients with fulminant hepatic failure [Carman et al., 1991; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Hawkins et al., 1994; Sato et al., 1995; Baumert et al., 1996; Chu et al., 1996], were not detected in patients with genotype A, low frequency of fulminant hepatic failure associated with genotype A infection may be attributed to the lack of these mutations. The high frequency of HBeAg in genotype A infection may also be related to low frequency of fulminant hepatic failure. However, interpretation on this data should be made carefully, because the number of patients studied was small. Further research is necessary to determine if the genotype itself affects the clinical course of acute hepatitis B.

In summary, (1) infection with HBV genotype A is common in patients with acute hepatitis in Japan; (2) patients with genotype A are more frequent in metropolitan areas and may be associated with particular sexual behavior; (3) patients with genotype A have a milder but longer course of infection, which may lead to increased risk of progression to chronic disease.

ACKNOWLEDGMENTS

We thank Ms. Kuniko Shibahara for her excellent technical assistance.

REFERENCES

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

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

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

Bowyer SM, van Staden L, Kew MC, Sim JG. 1997. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 78:1719–1729.

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

Chan HL GM, Lok ASF. 1999. Hepatitis B. In: Schiff ER SM, Maddrey WC, editors. Diseases of the liver, 8th edn. Philadelphia: Lippincott Williams & Wilkins. 763p.

Chan HLY LA, editor. 1999. Hepatitis B in adults. Philadelphia: W.B. Saunders Company. pp 291–308.

Chu CM, Yeh CT, Chiu CT, Sheen IS, Liaw YF. 1996. Precore mutant of hepatitis B virus prevails in acute and chronic infections in an area in which hepatitis B is endemic. J Clin Microbiol 34:1815–1818.

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

Ding X, Mizokami M, Ge X, Orito E, Iino S, Ueda R, Nakanishi M. 2002. Different hepatitis B virus genotype distributions among asymptomatic carriers and patients with liver diseases in Nanning, Southern China. Hepatol Res 22:37–44.

Fong TL, Di Bisceglie AM, Biswas R, Waggoner JG, Wilson L, Claggett J, Hoofnagle JH. 1994. High levels of viral replication during acute hepatitis B infection predict progression to chronicity. J Med Virol 43:155–158.

Hawkins AE, Gilson RJ, Beath SV, Boxall EH, Kelly DA, Tedder RS, Weller IV. 1994. Novel application of a point mutation assay: Evidence for transmission of hepatitis B viruses with precore mutations and their detection in infants with fulminant hepatitis B. J Med Virol 44:13–21.

Kamisango K, Kamogawa C, Sumi M, Goto S, Hirao A, Gonzales F, Yasuda K, Iino S. 1999. Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J Clin Microbiol 37:310–314.

Kao JH. 2002. Hepatitis B viral genotypes: Clinical relevance and molecular characteristics. J Gastroenterol Hepatol 17:643–650.

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

Kato H, Orito E, Sugauchi F, Ueda R, Gish RG, Usuda S, Miyakawa Y, Mizokami M. 2001. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. J Virol Methods 98:153–159.

Kato H, Orito E, Gish RG, Sugauchi F, Suzuki S, Ueda R, Miyakawa Y, Mizokami M. 2002. Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). J Virol 76:6131–6137.

Kato H, Orito E, Sugauchi F, Ueda R, Koshizaka T, Yanaka S, Gish RG, Kurbanov F, Ruzibakiev R, Kramvis A, Kew MC, Ahmad N, Khan M, Usuda S, Miyakawa Y, Mizokami M. 2003. Frequent coinfection with hepatitis B virus strains of distinct genotypes detected by hybridization with type-specific probes immobilized on a solid-phase support. J Virol Methods 110:29–35.

Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Takagi K, Miyakawa Y, Kumada H. 2002. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 68:522–528.

Koibuchi T, Hitani A, Nakamura T, Nojiri N, Nakajima K, Jyuji T, Iwamoto A. 2001. Predominance of genotype A HBV in an HBV-HIV-1 dually positive population compared with an HIV-1-negative counterpart in Japan. J Med Virol 64:435–440.

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

Kramvis A, Weitzmann L, Owiredu WK, Kew MC. 2002. Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. J Gen Virol 83:835–839.

Li JS, Tong SP, Wen YM, Vitvitski L, Zhang Q, Trepo C. 1993. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: Possible contribution of a single nucleotide in the precore region. J Virol 67:5402–5410.

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

Lok AS, Akarca U, Greene S. 1994. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. Proc Natl Acad Sci USA 91:4077–4081.

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

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

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

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

Ogawa M, Hasegawa K, Naritomi T, Torii N, Hayashi N. 2002. Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. Hepatol Res 23:167–177.

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

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

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. 2001a. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 34:590–594.

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

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

Sherlock S DJ. 1997. Virus hepatitis. In: Sherlock S DJ, editor. Diseases of the Liver and biliary system, 10th edn. London: Blackwell Scientific Publications. pp 265–392.

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

Sugauchi F, Chutaputti A, Orito E, Kato H, Suzuki S, Ueda R, Mizokami M. 2002a. Hepatitis B virus genotypes and clinical manifestation among hepatitis B carriers in Thailand. J Gastroenterol Hepatol 17:671–676.

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

Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, Gish RG, Tanaka Y, Kato T, Orito E, Ueda R, Miyakawa Y, Mizokami M. 2004. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 85:811–820.

Sugita S, Yoshioka Y, Itamura S, Kanegae Y, Oguchi K, Gojobori T, Nerome K, Oya A. 1991. Molecular evolution of hemagglutinin genes of H1N1 swine and human influenza A viruses. J Mol Evol 32:16–23.

Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. 2002. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. J Gastroenterol Hepatol 17:165–170.

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

Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, Mayumi M. 2000. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 87: 81–89.

Digestive Diseases and Sciences, Vol. 48, No. 4 (April 2003), pp. 821–823 (© 2003)

CASE REPORT

Hyperammonemic Encephalopathy in a Patient with Ureterosigmoidostomy and Acute Hepatitis

A Specific Case of Fulminant Hepatic Failure

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KEY WORDS: hyperammonemic encephalopathy; acute hepatitis; fulminant hepatic failure; ureterosigmoidostomy.

Ureterosigmoidostomy is known to be a useful surgical technique for patients undergoing bladder resection (1). However, various complications such as electrolyte imbalance, urinary tract infections, hyperchloremic metabolic acidosis, and colon neoplasia are recognized (2–4). In addition, although rare, hyperammonemic encephalopathy with or without coexistent liver disease has been reported (5–15). In those cases, diffusion of ammonia into the systemic circulation has been postulated. We report the case of a 63-year-old man with a ureterosigmoidostomy done 14 years previously who developed hyperammonemic encephalopathy a few days after admission for acut hepatitis.

CASE REPORT

A 63-year-old man, who had had a ureterosigmoidostomy 14 years previously for bladder cancer, was admitted to our hospital in early May 2001, complaining of appetite loss, mild fatigue, and fever that persisted for 10 days. Results of a physical examination were remarkable for normal vital and neurological signs, mild jaundice, and a palpable liver. His liver biochemistry studies on admission revealed a serum alkaline phosphatase level of 981 IU/liter (normal, 103–335 IU/liter, AST/ALT of 6630/5740 IU/liter (normal, 5–40/4–45 IU/liter), total/direct bilirubin of 6.4/4.4 mg/dl, albumin of 3.5 g/dl, PT international normalized ratio (INR) of 2.57, serum ammonia level of 115 μg/dl (normal, 20–70 μg/dl). His blood cell count was within the normal range and specific studies to detect viral infection or autoantibodies such as serum markers of viral hepatitis A, B, C, cytomegalovirus, and anti-nuclear antibodies (ANAs) were normal or negative. An ultrasound examination and CT scan of the abdomen showed a normal biliary tree, slightly enlarged liver, and thickened gallbladder wall consistent with acute hepatitis, and no dilatation of either the renal pelvis or the ureter was observed. Diagnosed as having acute hepatitis of unknown etiology, he was put on lactulose orally, and bed rest was recommended.

On the third day after admission, he suddenly lapsed into an encephalopathy in which he was confused and unresponsive to verbal stimuli. The AST/ALT was decreased to 2040/4660; however, the serum ammonia level had raisen to 465 μg/dl. Total bilirubin and PT-INR had raisen to 8.3 mg/dl and 3.01, respectively. At this point he was diagnosed as having suffered from fulminant hepatic failure and was transferred to the intensive care unit where he was treated with continuous hemodiafiltration under endotracheal and rectal intubation, put on lactulose, kanamycin, and polymyxin B sulfate from nasogastric tube, injected vitamin K intravenously and the liver transplantation was taken into account. However, a CT scan at that time revealed no atrophic change of the liver, but showed bilateral hydronephrosis and distension of the sigmoid colon and rectum with urinary stasis (Figure 1A,B). Moreover, a transjugular liver biopsy done on the seventh day after admission did not show severe dispersed necrosis (Figure 2), and there was no sign of intracranial hypertension. After rectal intubation, the serum ammonia level was dramatically decreased to 113 μg/dl within 24 hr, and distension of sigmoid colon and bilateral hydronephrosis gradually improved.

On the sixteenth day after admission, he was transferred to the general ward and recovered uneventfully, aside from hyperchloremic acidosis, which resolved after treatment with bicarbonate. The AST/ALT decreased to the normal range one month after admission and he was discharged two months later. Since that time he has been symptom-free, with liver biochemistry and serum ammonia level within the normal range, and has not required hospitalization.

DISCUSSION

Hyperammonemic encephalopathy is usually associated with severe liver dysfunction or portcaval shunts

Manuscript received March 28, 2002; revised manuscript received November 25, 2002; accepted December 2, 2002.

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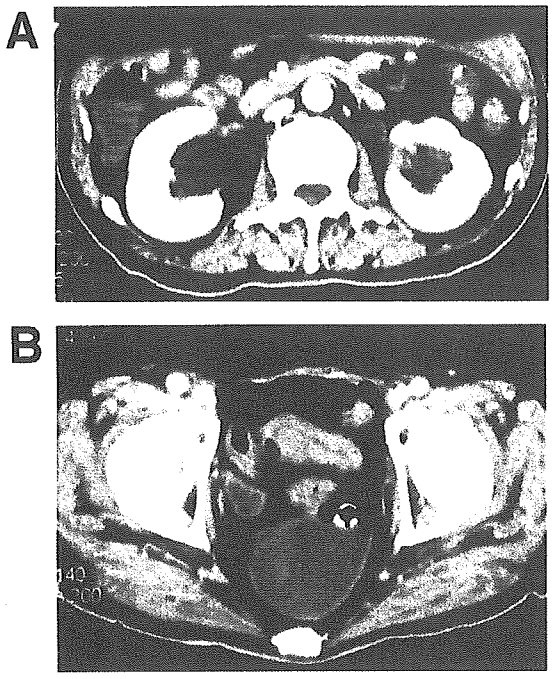

Fig 1. CT scan at the onset of hyperammonemic encephalopathy. The renal pelvis, ureter (A) and rectum (B) were distended with stasis of urine. A rectal tube was intubated for the drainage of urine.

(16, 17). This setting is also reported in the cases of acquired or congenital deficiency of hepatic enzymes of the Krebs urea cycle (18) and in some patients who have had urinary diversion with or without obvious liver disease (5–15). Serum ammonia may be elevated after a ureterosigmoidostomy, which provides a large amount of ammonia to the colon and reabsorption of ammonia would occur more extensively when there is urinary stasis in the colon (19, 20). Our patient did not have urinary stasis when he was admitted; however, the serum ammonia level was elevated markedly, concomitant with stasis of urine (Figure 1A,B), and was unresponsive to lactulose treatment. It seemed that bed rest induced urinary stasis because he had never suffered from hydronephrosis or hyperammonemia after the ureterosigmoidostomy, nor after recovery of hepatitis. Although diagnosed as "fulminant hepatic failure," this case might be specific or exceptional because liver biopsy did not show severe dispersed necrosis (Figure 2) and hyperammonemic encephalopathy might not have occurred if he had been intubated into the rectum immediately after admission. In patients developing encephalopathy, this pathophysiology should be considered rather than attribute the findings to decompensation or hepatic failure related to the hepatitis.

As for the treatment of hyperammonemia, some of the reported cases were treated with lactulose from rectal tube (9, 14), but it is possible that lactulose from a rectal tube would have a minimal effect when there is profound urinary stasis in the colon, because it would drain with urine rapidly after injection through the tube. The serum ammonia level of our patient was elevated despite treatment with lactulose orally; however, it declined dramatically only with rectal intubation for the drainage of urine.

Among four reported cases of hyperammonemic encephalopathy with ureterosigmoidostomy and obvious

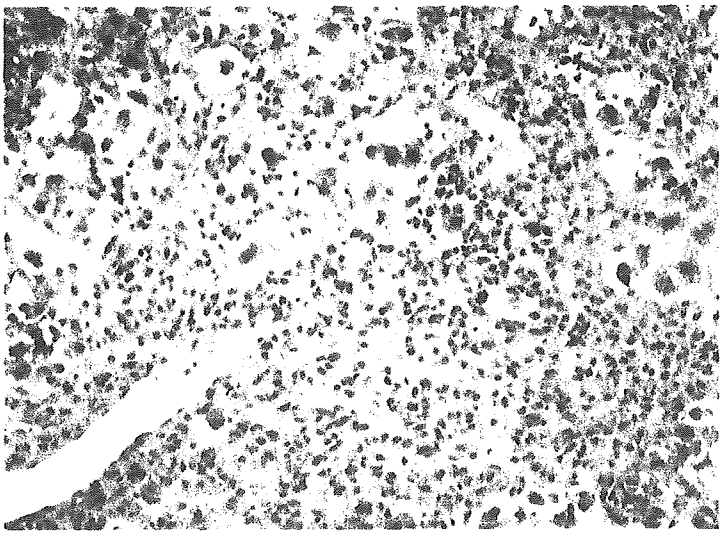

Fig 2. Liver biopsy on the seventh day after admission. Partial periportal lymphocytic and neutrophilic inflammatory infiltrates and swollen hepatocytes can be seen, but not severely dispersed necrosis. Hematoxylin and eosin, ×200.

coexistent liver disease (5–8), only one patient had acute liver injury in a case which was reported nearly half a century ago (8). Although liver transplantation is becoming one of the major strategies for the treatment of fulminant hepatic failure in recent years (21), evaluation of the patient's status by liver biopsy in the early stage and retrieval of the other causes of hyperammonemic encephalopathy would be important in order to circumvent unrequired surgical treatment. In addition, as a safe and effective technique, transjugular liver biopsy is recommended for those who have problems with percutaneous biopsy such as coagulopathy but require immediate diagnosis (22). We did a transjugular biopsy on our patient and obtained four independent specimens without any complications.

REFERENCES

1. Pannek J, Senge T: History of urinary diversion. Urol Int 60:1–10, 1998
2. Fichtner J: Follow-up after urinary diversion. Urol Int 63:40–45, 1999
3. Mottaz AE, Zingg EJ, Studer UE: Ureterosigmoidostomy in adults: long term results. Prog Urol 6:87–92, 1996
4. McDougal WS: Metabolic complications of urinary intestinal diversion. J Urol 147:1199–1208, 1992
5. Mortensen E, Lyng G, Juhl E: Ammonia-induced coma after ureterosigmoidostomy. Lancet 1:496, 1972
6. Egense J, Schwartz M: Recurrent hepatic coma following ureterosigmoidostomy. Scand J Gastroenterol 5(suppl 7):149–152, 1970
7. Silberman R: Ammonia intoxication following ureterosigmoidostomy in a patient with liver disease. Lancet 2:937–939, 1958
8. McDermott WV: Diversion of urine to the intestines as a factor in ammoniagenic coma. N Engl J Med 256:460–462, 1957
9. Van Laethem JL, Gay F, Franck N, Van Gossum A: Hyperammoniemic coma in a patient with ureterosigmoidostomy and normal liver function. Dig Dis Sci 37:1754–1756, 1992
10. Kaveggia FF, Thompson JS, Schafer EC, Fischer JL: Hyperammonemic encephalopathy in urinary diversion with urea-splitting urinary tract infection. Arch Intern Med 150:2389–2392, 1990
11. Cascino GD, Jensen JM, Nelson LA, Schutta HS: Periodic hyperammonemic encephalopathy associated with a ureterosigmoidostomy. Mayo Clin Proc 64:653–656, 1989
12. Gilbert GJ: Acute ammonia intoxication 37 years after ureterosigmoidostomy. South Med J 81:1443–1445, 1988
13. Kaufman JJ: Ammoniagenic coma following ureterosigmoidostomy. J Urol 131:743–745, 1984
14. Edwards RH: Hyperammonemic encephalopathy related to ureterosigmoidostomy. Arch Neurol 41:1211–1212, 1984
15. Mounger EJ, Branson AD: Ammonia encephalopathy secondary to ureterosigmoidostomy: a case report. J Urol 108:411–412, 1972
16. Lockwood AH, McDonald JM, Reiman RE, Gelbard As, Laughlin JS, Duffy TE, Plum F: The dynamics of ammonia metabolism in man. Effects of liver disease and hyperammonemia. J Clin Invest 63:449–460, 1979
17. McDermott WV, Adams RD: Episodic stupor associated with an Eck fistula in the human with particular reference to the metabolism of ammonia. J Clin Invest 33:1–9, 1954
18. Hsia YE: Inherited hyperammonemic syndromes. Gastroenterology 67:347–374, 1974
19. Boyce WH, Vest SA: The role of ammonia reabsorption in acid-base imbalance following uretercsigmoidostomy. J Urol 67:169–176, 1952
20. Mitchell AD, Valk WL: Hyperchloremic acidosis of ureterosigmoidostomies. J Urol 69:82–88, 1953
21. Gill RQ, Sterling RK: Acute liver failure. J Clin Gastroenterol 33:191–198, 2001
22. Bass NM, Yao FY: The role of the interventional radiologist. Transjugular procedures. Gastrointest Endosc Clin North Am 11:131–161, 2001

nosis must be confirmed by immunohistochemical staining. Metastatic disease is usually evident at presentation and is found in 27% of cases. Interestingly, poor survival has not been correlated with evidence of metastatic disease at time of surgery.[5] Patients followed conservatively without treatment have a 5-year survival rate of 28%.[2] The curative treatment options include local resection and orthotopic liver transplantation. Patients with radiographic and intraoperative appearance of a single lesion may develop early aggressive recurrence within 3 months of partial resection.[6] Therefore, partial resection should be offered with caution. Liver transplantation has been shown to improve 5-year survival to 71%.[5]

There are concerns regarding the appropriateness of liver transplantation given the shortage of cadaveric liver donors and the variable clinical course of hepatic epithelioid hemangioendothelioma.[6] The use of living donor liver transplantation may address these concerns by expanding the donor pool. We recommend that young, healthy patients without surgical contraindications can be considered for liver transplant surgery due to unpredictable natural history of hepatic epithelioid hemangioendothelioma. Further experience is needed to clearly establish the efficacy of living donor liver transplantation in the management of rare tumors, such as hepatic epithelioid hemangioendothelioma.

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REFERENCES

1. Weiss SW, Enzinger FM. Epithelioid hemangioendothelioma: a vascular tumor often mistaken for a carcinoma. *Cancer*. 1982;50:970–981.
2. Ohori NP, Yousem SA, Sonmez-Alpan E, et al. Estrogen and progesterone receptors in lymphangioleiomyomatosis, epithelioid hemangioendothelioma, and sclerosing hemangioma of the lung. *Am J Clin Pathol*. 1991;96:529–535.
3. Ishak KG, Sesterhenn IA, Goodman ZD, et al. Epithelioid hemangioendothelioma of the liver: a clinicopathologic and follow-up study of 32 cases. *Hum Pathol*. 1984;15:839–852.
4. Makhlouf HR, Ishak KG, Goodman ZD. Epithelioid hemangioendothelioma of the liver: a clinicopathologic study of 137 cases. *Cancer*. 1999;85:562–582.
5. Madariaga JR, Marino IR, Karavias DD, et al. Long-term results after liver transplantation for primary hepatic epithelioid hemangioendothelioma. *Ann Surg Oncol*. 1995;2:483–487.
6. Ben-Haim M, Roayaie S, Ye MQ, et al. Hepatic epithelioid hemangioendothelioma: resection or transplantation, which and when? *Liver Transpl Surg*. 1999;5:526–531.

Acute Hepatitis With *Salmonella paratyphi* A and Hepatitis E Virus Coinfection

To the Editor:

Paratyphoid fever is one of the infectious diseases that often affect the liver,[1] but it might be difficult for clinicians to determine the cause of hepatitis when there is coinfection with other hepatitis viruses. We observed a case of acute hepatitis with *Salmonella paratyphi* A and hepatitis E virus coinfection.

A 22-year-old man who had traveled in Nepal for 1 month until November 5, 2002, presented to our hospital on December 2, 2002 with the complaints of diarrhea and fever (≥38.0°C) persisting 3 weeks and 2 weeks, respectively. A physical examination revealed bradycardia (38 beats/min), but hepatosplenomegaly was not observed. Laboratory studies demonstrated little hepatic injury (aspartate aminotransferase [AST], 47 IU/L; alanine aminotransferase [ALT], 47 IU/L; alkaline phosphatase [ALP], 237 IU/L; lactic dehydrogenase [LDH], 324 IU/L), and the serum bilirubin level was within the normal range. The patient was admitted 1 week later because his symptoms had not improved and signs of hepatic injury were worse (AST, 2,394 IU/L; ALT, 3,730 IU/L; ALP, 607 IU/L; LDH, 1,930 IU/L), but the serum bilirubin level and the prothrombin time were within the normal range. Viral markers were negative for hepatitis A, B, C, G, TT virus, cytomegalovirus, and Epstein-Barr virus, but positive for hepatitis E virus (both immunoglobulin M and G anti-hepatitis E virus). The serum aminotransferase levels decreased immediately after admission; however, the diarrhea and high fever persisted. Four days after admission, *S. paratyphi* A was isolated from his blood and stool. His symptoms improved immediately after treatment with levofloxacin and he was discharged 3 weeks after the admission.

This is the second case report describing the *S. paratyphi* A and hepatitis E virus coinfection.[2] It is known that clinical features such as the incubation period and symptoms are similar in *Salmonella* hepatitis and viral hepatitis.[3–7] However, in *Salmonella* hepatitis, a high fever and bradycardia are detected more often, compared with the viral hepatitis, and the laboratory tests usually show higher serum levels of AST than that of ALT.[4] It is also reported that the ALT/LDH ratio may be the best discriminator of the two conditions as it is usually <4.0 in *Salmonella* hepatitis but >5.0 in acute viral hepatitis.[4] The cause of the hepatitis in our case is proposed to be due to the hepatitis E virus infection for the following reasons: 1) our patient showed little hepatic injury at the presentation despite persisting diarrhea, a high fever, and bradycardia, which are typical symptoms and signs for *Salmonella* infection; 2) the serum aminotransferase levels immediately decreased after admission without any antibiotics, even though the symptoms and signs noted above persisted; and 3) in *Salmonella* hepatitis the serum aminotransferase levels rarely increase to more than 1,000

units, and AST is usually higher than ALT.[4–6] The ALT/LDH ratio in our case was 2.0, more suggestive of *Salmonella* hepatitis than viral hepatitis[4]; however, this rule cannot be applied when present with coinfection. An extremely rare case of fulminant hepatic failure with typhoid and hepatitis E virus coinfection has been recently reported[7]; however, it was difficult to determine which infection predominantly contributed to the fulminant hepatic failure because typhoid infection alone can cause the encephalopathy in the form of tremors, abnormal behavior, irrelevant talking, and confusion, which are similar to hepatic encephalopathy.[8] Although hepatitis associated with *S. paratyphi* A is emphasized as an emerging clinical entity,[1] it should be also noted that coinfection of other hepatitis viruses may be present in a patient with liver dysfunction with paratyphoid fever, and in that case it is important to examine the clinical features carefully to determine the cause of the hepatitis and to treat it promptly and properly.

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REFERENCES

1. Hsueh PR, Kao JH, Chen YC, et al. Paratyphoid hepatitis: an emerging clinical entity. *Am J Med.* 2002;113:257–258.
2. Phadke AY, Desai HG. Hepatitis E virus and *Salmonella paratyphi* A coinfection. *Indian J Gastroenterol.* 1997;16:115–116.
3. Schwartz E, Jenks NP, Shlim DR. 'Typhoid hepatitis' or typhoid fever and acute viral hepatitis. *Trans R Soc Trop Med Hyg.* 1994;88:437–438.
4. El-Newihi HM, Alamy ME, Reynolds TB. Salmonella hepatitis: analysis of 27 cases and comparison with acute viral hepatitis. *Hepatology.* 1996;24:516–519.
5. Pramoolisinsap C, Viranuvatti V. Salmonella hepatitis. *J Gastroenterol Hepatol.* 1998;13:745–750.
6. Ramachandran S, Godfrey JJ, Perera MVF. Typhoid hepatitis. *JAMA.* 1974;230:236–240.
7. Pandey CK, Singh N, Kumar V, et al. Typhoid, hepatitis E, or typhoid and hepatitis E: the cause of fulminant hepatic failure—a diagnostic dilemma. *Crit Care Med.* 2002;30:376–378.
8. Ali G, Kamili MA, Shah MY, et al. Neuropsychiatric manifestations of typhoid fever. *J Assoc Physicians India.* 1992;40:333–335.

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Hepatology Research
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Hepatitis B virus genotype G is an extremely rare genotype in Japan

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Received 26 July 2004; received in revised form 13 September 2004; accepted 28 September 2004
Available online 14 November 2004

Abstract

Background: Hepatitis B virus (HBV) has been classified into seven genotypes (A–G). HBV genotypes have a geographically characteristic distribution. Since HBV genotype G (HBV/G) was identified recently, little is known about the distribution of HBV/G in Japan. The aim of this study was to clarify this issue.
Patients and methods: Seven hundred and twenty-one serum samples obtained from patients with HBV in Japan were investigated. The patients included 149 asymptomatic carriers, 325 with chronic hepatitis, 129 with liver cirrhosis, and 118 with hepatocellular carcinoma. Six HBV genotypes (A–F) were determined by restriction fragment length polymorphim targeting to the S region of the HBV genome. Furthermore, HBV/G was investigated by polymerase chain reaction with hemi-nested primers derived from an HBV/G-specific nucleotide sequence.
Results: Of the 721 serum samples investigated, 12 subjects were classified as having HBV/A, 88 HBV/B, 610 HBV/C, 3 HBV/D, and 1 HBV/F. Seven subjects had a mixed infection with distinct genotypes, two with HBV/A and HBV/D, and five with HBV/B and HBV/C. HBV/G was not identified among the 721 samples.
Conclusion: HBV/G was not identified in a large cohort of patients with HBV, either single or dual infection. HBV/G seems to be an extremely rare genotype in Japan.

Keywords: Distribution; Genotypes; Hepatitis B virus; Japan; Polymerase chain reaction; Restriction fragment length polymorphism

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doi:10.1016/j.hepres.2004.09.004

1. Introduction

Hepatitis B virus (HBV) infects approximately 350 million individuals worldwide and can cause a wide spectrum of liver disease [1]. HBV has been classified into seven genotypes based on an entire genome difference of more than 8% [2–4]. HBV genotypes have a geographically characteristic distribution [5]. HBV genotype A (HBV/A) and HBV/D are the most common genotypes worldwide, and account for the majority of cases in Europe and Africa. HBV/B and HBV/C are found in East Asia. HBV/E is confined to Africa, and HBV/F has been identified in indigenous populations of Central and South America. In 2000, a unique strain harboring a 36-base pair (bp) insertion into the core region was identified in France and was phylogenetically classified into the seventh genotype, G [4]. Thereafter, HBV/G was revealed to be distributed in San Francisco [6,7], Germany [8], Mexico [9], and Canada [10], and accounted for 1–5% in these areas. Although little is known about the virological and clinical characteristics of HBV/G, one of its unique characteristics is frequent coinfection with the other genotypes. In San Francisco, eight of the eight HBV/G patients were coinfected with HBV/A [6,7], and all of the HBV/G isolates from Canada were also coinfected with HBV/A, or HBV/A and HBV/C [10].

In Japan, HBV/C is the most common genotype, accounting for approximately 85% of all genotypes, and HBV/B follows with 12% [11–13]. However, little is known about the distribution of HBV/G in Japan. We have formerly investigated the 540 sera from patients with hepatitis B collected in and around Nagoya, and found that there were no HBV/G among them [14]. However, the serum samples in the study was obtained from a restricted area, a central part of Japan, therefore, further studies including serum samples collected from the other part of Japan had been required to conclude how often HBV/G distributed in Japan. Moreover, since HBV/G is frequently coinfected with the other genotypes, there is a possibility that HBV/G might exist as a minor population in the sera classified into the other six genotypes (A–F). At this time, to elucidate this issue, we conducted nationwide study of the distribution of HBV/G by analyzing sera obtained from patients with hepatitis B, including those whose genotypes were already known, using hemi-nested polymerase chain reaction (PCR) with HBV/G-specific primers. We also discussed the issues of HBV/G to date.

2. Materials and methods

2.1. Patients

Seven hundred and twenty-one serum samples were collected from patients with HBV in Japan. The patients resided in Hokkaido, Iwate, Yamagata, Niigata, Tokyo, Kanagawa, Nagano, Nagoya, Kyoto, Fukuoka, and Okinawa. The patients in this study were overlapped with some of the previous report [11]. They included 470 (65.1%) males and 251 (34.8%) females. The mean ± S.D. age was 43.6 ± 14.9 years (Table 1).

Table 1
Demographics of the 721 patients in this study

Sample	721
Gender (M:F)	470:251
Age (year)	43.6 ± 14.9
ALT (IU)	78.8 ± 115.8
ALP (IU)	240.8 ± 155.2
γ-GTP (IU)	52.2 ± 96.2
T. bil (mg/dl)	0.99 ± 1.60
HBeAg (%)	45.2
HBV DNA[a] (LGE/ml)	5.69 ± 1.84
Diagnosis	
Asymptomatic carrier	149
Chronic hepatitis	325
Liver cirrhosis	129
Hepatocellular carcinoma	118

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma-glutamyl transpeptidase; LGE, log genome equivalents; T. bil, total bilirubin; TMA, transcription-mediated amplification.

[a] Value was calculated using available data of transcription-mediated amplification of 255 subjects.

2.2. Detection of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) and HBV DNA level

HBsAg was detected by a particle-agglutination test using a commercial kit (Serodia: Fujirebio, Tokyo, Japan), and HBeAg was detected by ELISA using a commercial kit (Serodia: Kokusai-shiyaku, Tokyo, Japan), following the manufacturer's recommendations. Levels of HBV DNA were determined by the transcription-mediated amplification (TMA) method (Chugai Industry, Tokyo, Japan), and the results were expressed as log genome equivalents (LGE) per millilitre.

2.3. Determination of six HBV genotypes (A–F) by restriction fragment length polymorphism (RFLP)

DNA was extracted from 100 μl of serum samples using commercial kits (Smitest EX R&D: Genome Science, Fukushima, Japan) under manufacturer's recommendation. The extracted DNA was amplified in a 50-μl reaction mixture containing 0.5 μM of a sense primer MF1 (5′-YCC TGC TGG TGG CTC CAG TTC-3′: nt. 55–75), 0.5 μM of an antisense primer MR2 (5′-AAG CCA NAC ART GGG GGA AAG C-3′: nt. 730–709), 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Co. Ltd., Tokyo, Japan), 0.2 mM each dNTPs, 3 mM $MgCl_2$, and 1× AmpliTaq Gold Buffer. The reactions were performed in a GeneAmp PCR system 9600 thermocycler. The sample was denatured at 96 °C for 9 min, and subjected to 40 cycles of PCR (95 °C for 1 min; 60 °C for 1 min; 72 °C for 1 min) followed by 72 °C for 5 min at final extension in a 96-well cycler (GeneAmp 9600; Perkin-Elmer, Norwalk, CT, USA). The amplified product

was subjected to the second round PCR with a sense primer MF2 (5′-GTC TAG ACT CGT GGT GGA CTT CTC TC-3′: nt. 246–271) and MR2 under the same condition as the first round PCR. The second round PCR product with the length of 485 bp was subjected to the digestion with five kinds of restriction enzymes. Genotype B could be distinguished by digestion with *Ear*I because of no recognition site of it was existed. Similarly, genotype C also could be distinguished by digestion with *Alw*I, as no recognition site of it was found within the amplified product. Only genotype E had a recognition site of *Nci*I, and only genotype F had no recognition site of *Hph*I. Finally, the distinction between genotypes A and D were done by digestion with *Nla*IV. Genotype A has a recognition site of *Nla*IV, result in the generation of fragments of 220 and 265 bp. While genotype D had two recognition site of *Nla*IV, result in generation of fragments of 34, 186, and 265 bp. Therefore, genotypes A and D were distinguished by if each of 220 and 186 bp were observed, respectively. The digested amplicon were run on 3% agarose gel stained with ethidium bromide and observed under UV light [15].

2.4. Identification of HBV/G

Nucleic acids extracted from serum were subjected to PCR with hemi-nested primers designed on the 36-bp insertion in the C gene of HBV/G genomes. In brief, the DNA was amplified by the first round of PCR for 40 cycles with HBHKF1 (sense: 5′-ACG GGG CGC ACC TCT CTT TAC-3′ [nt. 1519–1539]) and HBHKR2 that involved the 36-bp insertion characteristic of HBV/G (antisense: 5′-AGC CAA AAA GGC CAT ATG GCA-3′ [nt. 17–37 in the core gene of HBV/G]) in the presence of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The second round of PCR was performed for 40 cycles on the product of the first-round PCR with HBHKF2 (sense: 5′-GCA CTT CGT TTC ACC TCT GCA-3′ [nt. 1581–1601]) and HBHKR2. Then, the products were examined for fragments of 357 bp [15].

3. Results

3.1. Demographics, laboratory findings, and diagnosis of the patients

The mean value of alanine aminotransferase (ALT), alkaline phosphatase, gamma-glutamyl transpeptidase, and total bilirubin in the sera was 78.8 ± 115.8 IU, 240.8 ± 155.2 IU, 52.2 ± 96.2 IU, 0.99 ± 1.60 mg/dl, respectively (Table 1). Three hundred and twenty-six patients (45.2%) were positive for HBeAg. The mean value of HBV DNA measured by TMA was 5.69 ± 1.84 LGE per millilitre. One hundred and forty-nine patients (20.1%) were diagnosed as asymptomatic carriers, 325 (45.1%) with chronic hepatitis, 129 (17.9%) with liver cirrhosis, and 118 (16.4%) with hepatocellular carcinoma.

Table 2
Six genotypes (A–F) and HBV genotype G in 721 subjects from Japan

Genotype	No.	No. of HBV genotype G
A	12	0
A+D	2	0
B	88	0
B+C	5	0
C	610	0
D	3	0
F	1	0

3.2. HBV/G among 721 serum samples

Of the 721 serum samples investigated, 12 subjects were classified as having HBV/A, 88 HBV/B, 610 HBV/C, 3 HBV/D, and 1 HBV/F (Table 2). Seven subjects had a mixed infection with distinct genotypes, two with HBV/A and HBV/D, and five with HBV/B and HBV/C. HBV/G was not identified among the 721 samples.

4. Discussion

Several lines of evidence about the clinical significance of HBV genotypes have been accumulated in recent years. HBV/C causes more severe liver diseases than HBV/B by prolonging active hepatitis accompanying HBeAg production [16,17]. In a Western study, the rate of sustained remission after seroconversion was higher in genotype A than in genotype D hepatitis in patients who seroconverted to anti-HBe, and mortality related to liver disease was more frequent in genotype F than in genotype A or genotype D hepatitis [18]. Clinical data concerning HBV/G are very limited. One previous study analyzed 165 patients living in San Francisco and showed that the ALT level was higher in HBV/G than in HBV/C, and HBeAg was more prevalent in HBV/G than in HBV/C or HBV/D [7]. Further studies with a large sample size are warranted to confirm these findings.

Coinfection with distinct genotypes was seen also in other than HBV/G. In this study, coinfections with HBV/A and HBV/D as well as HBV/B and HBV/C were observed. In the previous study, analyzed 256 sera from the USA, Japan, Uzbekistan, Bangladesh, South Africa, and Cameroon, coinfection with distinct genotypes was identified in 28 subjects (10.9%) [19]. The occurrence of coinfection with distinct genotypes is important in virological aspects. It is reported that genomic recombination between distinct genotypes resulted in hybrid HBV strains, which causes distinct degree of liver diseases [20,21]. In such cases, genomic recombination never occurs without coinfection with distinct genotypes. However, clinical implication of coinfection with distinct genotypes per se still remains unanswered.

Ten years before the classification of HBV/G by Stuyver et al. [4], a unique strain with a 36-nucleotide insertion into the core region, which is known to a characteristic of HBV/G nowadays [22], was isolated from a homosexual man with hu-

man immunodeficiency virus infection [23]. Laboratory findings of his serum showed a few curious values. One was that HBeAg was detected in his serum in spite of a stop codon existing in the precore region of its genome, generally aborting the production of HBeAg at the stage of translation. Stuyver et al. also observed the same phenomenon, detection of HBeAg despite the stop codon in the precore region, and speculated that HBV/G might harbor another mechanism for producing HBeAg. Two years later, the mystery was solved by demonstration of coinfection with HBV/A in four of four sera with HBV/G [6]. It was explained that the HBeAg in the sera was produced by the coinfected precore wild type HBV/A. Furthermore, it was revealed that eight of the eight HBV/G patients from San Francisco were coinfected with HBV/A [7], and three of the three HBV/G patients were coinfected with HBV/A, or HBV/A and HBV/C in Canada [10]. These findings of the high frequency of coinfection of HBV/G with other genotypes give rise to another question, of whether HBV/G is competent to replicate by itself. An inoculation experiment in chimpanzees or an expression study in cultured cells would be required to answer this question.

The entire genome sequence of HBV/G has been reported from France [4,24], the USA [22], and Germany [8] so far. Interestingly, the sequence homology of these strains was surprisingly high. In one study in the USA, 10 HBV/G isolates, including 8 from San Francisco as well as 2 from France (FR1 [4] and B1-89 [24]), had a sequence homology of 99.3–99.8% among themselves [22]. Furthermore, another report from Germany showed that the HBV/G isolate (235/01) was nearly identical (sequence homology of the entire length was 99.7%) to both B1-89 and FR1 [8]. There are a few possible explanations for this finding. One possibility is that there are epidemiological links among French, German, and American HBV/G. A patient with HBV/G from Germany [8] and a homosexual male patient with HBV/G from San Francisco [23] were both positive for human immunodeficiency virus type-1. Thus, HBV/G might spread among a specific population, such as homosexual men or intravenous drug users. This would be also associated with the fact that HBV/G was not found among the patients in the current study, in which homosexual and intravenous drug were not included. The other possibilities are that HBV/G has a high genetic stability or was introduced into humans very recently. The mutation rate of HBV has been estimated to be 4.57×10^{-5} per site per year [25]. Thus, HBV/G might have an exceptionally low mutation rate under specific conditions, or the time since its introduction into humans might not have been long enough to gain a genetic diversity like that of the other six genotypes. To elucidate this issue, more HBV/G isolates from a wide variety of areas should be investigated.

In conclusion, HBV/G was investigated in a large cohort of patients with HBV from various areas in Japan, but no HBV/G isolate was identified, in either single or dual infection. The finding of the current nationwide study, the same as that of the previous study investigated the patients in a restricted area, indicates that HBV/G is extremely rare in Japan. Further studies with a large sample size from various areas in the world are required to further reveal the virological and clinical characteristics of HBV/G.

Acknowledgements

Dr. Masashi Mizokami was given a Grant by the Ministry of Health, Labor, and Welfare of Japan (H13-kaken-2).

References

[1] Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733–45.
[2] Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69:2575–83.
[3] Norder H, Ebert JW, Fields HA, Mushahwar IK, Magnius LO. Complete sequencing of a gibbon hepatitis B virus genome reveals a unique genotype distantly related to the chimpanzee hepatitis B virus. Virology 1996;218:214–23.
[4] Stuyver L, De Gendt S, Van Geyt C, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67–74.
[5] Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. Intervirology 2003;46:329–38.
[6] Kato H, Orito E, Gish RG, et al. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. Hepatology 2002;35:922–9.
[7] Kato H, Gish RG, Bzowej N, et al. Eight genotypes (A–H) of hepatitis B virus infecting patients from San Francisco and their demographic, clinical, and virological characteristics. J Med Virol 2004;73:516–21.
[8] Vieth S, Manegold C, Drosten C, Nippraschk T, Gunther S. Sequence and phylogenetic analysis of hepatitis B virus genotype G isolated in Germany. Virus Genes 2002;24:153–6.
[9] Sanchez LV, Maldonado M, Bastidas-Ramirez BE, Norder H, Panduro A. Genotypes and S-gene variability of Mexican hepatitis B virus strains. J Med Virol 2002;68:24–32.
[10] Osiowy C, Giles E. Evaluation of the INNO-LiPA HBV genotyping assay for determination of hepatitis B virus genotype. J Clin Microbiol 2003;41:5473–7.
[11] Orito E, Ichida T, Sakugawa H, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 2001;34:590–4.
[12] Joh R, Hasegawa K, Ogawa M, et al. Genotypic analysis of hepatitis B virus from patients with fulminant hepatitis: comparison with acute self-limited hepatitis. Hepatol Res 2003;26:119–24.
[13] Lin ZM, Yatsuhashi H, Daikoku M, et al. Hepatitis B virus of genotype C persistence after recovery from acute hepatitis B virus infection in Japan. Hepatol Res 2003;25:244–53.
[14] Kato H, Orito E, Sugauchi F, et al. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. J Virol Meth 2001;98:153–9.
[15] Mizokami M, Nakano T, Orito E, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 1999;450:66–71.
[16] Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000;118:554–9.
[17] Orito E, Mizokami M, Sakugawa H, et al. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. Hepatology 2001;33:218–23.

[18] 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;123:1848–56.
[19] Kato H, Orito E, Sugauchi F, et al. 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 Meth 2003;110:29–35.
[20] Sugauchi F, Orito E, Ichida T, et al. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 2002;76:5985–92.
[21] Sugauchi F, Orito E, Ichida T, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124:925–32.
[22] Kato H, Orito E, Gish RG, et al. Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). J Virol 2002;76:6131–7.
[23] Bhat RA, Ulrich PP, Vyas GN. Molecular characterization of a new variant of hepatitis B virus in a persistently infected homosexual man. Hepatology 1990;11:271–6.
[24] Tran A, Kremsdorf D, Capel F, et al. Emergence of and takeover by hepatitis B virus (HBV) with rearrangements in the pre-S/S and pre-C/C genes during chronic HBV infection. J Virol 1991;65:3566–74.
[25] Orito E, Mizokami M, Ina Y, et al. Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. Proc Natl Acad Sci USA 1989;86:7059–62.

Journal of Viral Hepatitis, 2005, 12, 154–159 doi:10.1111/j.1365-2893.2005.00559.x

Long-term follow-up of chronic hepatitis B after the emergence of mutations in the hepatitis B virus polymerase region

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Received November 2003; accepted for publication March 2004

SUMMARY. Treatment of chronic hepatitis B has been greatly improved by the use of lamivudine, but mutations occur in the polymerase region of hepatitis B virus (HBV) and lamivudine-resistant mutants frequently develop. The emergence of lamivudine-resistant strains of HBV is a problem for treating chronic hepatitis B using lamivudine. We observed biochemical and virological changes in 15 patients with chronic hepatitis B for a median period of 29 months (range: 4–42 months) after the emergence of lamivudine-resistant mutants of HBV. Patterns of mutation of the polymerase gene were examined by sequencing the LLAQ motif in domain B and the YMDD motif in domain C. Exacerbation of liver dysfunction occurred in 14 (93.3%) of the 15 patients at a median of 4 months after the emergence of mutations. However, exacerbation of liver dysfunction was observed only in four patients (26.7%) at the time of appearance of the first mutations and in 80.0% of the patients at the time of appearance of the second mutations. Increase in serum alanine aminotransferase (ALT) levels was significantly greater at the time of appearance of second mutations ($P = 0.0096$). In most cases, wild-type HBV was mutated with the substitution of only rtM204I at first, and rtL180M/M204I mutations and then rtL180M/M204V mutations subsequently appeared. Further mutations of the polymerase region caused clinical deterioration. Thus as mutations emerge in the polymerase region, the clinical outcome deteriorates. Thus, monitoring the patterns of mutation of the polymerase gene is useful when using lamivudine for treating HBV.

Keywords: breakthrough, hepatitis B virus, lamivudine, LLAQ, mutation, YMDD.

INTRODUCTION

Lamivudine is a nucleoside analogue that suppresses the replication of hepatitis B virus (HBV) by inhibiting the viral RNA-dependent DNA polymerase. Treatment of chronic hepatitis B has been greatly improved by the use of lamivudine, and the rates of seroconversion (loss of HBe antigen and appearance of anti-HBe) in HBe antigen-positive patients have been reported to be 16–22% after 1 year and 35–40% after 3 years of lamivudine therapy [1–4]. Moreover, in HBe antigen-negative patients, normalization of alanine aminotransferase (ALT) and suppression of serum HBV-DNA to undetectable levels have been achieved [5]. However, it has been reported that lamivudine-resistant HBV mutations of the polymerase region develop in 30% of patients after 1 year and in 49–57% of patients after 3 years of lamivudine therapy [3,6]. Breakthrough hepatitis induced by lamivudine-resistant mutations is sometimes difficult to treat and can be fatal, and is one of the biggest problems in lamivudine treatment of chronic hepatitis B. Although new nucleoside analogues such as adefovir dipivoxil and entecavir used in the United States, Europe, Australia and some Asian countries have demonstrated clinical activity against lamivudine-resistant strains of HBV [7–9], lamivudine is still a key drug in the treatment of chronic hepatitis B. Elucidation of the clinical course of hepatitis B after the emergence of lamivudine-resistant mutations is important. In this paper, we report the long-term biochemical and virological changes in HBV after the emergence of mutations in chronic hepatitis B patients.

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis C virus; PCR, polymerase chain reaction; IFN, interferon.

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PATIENTS AND METHODS

Patients

During the period from March 1999 to February 2002, 40 patients with chronic hepatitis B were treated with lamivudine (100 mg/day) at the Hokkaido University Hospital, and HBV mutations emerged in 15 (37.5%) patients.

Table 1 Patients' characteristics at the start of lamivudine treatment

Total	$n = 15$
Age median (range)	36 (23–59)
Sex	
Male	11 (73.3%)
Female	4 (26.7%)
Background liver disease	
Chronic hepatitis	13 (86.7%)
Cirrhosis	2 (13.3%)
Knodell's Histologic Activity Index ($n = 10$)	
Necroinflammatory score	6.9 ± 3.1
Fibrosis score	1.9 ± 1.3
HBeAg positive	14 (93.3%)
ALT	407 ± 439 (IU/L)
HBV-DNA	7.7 ± 1.3 (LGE/mL)

Age is expressed as median (range) and values of Knodell's Histologic Activity Index and ALT and HBV-DNA are expressed as mean ± SD.

Laboratory testing

Serum ALT levels and HBe antigen, anti-HBe and serum HBV-DNA levels were checked biweekly or at least once a month. HBe antigen and anti-HBe levels were determined using radioimmunoassay kits (Abbot, North Chicago, IL, USA), and serum HBV-DNA levels were measured using transcription-mediated amplification (TMA) assay kits (Chugai Diagnostic Science Co., Ltd, Tokyo, Japan).

Sequencing of the polymerase region

DNA was extracted from 200 μL of serum of each patient by using a QIAamp DNA blood kit (Qiagen, Chatswoth, CA, USA). Five microlitres of DNA template was mixed with 12.5 μL of PCR Master Mix (Promega, Madison, WI, USA), 0.4 μM of sense and antisense primers, and 5.5 μM nuclease-free water for amplification by PCR. The sense primer for PCR was 5′-TGGCTATCGCTGGATGTGTCT-3′ and the antisense primer was 5′-TTGTTCAGTGGTTCG-TAGGGC-3′. The conditions of polymerase chain reaction (PCR) were as follows: 94 °C for 2 min for the initial incubation, 94 °C for 30 s for denaturing, 57 °C for 30 s for annealing, 72 °C for 1′ min for extension for 35 cycles, and a final extension step of 72 °C for 5 min. The DNA product was purified by using a QIAquick PCR Purification Kit (Qiagen). The PCR products were reacted by using an ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequence primer of 5′-CCCTCATGTTGCTGTA-CAAAACCT-3′. Then sequence reaction products were purified by using a DyeEx Spin Kit (Qiagen) and sequenced by using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). We checked two motifs of the HBV polymerase region, the leucine–leucine–alanine–glutamine (LLAQ) motif from codon rt179 to codon rt182 in domain B of the polymerase region, and the tyrosine–methionine–aspartate–aspartate (YMDD) motif from codon rt203 to codon rt206 in domain C. Emergence of lamivudine-resistant mutations was defined as detection of LLAQ and/or YMDD motif mutations. Sequencing of the polymerase region was performed at intervals of 2 weeks to 3 months partly retrospectively, and serial changes in mutation patterns of the HBV polymerase region were observed. In this study, the first mutation was defined as any mutation in the polymerase region detected for the first time and the second or further mutations were defined when new patterns of mutations emerged after previous mutations.

Statistical analysis

Mann–Whitney's *U*-test was used to compare the periods to the emergence of mutations, and Student's *t*-test test was used to compare serum ALT and HBV-DNA levels. All *P*-values were two-sided, and $P < 0.05$ was considered to be statistically significant. All serum HBV-DNA levels less than the limit of detection (<3.7 LGE/mL) were analysed as being 3.7 LGE/mL, and levels above the upper limit of detection (more than 8.8 LGE/mL) were analysed as being 8.8 LGE/mL.

RESULTS

Patients

We observed biochemical and virological changes, including patterns of mutations in the polymerase region, in those 15 lamivudine-resistant patients. Baseline characteristics of the patients at the start of lamivudine treatment are shown in Table 1. The 15 patients included 11 males and four females with a median age of 36 years (range: 23–59). Liver biopsies were performed in 10 patients. In those 10 patients, the necroinflammatory score of hepatitis was 6.9 ± 3.1 (mean ± SD) and the fibrosis score was 1.9 ± 1.3 in the Knodell's Histologic Activity Index [10]. HBe antigen was positive in 14 (93.3%) of the 15 patients. The mean serum ALT level and mean serum HBV-DNA level in the 15 patients were 407 ± 439 IU/L and 7.7 ± 1.3 log genome equivalent (LGE)/mL, respectively. The median follow-up periods were 35 months (range: 27–50 months) from the start of lamivudine treatment and 29 months (range: 4–42 months) after the emergence of lamivudine-resistant mutations.

Period to the emergence of mutations

The median period from the start of lamivudine therapy to emergence of mutations was 16 months (range: 5–34). The

median period was 22 months (range: 12–30 months) in patients with HBV-DNA levels <7.7 LGE/mL (mean serum HBV-DNA level of 15 patients at the start of lamivudine therapy), and it was 15 months (range: 7–18) in patients with higher levels. Patients with higher serum HBV-DNA levels developed mutations more rapidly ($P = 0.0056$).

Patterns of mutation in the HBV polymerase region

Mutation patterns of the polymerase region changed serially (Table 2). The median number of changes in mutation pattern during the follow-up period was 2 (range: 1–4). In nine (60.0%) of the 15 patients, the first mutation pattern was wild type in domain B and YIDD (rtM204I) in domain C. The first mutation patterns of the LLAQ motif in domain B were LMAQ (rtL180M) in five patients (83.3%) and LLTQ (rtA181T) in one patient (16.7%), and those of the YMDD motif in domain C were YIDD (rtM204I) in 10 patients (76.9%) and YVDD (rtM204V) in three patients (23.1%). Only four patients (26.7%) showed liver dysfunction at the time of the appearance of the first mutations. In 10 patients, further mutations appeared in the polymerase region 7 months (range: 1–27 months) after the appearance of the first mutations, and exacerbation of liver dysfunction occurred in eight (80.0%) of the 10 patients. Second or further mutations occurred in domain B and/or domain C, and each mutation caused exacerbation of liver dysfunction. In most cases, mutation with substitution of only rtM204I appeared at first, and mutation with rtL180M/M204I and then mutation with rtL180M/M204V subsequently emerged. Further mutations of the polymerase region caused worse clinical outcomes (Table 3).

Serum ALT and HBV-DNA levels

Exacerbation of liver dysfunction was observed frequently. Serum ALT levels remained within the normal range in only one patient (6.7%), and they were lower than 100 IU/L in five patients (33.3%), 100–500 IU/L in four patients (26.7%), and exceeded 500 IU/L in five patients (33.3%). The median period from the emergence of mutations to the start of exacerbation of liver dysfunction was 4 months (range: 2–10), and the peak serum ALT level was 367.8 ± 385.8 IU/L (mean ± SD). The peak serum ALT level after the appearance of the first mutations was 191.8 ± 281.6 IU/L and that after the appearance of the second mutations was 570.6 ± 390.3 IU/L, exacerbation of liver dysfunction being significantly more

Table 3 Patterns of mutations in the polymerase region and exacerbation of liver dysfunction

Domain C: wild Domain B: wild	→	YIDD wild	→	YIDD LMAQ	→	YVDD LMAQ
n		12		3		10
Extraction of liver dysfunction (ALT >200 IU/L)		4 (33.3%)		2 (66.7%)		7 (70.0%)

Table 2 Mutation patterns of the HBV polymerase region

case	First mutation	Second mutation	Third mutation	Fourth mutation	Follow-up period (months)
1	LLAQ YIDD*	LMAQ YVDD*			42
2	LLTQ YMDD	LMTQ YVDD	LMAQ YVDD		36
3	LLAQ YIDD				35
4	LLAQ YIDD	LMAQ YIDD*	LLAQ YIDD		33
5	LLAQ YIDD	LMAQ YVDD*	LLAQ YIDD		32
6	LLAQ YIDD*	LMAQ YIDD*	LMAQ YMDD†	LMAQ YVDD*	31
7	LMAQ YVDD				29
8	LLAQ YIDD†	LLAQ YMDD	VLAQ YMDD		26
9	LMAQ YIDD	LMAQ YVDD†			24
10	LLAQ YIDD				19
11	LLAQ YIDD				19
12	LLAQ YIDD	LMAQ YVDD*			17
13	LMAQ YVDD	LLAQ YIDD*			13
14	LMAQ YVDD†				11
15	LMAQ YMDD	LMAQ YVDD†			8

*Liver dysfunction: ALT >500 IU/L; †Exacerbation of: ALT >200 IU/L.
Values with '*' and '†' mean that the mutations were accompanied with exacerbation of liver dysfunction. Values with '†' mean ALT levels of 200–500 IU/L and '*' mean more than 500 IU/L.

severe after the appearance of second mutations ($P = 0.0096$).

The mean serum HBV-DNA level was 5.00 ± 0.39 LGE/mL (mean ± SE) at the first detection of mutations. The peak value of serum HBV-DNA levels was 6.27 ± 0.59 LGE/mL after the appearance of the first mutations, and it increased further after the appearance of the second mutations, peaking at 7.18 ± 0.54 LGE/mL (Fig. 1).

Clinical course

The clinical courses of 11 patients who did not use other antiviral drugs such as interferon (IFN) or new nucleoside analogues after the emergence of mutations were observed. The median follow-up period was 30 months (range: 8–42). Serum ALT levels remained within the normal range in only one patient and normalized in five patients after temporary worsening. In two patients, seroconversion of HBe antigen to anti-HBe occurred after temporary worsening of liver function, and serum HBV-DNA levels were decreased to undetectable levels. However, the exacerbation of liver dysfunction continued in five patients.

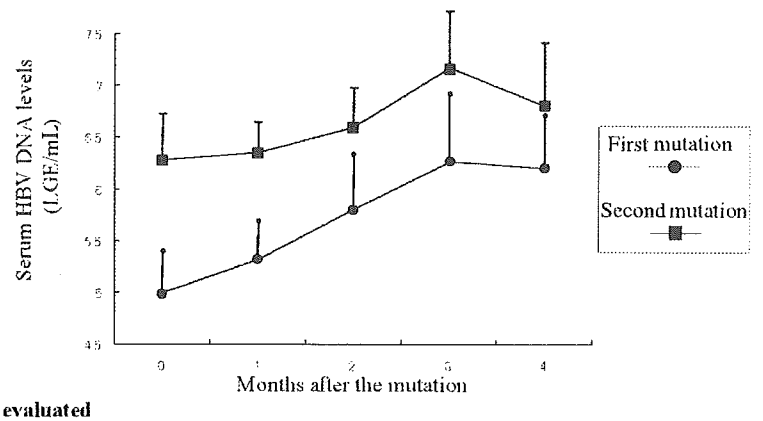
Four patients with severe breakthrough hepatitis were treated with IFN, and the median follow-up period for those patients was 6.5 months (range: 3–14). Six million units of IFN-beta was administered everyday for the first 4 weeks and thereafter three times a week for 20 weeks. Serum ALT levels were normalized in three patients, and serum HBV-DNA levels decreased to undetectable levels in two patients. One of the three patients who had HBe antigen before IFN treatment achieved seroconversion.

DISCUSSION

Lamivudine is a nucleoside analogue that suppresses replication of HBV by inhibiting the viral RNA-dependent DNA polymerase. It has been reported that resistance to lamivudine often develops after 6 months of treatment [11,12] Mutations occur in the polymerase region of HBV-DNA, and HBV becomes resistant to lamivudine. There have been many reports on mutations in the polymerase region and viral resistance, most of them focusing on the YMDD motif from codon rt203 of domain C and the LLAQ motif from codon rt179 of domain B of the HBV polymerase region [13–16]. In this study, we observed changes in serum ALT and HBV-DNA levels, HBe antigen, and anti-HBe in relation to serial changes in mutation patterns of the HBV polymerase region for a median period of 29 months after the emergence of mutations.

Serum HBV-DNA levels increased as soon as the first mutation occurred in the polymerase region, indicating that monitoring of serum HBV-DNA levels is important and that serum HBV-DNA level can be used as a predictive factor for the appearance of mutations as reported previously [17]. On the contrary, increases in serum ALT levels were delayed and ALT level peaked at a median of 4 months after the emergence of mutations. It has been reported that biochemial breakthrough phenomena are usually observed several months after the first detection of strains resistant to lamivudine [18,19]. This time lag is thought to be due to the duration until the occurrence of second or further mutations in the polymerase region.

In our series, rtL179V, rtL180M and rtA181T mutations were observed in domain B, and rtM204I and rtM204V mutations were observed in domain C. These mutations are almost the same as those reported previously [20,21]. In most cases, a mutation with only rtM204I appeared at first, and rtL180M/M204I mutations and then rtL180M/M204V mutations appeared subsequently in the polymerase region. Exacerbation of liver dysfunction at the time of appearance of the first HBV mutations occurred in only 26.7% of our patients. However, when further mutations appeared in 10 patients (66.7%) a median of 7 months after the appearance



No. evaluated

First mutation	15	15	14	12	11
Second mutation	10	10	9	8	7

Fig. 1 Changes of serum HBV-DNA levels after the emergence of mutations. The changes of serum HBV DNA levels after the first mutations to the second were shown by closed circles and after the second mutations to the third by closed squares. Serum HBV-DNA levels were measured by TMA assay. Vertical bars mean standard errors.

of first mutations, worsening of liver impairment occurred in 80.0% of the patients. Further mutations resulted in worsening of clinical courses. Although mutations of the YMDD motif in domain C have stronger effects on resistance to lamivudine than those of the LLAQ motif in domain B, it has been reported that single C-domain mutants have remarkedly decreased abilities of replication [22–24] and that B-domain mutation rtL180M rescues the defective replication competence of domain C mutants [25,26]. In another study, the effect of the addition of rtL180M mutation was examined by using a three-dimensional homology model of the catalytic core of HBV, and it was also shown that the rtM204V mutant is more resistant to lamivudine than the rtM204I mutant and that rtL180M substitution makes each mutant more resistant to lamivudine *in vitro* [27]. These results explain our finding that exacerbation of liver dysfunction occurred more frequently in cases with further mutations because rtM204V and/or rtL180M mutations were detected more frequently in our series when further mutations occurred in the polymerase region. Although small amounts of HBV mutants could not be detected because of the detection limit of direct sequencing, our results showed the significant correlation between accumulation of mutations and exacerbation of liver dysfunction. Direct sequencing was a useful method to detect mutations in the polymerase region clinically. But it is more effective to detect especially second or further mutations by more sensitive methods such as peptide nucleic acid mediated PCR clamping [28,29] because these mutations frequently caused severe liver dysfunction.

Exacerbation of liver dysfunction occurred without increase in serum HBV-DNA level after the emergence of mutations in some of our patients, indicating that some other factors may also lead to exacerbation of liver dysfunction. The polymerase gene of HBV overlaps with the surface antigen gene, and mutations in the polymerase region result in a change in the relevant amino acid of the surface antigen. It is possible that changes in the amino acid of the surface antigen may induce exacerbation of liver dysfunction.

In 11 patients, antiviral drugs other than lamivudine were not used. Fatal cases after discontinuation of lamivudine treatment have been reported [30], and it has been reported that lamivudine still suppresses the replication of wild-type HBV-DNA after the emergence of mutations [11,31]. We therefore continued lamivudine treatment after the appearance of lamivudine-resistant mutations. Serum ALT levels were normalized in six patients, and two of those six patients achieved seroconversion after temporary worsening. Thus, good clinical courses following temporary worsening were observed in some cases, indicating that observation without using other antiviral drugs is one of choices for patients with breakthrough hepatitis. However, exacerbation of liver dysfunction continued in five of our patients, and HBe antigen reappeared in two of them who had anti-HBe before the emergence of mutations. It has been reported that breakthrough hepatitis can be fatal in patients with advanced liver disease [32]. Patients with continuous liver dysfunction after emergence of mutations and patients with advanced liver disease should therefore be treated with new antiviral drugs such as adefovir dipivoxil.

Although the follow-up period was short, a good clinical response to IFN in patients with breakthrough hepatitis was obtained. Suzuki *et al.* [33] reported that IFN therapy was effective for lamivudine-resistant HBV mutants and that it may induce virological and clinical improvement accompanied by seroconversion. Although IFN has some side-effects and it is difficult to use for cirrhotic patients, it is one of the treatment options for breakthrough hepatitis. However, it has also been reported that serum HBV-DNA levels increased again and that hepatitis reccurred after reducing the dose of IFN [33]. Indeed, hepatitis recurred after cessation of IFN treatment in one of our patients. Care should therefore be taken in reducing the dose of IFN or terminating IFN treatment.

CONCLUSIONS

Exacerbation of liver dysfunction was observed frequently after the emergence of mutations in the HBV polymerase region. In most cases, the mutation pattern was a substitution of only rtM204I at first, and rtL180M/M204I mutations and then rtL180M/M204V mutations subsequently appeared. Exacerbation of liver dysfunction became more severe as more mutations occurred in the polymerase region. These results suggest that monitoring the patterns of mutation of the polymerase gene is useful when using lamivudine for treatment of HBV.

In some cases, serum ALT levels were normalized and seroconversion was achieved after temporary worsening of liver impairment, indicating that treatment is not necessarily required for all cases after the appearance of lamivudine-resistant mutations.

REFERENCES

1 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; 339: 61–68.
2 Dienstag JL, Schiff ER, Wright TL *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; 341: 1256–1263.
3 Leung NW, Lai CL, Chang TT *et al.* on behalf of the Asia Hepatitis Lamivudine Study Group. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. *Hepatology* 2001; 33: 1527–1532.
4 Da Silva LC, Pinho JR, Sitnik R, Da Fonseca LE, Carrilho FJ. Efficacy and tolerability of long-term therapy using high lamivudine doses for the treatment of chronic hepatitis B. *J Gastroenterol* 2001; 36: 476–485.