core-promoter mutation might lead to fulminant hepatitis.

That HBV DNA levels were lower in patients with fulminant than acute hepatitis, despite a high replication capacity of HBV/Bj incriminated in the development of fulminant hepatic failure, may seem surprising. Because destruction of hepatocytes proceeds swiftly in patients with fulminant hepatitis, hepatic mass for HBV to thrive would have been extremely reduced in them at presentation. As a consequence, some patients with fulminant hepatitis B are without serum HBsAg; they are diagnosed by high-titered IgM anti-HBc.[41] On the contrary, HBV DNA levels were higher in the patients with HBV/Ae than Bj (Table 1); those with Ae tend to delay reducing HBV DNA, some of whom have chronic outcome. Combined, correlating HBV DNA levels with the clinical outcome in acute HBV infection would be difficult.

A wide variation has been seen in the rate of persistence after acute HBV infection in adulthood. No chronic outcomes of acute hepatitis B were seen in female recipients of red blood cells contaminated with HBV (0/28)[42] or patients in an acupuncture-associated outbreak (0/35).[43] In marked contrast, they ranged from 0.2% (14/715) in Greece[44] through 2.7% (1/37) in university students in Taiwan[45] to 10.4% (5/8) in Alaskan Eskimos[46] and 12.1% (7/58) in Germany.[47] HBV genotypes are implicated in a high rate of persistence in European countries where HBV/A is predominant.[48] In Japan, also, adulthood infection tends to persist longer with HBV/A than B or C (23% 3/13 vs. 13% 1/8 or 12% 3/25).[49] In the current series on 256 patients with acute hepatitis B in Japan who were followed rigorously, HBV infection persisted in only three (1%), representing 2 of the 32 (6%) with HBV/Ae and 1 of the 21 (5%) with Ba. Hence, 99% of patients lost their HBsAg by 6 months. Persistence of HBV observed in the patients with HBV/Ae (6%) is less frequent than that in 4 of the 31 (13%) patients with Ae from a hospital in metropolitan Tokyo.[49] The difference would be ascribable, at least in part, to lamivudine given to some patients in this study (18%). All patients treated with lamivudine recovered from acute hepatitis, whereas none of the three patients with chronic outcome had received antiviral treatment during their acute phase of illness, indicating that lamivudine might be able to prevent the chronic outcome. Likewise, some patients from metropolitan Tokyo, in whom HBV persisted,[49,50] had received immunosuppressants in the acute phase of infection before referral to their hospital.

Using cell culture and chimeric mice models for the replication system of different genotype/subgenotype clones, we have observed that the replication of HBV is the highest for HBV/Bj or C and the lowest for Aa/Ae (Sugiyama M et al., manuscript in submission). It is probable that the propensity of HBV/A infection to chronicity would be due to less intensive immune response against its slow viral dynamics. Taken together, the infection with HBV/A appears to persist longer than those with the other genotypes; this needs to be confirmed by further investigation in patients from various countries.

In conclusion, persistence of HBV after acute infection is rare and occurs more often in patients infected with HBV/Ae than others. Fulminant outcome is frequent in hospitalized patients and associated with HBV/Bj accompanied by the lack of serum HBeAg as well as high replication due to precore stop-codon mutation (G1896A), a finding supported by an *in vitro* replication model.

References

1. Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733-1745.
2. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;83:2059-2073.
3. Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol 1992;73:1201-1208.
4. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69: 2575-2583.
5. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67-74.
6. Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. HEPATOLOGY 2002;35:1274-1276.
7. Kao JH. Hepatitis B viral genotypes: clinical relevance and molecular characteristics. J Gastroenterol Hepatol 2002;17:643-650.
8. Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. Intervirology 2003;46:329-338.
9. Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, et al. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 2004;85:811-820.
10. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, 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;76:5985-5992.
11. Huy TT, Ushijima H, Quang VX, Win KM, Luengrojanakul P, Kikuchi K, et al. Genotype C of hepatitis B virus can be classified into at least two subgroups. J Gen Virol 2004;85:283-292.
12. Tanaka Y, Orito E, Yuen MF, Mukaide M, Sugauchi F, Ito K, et al. Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism. Hepatol Res 2005;33:216-224.
13. Kramvis A, Kew MC. Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. J Viral Hepatol 2005;12:456-464.
14. Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315-321.
15. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124: 925-932.

16. Tanaka Y, Hasegawa I, Kato T, Orito E, Hirashima N, Acharya SK, et al. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. HEPATOLOGY 2004;40:747-755.
17. Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus: large-scale analysis using a new genotyping method. J Infect Dis 1997;175:1285-1293.
18. Mitamura K. Epidemiology of HDV infection in Japan. Prog Clin Biol Res 1991;364:81-87.
19. Trey C, Lipworth L, Chalmers TC, Davidson CS, Gottlieb LS, Popper H, et al. Fulminant hepatic failure. presumable contribution of halothane. N Engl J Med 1968;279:798-801.
20. Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, et al. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 2000;87:81-89.
21. Kato H, Orito E, Gish RG, Bzowej N, Newsom M, Sugauchi F, et al. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. HEPATOLOGY 2002;35:922-929.
22. Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 1997;78:1719-1729.
23. Hasegawa I, Tanaka Y, Kramvis A, Kato T, Sugauchi F, Acharya SK, et al. Novel hepatitis B virus genotype A subtyping assay that distinguishes subtype Aa from Ae and its application in epidemiological studies. J Virol 2004;78:7575-7581.
24. Sugauchi F, Kumada H, Sakugawa H, Komatsu M, Niitsuma H, Watanabe H, et al. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. Clin Infect Dis 2004;38:1222-1228.
25. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;38:2899-2903.
26. Noto H, Terao T, Ryou S, Hirose Y, Yoshida T, Ookubo H, et al. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980-1994. J Gastroenterol Hepatol 2003;18:943-949.
27. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, 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;33:218-223.
28. Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshiba M, et al. Fulminant hepatitis B: induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. Gastroenterology 1991;100:1087-1094.
29. Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 1991;324:1699-1704.
30. Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, et al. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med 1995;122:241-248.
31. Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med 1991;324:1705-1709.
32. Karayiannis P, Alexopoulou A, Hadziyannis S, Thursz M, Watts R, Seito S, et al. Fulminant hepatitis associated with hepatitis B virus e antigen-negative infection: importance of host factors. HEPATOLOGY 1995;22: 1628-1634.
33. Laskus T, Persing DH, Nowicki MJ, Mosley JW, Rakela J. Nucleotide sequence analysis of the precore region in patients with fulminant hepatitis B in the United States. Gastroenterology 1993;105:1173-1178.
34. Laskus T, Rakela J, Nowicki MJ, Persing DH. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. Gastroenterology 1995;109:1618-1623.
35. Liang TJ, Hasegawa K, Munoz SJ, Shapiro CN, Yoffe B, McMahon BJ, et al. Hepatitis B virus precore mutation and fulminant hepatitis in the United States. A polymerase chain reaction-based assay for the detection of specific mutation. J Clin Invest 1994;93:550-555.
36. Parekh S, Zoulim F, Ahn SH, Tsai A, Li J, Kawai S, et al. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. J Virol 2003;77:6601-6612.
37. Imamura T, Yokosuka O, Kurihara T, Kanda T, Fukai K, Imazeki F, et al. Distribution of hepatitis B viral genotypes and mutations in the core promoter and precore regions in acute forms of liver disease in patients from Chiba, Japan. Gut 2003;52:1630-1637.
38. Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 1996;70:5845-5851.
39. Ogata N, Miller RH, Ishak KG, Purcell RH. The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology 1993;194:263-276.
40. Bocharov G, Ludewig B, Bertoletti A, Klenerman P, Junt T, Krebs P, et al. Underwhelming the Immune Response: effect of slow virus growth on CD8+-T-lymphocyte responses. J Virol 2004;78:2247-2254.
41. Shimizu M, Ohyama M, Takahashi Y, Udo K, Kojima M, Kametani M, et al. Immunoglobulin M antibody against hepatitis B core antigen for the diagnosis of fulminant type B hepatitis. Gastroenterology 1983;84:604-610.
42. Rinker J, Galambos JT. Prospective study of hepatitis B in thirty-two inadvertently infected people. Gastroenterology 1981;81:686-691.
43. Kent GP, Brondum J, Keenlyside RA, LaFazia LM, Scott HD. A large outbreak of acupuncture-associated hepatitis B. Am J Epidemiol 1988; 127:591-598.
44. Tassopoulos NC, Papaevangelou GJ, Sjogren MH, Roumeliotou-Karayannis A, Gerin JL, Purcell RH. Natural history of acute hepatitis B surface antigen-positive hepatitis in Greek adults. Gastroenterology 1987;92: 1844-1850.
45. Beasley RP, Hwang LY, Lin CC, Ko YC, Twu SJ. Incidence of hepatitis among students at a university in Taiwan. Am J Epidemiol 1983;117:213-222.
46. McMahon BJ, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP, et al. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J Infect Dis 1985;151:599-603.
47. Schomerus H, Wiedmann KH, Dolle W, Peerenboom H, Strohmeyer G, Balzer K, et al. (+)-Cyanidanol-3 in the treatment of acute viral hepatitis: a randomized controlled trial. HEPATOLOGY 1984;4:331-335.
48. Sherlock S. The natural history of hepatitis B. Postgrad Med J 1987;63: S7-S11.
49. Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, et al. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 2002;68: 522-528.
50. Suzuki Y, Kobayashi M, Ikeda K, Suzuki F, Arfase Y, Akuta N, et al. Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. J Med Virol 2005;76:33-39.

ELSEVIER

Journal of Clinical Virology 35 (2006) 250–256

JOURNAL OF CLINICAL VIROLOGY

www.elsevier.com/locate/jcv

Association of virus infected-T cell in severe hepatitis caused by primary Epstein-Barr virus infection

Shinya Hara[a], Yo Hoshino[b], Takehito Naitou[c], Kenichi Nagano[c], Masakatsu Iwai[d], Kazuyuki Suzuki[d], Kazuhide Yamamoto[e], Tetsuro Nagasaka[f], Tsuneo Morishima[g], Hiroshi Kimura[b,*]

[a] *Department of Pediatrics, Toyota Memorial Hospital, Toyota, Japan*
[b] *Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*
[c] *Departments of Internal Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[d] *First Department of Internal Medicine, Iwate Medical University School of Medicine, Iwate, Japan*
[e] *First Department of Internal Medicine, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan*
[f] *Departments of Clinical Pathology, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[g] *Department of Pediatrics, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan*

Received 13 April 2005; received in revised form 7 July 2005; accepted 12 July 2005

Abstract

Background: Infectious mononucleosis owing to primary Epstein-Barr virus (EBV) infection sometimes causes hepatitis, which is usually self-limiting with mildly elevated transaminases, but can rarely develop into severe hepatitis with jaundice.
Objective: To clarify the pathogenesis of severe hepatitis by primary EBV infection.
Methods: We experienced four cases of severe hepatitis with jaundice caused by primary EBV infection. These cases were analyzed virologically and histologically, and compared with infectious mononucleosis patients without jaundice.
Results and discussion: Using real-time PCR, more EBV-DNA was detected in peripheral blood from patients with severe hepatitis, as compared to those without jaundice. Furthermore, $CD3^+$, $CD4^+$ or $CD8^+$ cells contained more EBV DNA than did other cell populations, indicating that in severe hepatitis, T cells harbor most of the EBV. By contrast, mainly B cells were infected in infectious mononucleosis patients without jaundice. The liver was biopsied in three of the four cases. An in situ hybridization study showed that EBV infected lymphocytes, not hepatocytes. In addition, in one patient, it was confirmed that the infected lymphocytes were $CD8^+$ T cells. These results suggest that a large EBV burden and T cell infection may play major roles in the mechanism of severe hepatitis caused by primary EB virus infection.

Keywords: EBV; Primary infection; Hepatitis; T cells

1. Introduction

Epstein-Barr virus (EBV) is ubiquitous in humans and is the causative agent of infectious mononucleosis. Although primary EBV infection in children is usually asymptomatic, some children or young adults manifest infectious mononucleosis with typical symptoms of fever, pharyngitis, lymphadenopathy, hepatosplenomegaly and atypical lymphocytosis (Sumaya and Ench, 1985). Infectious mononucleosis is caused by an intense cytotoxic T lymphocyte response to eliminate EBV-infected B cells (Rickinson and Kieff, 2001). This disease is usually benign, and self-limiting. In the acute phase of infectious mononucleosis, elevated transaminases are found in 80% of patients, while jaundice is noted in only 5.0–6.6% (Markin, 1994; White and Juel-Jensen, 1984).

Abbreviations: ALT, alanine aminotransferase; EBER1, Epstein-Barr encoded small RNA 1; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LMP, latent membrane protein; PBMCs, peripheral blood mononuclear cells; VCA, viral capsid antigen; WBC, white blood cells

* Corresponding author. Tel.: +81 52 744 2294; fax: +81 52 744 2974.
E-mail address: hkimura@med.nagoya-u.ac.jp (H. Kimura).

1386-6532/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jcv.2005.07.009

Hepatitis owing to primary EBV infection is usually mild and self-limited, although the mechanism is unclear. Rarely, it results in hepatic failure with severe jaundice in fatal infectious mononucleosis (Markin et al., 1987; Tazawa et al., 1993).

We previously reported on one severe hepatitis patient with primary EBV infection. In that case, EBV did not infect hepatocytes, but infected T cells (Kimura et al., 2001b). In this study, we report three additional cases of severe hepatitis with jaundice caused by primary EBV infection. We performed virological and histological studies of the four cases, and compared them with results for infectious mononucleosis patients without jaundice.

2. Patients and methods

2.1. Patients

Four female patients, aged 2–22 years old (mean age: 15.5 years) with severe hepatitis caused by EBV, were enrolled in this study (Table 1). Severe hepatitis was defined on the basis of clinical jaundice. All four were previously healthy and had no prior serious infections, and none had contributory family histories. Two were hospitalized in the University Hospital of Nagoya University Graduate School of Medicine, one in the University Hospital of Okayama University Graduate School of Medicine, and one University Hospital of Iwate Medical University. They all had several clinical symptoms typical of patients with infectious mononucleosis, including fever, lymphadenopathy, pharyngitis and hepatosplenomegaly. The percentage of atypical lymphocytes exceeded 20% in three patients and that of normal and atypical lymphocytes exceeded 60% of the white blood cells (WBC) in all of them (Table 1). The Paul-Bunnell test was performed on serum samples from two patients, but was negative. In the EBV-specific serology, viral capsid antigen (VCA)-IgG antibody was positive and EB nuclear antigen (EBNA) antibody was negative in all cases. VCA-IgM was positive in three cases, indicating the presence of primary EBV infection. Antibodies against hepatitis viruses A, B and C, and human immunodeficiency virus were negative. Cytomegalovirus DNA was not detected in their blood.

Each patient received different treatments. In case 1, acyclovir was administered intravenously without clinical improvement, then vidarabine was administered, which led to improvement. Administration of methylprednisolone pulse therapy combined with acyclovir led to clinical improvement in case 3. Case 4 was treated with γ-globulin and acyclovir without clinical improvement; subsequent administration of methylprednisolone pulse therapy led to a remarkable clinical improvement. Glycyrrhizic acid was administered to all of the patients. Cases 1 and 3 were hospitalized for about 1 month, while cases 2 and 4 were hospitalized for about 2 months. They were all discharged without sequelae.

Table 1
Characteristics of the patients with severe hepatitis

	Case 1	Case 2	Case 3	Case 4
Age	2	17	22	21
Sex	F	F	F	F
Symptoms				
Fever	+	+	+	+
Lymphadenopathy	−	+	+	+
Pharyngitis	+	−	+	+
Hepatosplenomegaly	+	+	+	+
Rash	−	−	−	−
Laboratory data				
WBC count (μl^{-1})	14700	2400	16000	12700
Lymphocytes (%)	64	43	27	53
Atypical lymphocytes (%)	0	27	44	21
Hemoglobin (g/dl)	11	13	12	14
Platelet count ($\times 10^4$ μl^{-1})	13.9	6.5	7.4	18.6
EBV-related antibody				
Anti-VCA-IgG	2560	160	1280	1280
Anti-VCA-IgM	<10	10	20	40
Anti-EBNA	<10	<10	<10	<10
Viral load at peak				
PBMCs (copies/μg DNA)	13000	470[a]	3700	1920[a]
Plasma (copies/ml)	530	290000	32000	43

Note: WBC, white blood cells; VCA, viral capsid antigen; EBNA, Epstein-Barr nuclear antigen; PBMCs, peripheral blood mononuclear cells.
[a] PBMCs were not available in their acute phase.

2.2. Controls

As controls, 21 infectious mononucleosis patients without clinical jaundice were enrolled. They aged 1–21 years old (mean age: 6.8 years). All fulfilled at least three of the following criteria for infectious mononucleosis: (1) fever, (2) tonsillopharyngitis, (3) cervical adenopathy, (4) hepatomegaly or splenomegaly, and a peripheral WBC count of (a) at least 50% or 5000 lymphocytes per microliter and (b) at least 10% or ≥1000 atypical lymphocytes per microliter. Moreover, they met one or more of the following serologic criteria: (1) early detection of VCA-IgM, (2) four-fold or greater VCA-IgG titer rise during the course of the disease and (3) early presence of VCA-IgG with early absence and later emergence of EBNA (Sumaya and Ench, 1985).

2.3. Samples

Blood samples were collected after obtaining informed consent from the patients or their parents. Plasma was separated from whole blood by centrifugation. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient with Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). Alanine aminotransferase (ALT), total bilirubin and EBV-DNA in plasma were examined sequentially. ALT was measured at no less than three points and EBV-DNA was quantified at no less than two points in all patients and controls.

2.4. Real-time quantitative PCR assay using a fluorogenic probe

Both PBMCs and plasma were used for the real-time PCR assay. DNA was extracted from either 1×10^6 cells or 200 μl of plasma using a QIAamp Blood Kit (Qiagen, Hilden, Germany) and eluted in 50 μl distilled water; 10 μl solution were used for the real-time PCR assay, which was performed with a TaqMan PCR kit and a Model 7700 Sequence Detector (Applied Biosystems, Foster, USA) as previously described (Kimura et al., 1999). The copy number of virus DNA was expressed as copies/μg DNA in PBMCs and copies/ml in plasma samples.

2.5. Determination of EBV-infected cells

The PBMCs from three severe hepatitis patients were fractionated into $CD3^+$, $CD16^+$ and $CD19^+$ cells using an immunobeads method (DynaBeads; Dynal AS, Oslo, Norway), as reported previously (Ito et al., 2001; Kimura et al., 2005, 2001a). In two cases, the PBMCs were further fractioned into $CD4^+$ and $CD8^+$ cells. As controls, the PBMCs obtained from six patients with infectious mononucleosis were fractionated into $CD3^+$, $CD16^+$ and $CD19^+$ cells. The purity of each subpopulation was confirmed by flow cytometry analysis (Ito et al., 2001). The immunomagnetic beads were detached from isolated cells by Detachabeads (Dynal AS). Aliquots of 1×10^5 isolated cells were incubated with Opticlone CD4-fluorescein isothiocyanate/CD8-phycoerythrin or CD3-fluorescein isothiocyanate/CD19-phycoerythrin or CD16-fluorescein isothiocyanate/CD56-phycoerythrin (Immunotech, Marseilles, France). Cell samples were analyzed on FACScan (Becton Dickinson, Mountain View, USA) using CellQuest software (Becton Dickinson). Live cell gating was performed and 5000 events were acquired for each analysis. All purity results were greater than 92% (range: 92–97%). EBV-DNA was quantified in all of the fractionated cells using real-time quantitative PCR. As an internal reference, human β-actin gene was also quantified in the fractionated cells using TaqMan β-actin Control Reagent (Applied Biosystems). The copy number of β-actin DNA was expressed as copies/μg DNA in the fractionated cells.

2.6. Histology

Liver tissue was fixed in 10% formalin, dehydrated in alcohol and embedded in paraffin. Samples were sectioned at 4-μm thickness. Routine hematoxylin- and eosine-stained sections were made for histologic examination. The in situ hybridization assay was performed using the Epstein-Barr encoded small RNA 1 (EBER 1; Dako A/S, Glostrup, Denmark), as previously described (Kimura et al., 2001b). 5-Bromo-4-chloro-3-indoyl phosphate (BCIP) was used for visualization. Immunostaining was performed using the streptavidin–biotin peroxidase complex method using monoclonal antibodies against CD45RO, CD4, CD8, CD20 and CD56 (Kimura et al., 2001b). Diaminobenzydine was used for visualization.

In one case, for double labeling with immunostaining and in situ hybridization, the immunohistochemical studies were performed before in situ hybridization using a previously published technique with monoclonal antibodies against CD4, CD8 and CD56 (Sheibani and Tubbs, 1984).

2.7. Statistical analysis

EBV DNA copy number was compared between the severe hepatitis group and infectious mononucleosis without jaundice group by using analysis of co-variance. Days after onset were used as covariate. Since viral load tends to change in a logarithmic fashion, statistical analysis was completed after logarithmic (base 10) transformation. Statistical analyses were performed by using software StatView 5.0.1 (SAS Institute Inc., Cary, USA).

3. Results

ALT was measured sequentially and compared between patients with severe hepatitis and those without jaundice (Fig. 1). In 4 of 21 patients without jaundice, ALT levels were normal throughout the clinical course. Peak ALT levels in patients with severe hepatitis ranged from 239 to 1426 (average: 886) IU/l, and were much higher than levels in patients without jaundice (range: 21–443, average: 117 IU/l). ALT levels peaked 5–11 (average: 7.3) days after the onset of the disease in patients with severe hepatitis as compared to 2–17 (average: 6.9) days after onset in control patients. In patients without jaundice, ALT levels decreased to normal within 1 month, whereas ALT levels remained elevated for more than 1 month in patients with severe hepatitis (Fig. 1). As Fig. 2 shows, total bilirubin was markedly elevated in the patients with severe hepatitis, peaking at 8.7–19.8 mg/dl 17–29 days after the onset of the disease. Jaundice lasted about 2 months.

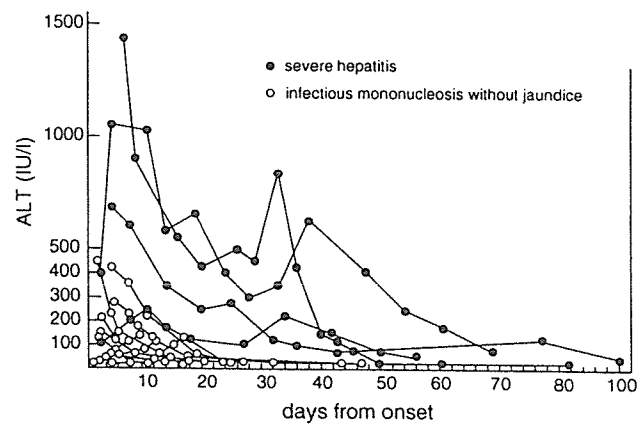


Fig. 1.

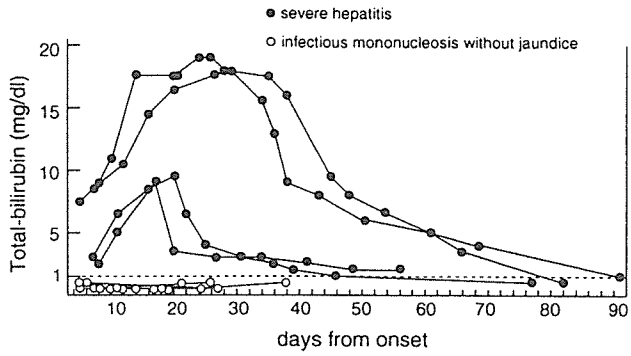


Fig. 2.

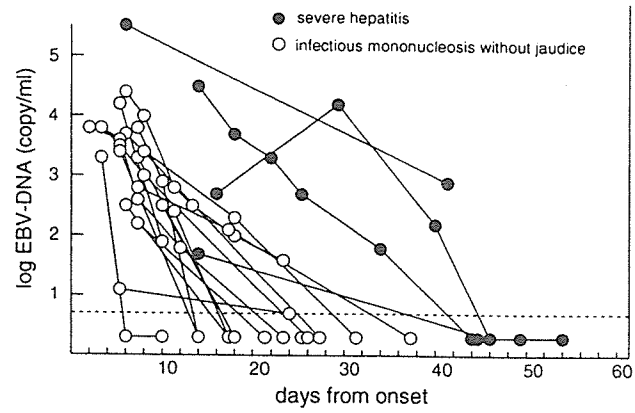


Fig. 3.

Fig. 3 shows sequential data on EBV-DNA in plasma. In infectious mononucleosis patients without jaundice, EBV-DNA copy numbers decreased and became undetectable within 1 month after onset. The mean copy number of EBV-DNA at the onset was $10^{3.3}$ (range: $10^{1.1-4.4}$) copies/ml. On the other hand, the mean copy number at the onset was $10^{4.0}$ (range: $10^{1.7-5.5}$) copies/ml in patients with severe hepatitis. Copy numbers were higher in patients with severe hepatitis and decreased more slowly than in patients without jaundice ($P = 0.02$ by analysis co-variance).

Infected cells were determined using fractionated PBMCs followed by quantitative PCR (Table 2). $CD3^+$, $CD4^+$ or $CD8^+$ cells contained more EBV-DNA than did other cell populations, indicating that EBV infected mainly T cells in patients with severe hepatitis. By contrast, $CD19^+$ cells harbored most of the EBV in infectious mononucleosis patients without jaundice, indicating that mainly B cells were infected. In parallel, human β-actin gene was quantified in the fractionated cells to investigate the quality and quantity of DNA (Table 2). The number of β-actin gene was similar among fractionated cell populations in each patient.

The liver was biopsied in three of the four patients with severe hepatitis. Table 3 summarizes the histological findings.

Table 3
Histological findings of the liver biopsy in patients with severe hepatitis

	Case 1	Case 2	Case 4
Histology			
Spotty necrosis	++	+	++
Lymphoid infiltration of the portal tract	++	+	+
Destruction of the limiting plate	+	+	–
Sinusoidal lymphoid infiltration	++	–	++
Fatty degeneration of liver cells	+	+	+
Cholestasis	+	+	–
Vasculitis	+	–	–
Fibrosis	–	–	–
EBER-1 in situ hybridization			
Lymphocytes	+	+	n.d.
Hepatocytes	–	–	n.d.

Note: EBER-1: Epstein-Barr-encoded small RNAs, n.d.: not done.

In all cases, mononuclear lymphocytes infiltrated the portal and lobular areas. Spotty necrosis of the liver parenchyma and fatty degeneration of liver cells were also seen in all. In the two cases, destruction of the limiting plate was noted. EBER-1 RNA was positive in infiltrating lymphocytes, but negative in hepatocytes in two patients in whom the in situ

Table 2
Determination of Epstein-Barr virus (EBV)-infected cells using peripheral blood mononuclear cells

	EBV-DNA copy number (copies/μg DNA)						β-Actin DNA copy number (log_{10} copies/μg DNA)					
	Unfractionated cells	Fractionated cells					Unfractionated cells	Fractionated cells				
		$CD3^+$	$CD4^+$	$CD8^+$	$CD16^+$	$CD19^+$		$CD3^+$	$CD4^+$	$CD8^+$	$CD16^+$	$CD19^+$
Severe hepatitis												
Case 1	8600	**12000**	7800	**14000**	n.d.	5400	5.9	5.4	5.0	5.3	n.d.	5.1
Case 2	470	**580**	n.d.	n.d.	250	360	5.6	5.8	n.d.	n.d.	6.0	5.7
Case 3	1700	60	**2800**	470	1400	710	5.4	5.8	5.0	5.9	5.7	5.5
Infectious mononucleosis without jaundice												
Case A	580	260	n.d.	n.d.	190	**1600**	5.6	5.7	n.d.	n.d.	5.8	5.9
Case B	1100	120	n.d.	n.d.	40	**2300**	6.0	6.2	n.d.	n.d.	6.3	6.9
Case C	280	310	n.d.	n.d.	450	**4400**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Case D	2800	760	n.d.	n.d.	1800	**3100**	5.5	5.7	n.d.	n.d.	5.8	5.9
Case E	270	130	n.d.	n.d.	270	**1300**	6.0	5.9	n.d.	n.d.	6.0	6.0
Case F	2900	1300	n.d.	n.d.	**3100**	**4300**	5.6	5.8	n.d.	n.d.	5.9	5.9

Note: n.d., not done. Fractions in bold letters indicate that EBV-DNA was concentrated after fractionation.

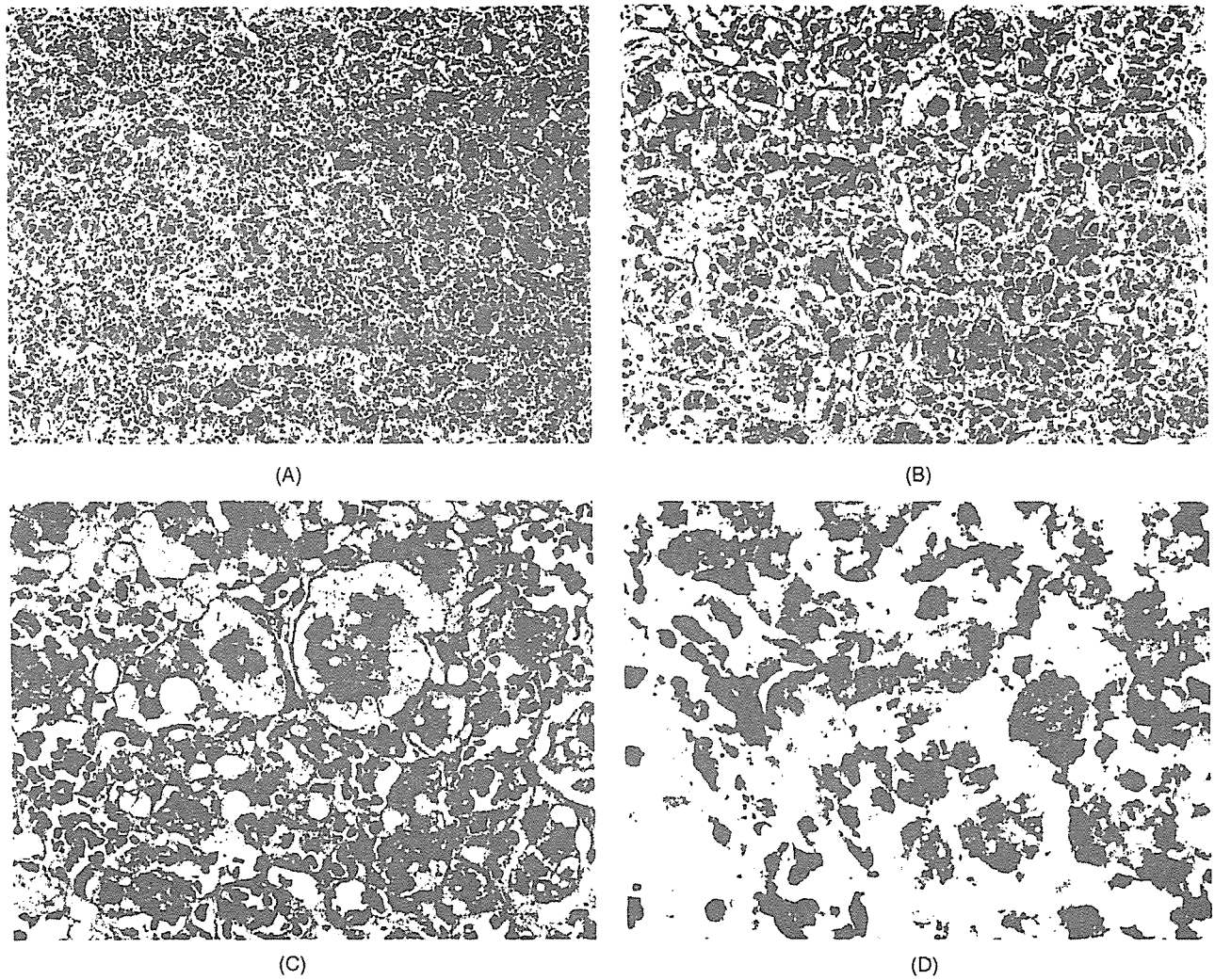


Fig. 4.

hybridization assay was performed. In case 1, double labeling with EBER-1 and surface marker analysis showed that most of the EBER-1-positive cells were $CD8^+$ T cells, as we previously reported (Kimura et al., 2001b). Histology of the case (Case 1) is shown in Fig. 4 as a representative case.

4. Discussion

We present four cases of severe hepatitis with primary EBV infection. Relative to infectious mononucleosis patients without jaundice, patients with severe hepatitis had higher, more prolonged elevation of ALT and total bilirubin levels and larger viral loads in their blood. It is unlikely that they had congenital or acquired immunodeficiency, as they were previously healthy and are now well after recovering from their hepatitis.

In infectious mononucleosis, B cells harbor EBV (Rickinson and Kieff, 2001). We found that in severe hepatitis patients, EBV infected mainly T cells, whereas in patients without jaundice EBV infected B cells. Peripheral blood or bone marrow T cells harbor EBV in life-threatening infectious mononucleosis (Baumgarten et al., 1994; Iijima et al., 1992), EBV-associated hemophagocytic syndrome (Kawaguchi et al., 1993; Su et al., 1994) and chronic active EBV infection (Kimura et al., 2001a; Quintanilla-Martinez et al., 2000; Yuge et al., 2004), all of which have high mortality rates, with multiple organ failure, including hepatic failure. Many papers concerning these EBV-associated severe diseases have emanated from the East Asia. The genetic backgrounds of East Asia people may be associated with the functions of virus-specific or non-specific lymphocytes that allow the expansion of EBV-infected T cells. On the one hand, EBV-infected T cells may escape the host's immunity and thereby proliferate, because they express fewer and less antigenic viral proteins than do B cells (Rickinson and Kieff, 2001).

In this study, we used immunomagnetic cell isolation to fractionate PBMCs, followed by quantitative PCR. Although this method is very convenient and rapid, its disadvantage is the relatively low purity of the selected cells. EBV-infected

cells can contaminate uninfected cell fractions, and the real-time PCR assay is sufficiently sensitive to detect the contaminating genome. Therefore, this method can be used only to determine the main infected cell population. The low levels of EBV-DNA seen in other populations do not always indicate that they are infected with EBV (Kimura et al., 2005). For example, 120 copies of EBV-DNA/μg DNA was detected in the $CD3^+$ fraction from case B (Table 2). The number nearly equals to that of 5% of the $CD19^+$ fraction (2300 copies/μg DNA). If 5% of B cells contaminated the $CD3^+$ fraction, 120 copies of EBV DNA/μg DNA would be detected in T cells. Thus, EBV DNA in the $CD3^+$ or $CD16^+$ fraction does not mean that T or NK cells are infected with EBV in infectious mononucleosis. Another drawback in this method is that the immunomagnetic sorting can be incomplete if surface expression of targeted antigens was decreased. It is possible that some surface marker molecules might be down-regulated in EBV-infected cells. In fact, there was a discrepancy between the $CD3^+$ cell fraction and the $CD4^+$ cell fraction in case 3.

The histological features of acute infectious mononucleosis are characterized by a mild increase in portal inflammation that consists primarily of lymphocytes (Markin, 1994). The bile ducts, hepatic arterioles and portal venules are usually unaffected. The hepatic parenchyma may show mild hepatocellular ballooning (Markin, 1994). In our cases of severe hepatitis caused by primary EBV infection, spotty necrosis of the liver parenchyma was seen in all patients and destruction of the limiting plate was seen in two patients. These findings indicate that the liver was more inflamed in severe hepatitis than in infectious mononucleosis without jaundice. Interestingly, the in situ hybridization assay showed that lymphocytes, not hepatocytes, were infected with EBV. Furthermore, the cells were $CD8^+$ T cells, which is in accord with the results for peripheral blood.

The mechanism of EBV-induced hepatitis remains unclear. T cell infection might be associated with the pathogenesis of severe hepatitis, although this might be a bystander event associated with more severe infection or genetic predisposition. A recent animal model showed that activated $CD8^+$ T cells are trapped in the liver selectively, primarily by intracellular adhesion molecule 1, which is expressed constitutively on sinusoidal endothelial cells and Kupffer cells (Mehal et al., 1999). In EBV-associated hepatitis, EBV-infected $CD8^+$ T cells, presumably activated T cells, may accumulate in the liver. A series of experiments showed that certain soluble products of the immune response, especially interferon γ, tumor necrosis factor α and Fas ligand, induce hepatitis (Bradham et al., 1998; Kondo et al., 1997; Kusters et al., 1996). In EBV-associated hepatitis, these products, which are produced by either EBV-infected $CD8^+$ T cells or infiltrating cytotoxic T lymphocytes, may induce hepatocyte injury. Although further studies are necessary to clarify the precise mechanisms, our observations suggest that a large EBV burden and T cell infection play major roles in severe hepatitis following primary EBV infection.

Acknowledgment

This work was supported by a grant (16591022) from the Ministry of Education, Culture, Sport, Science and Technology of Japan.

References

Baumgarten E, Herbst H, Schmitt M, Seeger KH, Schulte-Overberg U, Henze G. Life-threatening infectious mononucleosis: is it correlated with virus-induced T cell proliferation? Clin Infect Dis 1994;19(1):152–6.

Bradham CA, Plumpe J, Manns MP, Brenner DA, Trautwein C. Mechanisms of hepatic toxicity. I. TNF-induced liver injury. Am J Physiol 1998;275(3 Pt. 1):G387–92.

Iijima T, Sumazaki R, Mori N, Oka K, Nagai Y, Shibazaki M, et al. A pathological and immunohistological case report of fatal infectious mononucleosis, Epstein-Barr virus infection, demonstrated by in situ and Southern blot hybridization. Virchows Arch A Pathol Anat Histopathol 1992;421(1):73–8.

Ito Y, Kimura H, Hara S, Kido S, Ozaki T, Nishiyama Y, et al. Investigation of varicella-zoster virus DNA in lymphocyte subpopulations by quantitative PCR assay. Microbiol Immunol 2001;45(3):267–9.

Kawaguchi H, Miyashita T, Herbst H, Niedobitek G, Asada M, Tsuchida M, et al. Epstein-Barr virus-infected T lymphocytes in Epstein-Barr virus-associated hemophagocytic syndrome. J Clin Invest 1993;92(3):1444–50.

Kimura H, Hoshino Y, Hara S, Sugaya N, Kawada J, Shibata Y, et al. Differences between T cell-type and natural killer cell-type chronic active Epstein-Barr virus infection. J Infect Dis 2005;191(4):531–9.

Kimura H, Hoshino Y, Kanegane H, Tsuge I, Okamura T, Kawa K, et al. Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. Blood 2001a;98(2):280–6.

Kimura H, Morita M, Yabuta Y, Kuzushima K, Kato K, Kojima S, et al. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol 1999;37(1):132–6.

Kimura H, Nagasaka T, Hoshino Y, Hayashi N, Tanaka N, Xu JL, et al. Severe hepatitis caused by Epstein-Barr virus without infection of hepatocytes. Hum Pathol 2001b;32(7):757–62.

Kondo T, Suda T, Fukuyama H, Adachi M, Nagata S. Essential roles of the Fas ligand in the development of hepatitis. Nat Med 1997;3(4):409–13.

Kusters S, Gantner F, Kunstle G, Tiegs G. Interferon gamma plays a critical role in T cell-dependent liver injury in mice initiated by concanavalin A. Gastroenterology 1996;111(2):462–71.

Markin RS. Manifestations of Epstein-Barr virus-associated disorders in liver. Liver 1994;14(1):1–13.

Markin RS, Linder J, Zuerlein K, Mroczek E, Grierson HL, Brichacek B, et al. Hepatitis in fatal infectious mononucleosis. Gastroenterology 1987;93(6):1210–7.

Mehal WZ, Juedes AE, Crispe IN. Selective retention of activated CD8+ T cells by the normal liver. J Immunol 1999;163(6):3202–10.

Quintanilla-Martinez L, Kumar S, Fend F, Reyes E, Teruya-Feldstein J, Kingma DW, et al. Fulminant EBV(+) T-cell lymphoproliferative disorder following acute/chronic EBV infection: a distinct clinicopathologic syndrome. Blood 2000;96(2):443–51.

Rickinson AB, Kieff E. Epstein-Barr virus. In: Knipe DM, Howly PM, editors. Virology, vol. 2, fourth ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 2575–627.

Sheibani K, Tubbs RR. Enzyme immunohistochemistry: technical aspects. Semin Diagn Pathol 1984;1(4):235–50.

Su IJ, Chen RL, Lin DT, Lin KS, Chen CC. Epstein-Barr virus (EBV) infects T lymphocytes in childhood EBV-associated hemophagocytic syndrome in Taiwan. Am J Pathol 1994;144(6):1219–25.

Sumaya CV, Ench Y. Epstein-Barr virus infectious mononucleosis in children. I. Clinical and general laboratory findings. Pediatrics 1985;75(6):1003–10.

Tazawa Y, Nishinomiya F, Noguchi H, Takada G, Tsuchiya S, Sumazaki R, et al. A case of fatal infectious mononucleosis presenting with fulminant hepatic failure associated with an extensive CD8-positive lymphocyte infiltration in the liver. Hum Pathol 1993;24(10):1135–9.

White NJ, Juel-Jensen BE. Infectious mononucleosis hepatitis. Semin Liver Dis 1984;4(4):301–6.

Yuge A, Kinoshita E, Moriuchi M, Ohno Y, Haga H, Moriuchi H. Persistent hepatitis associated with chronic active Epstein-Barr virus infection. Pediatr Infect Dis J 2004;23(1):74–6.

Journal of Medical Virology 78:476–484 (2006)

Analysis of the Full-Length Genome of Genotype 4 Hepatitis E Virus Isolates From Patients With Fulminant or Acute Self-Limited Hepatitis E

Jun Inoue,[1,2] Tsutomu Nishizawa,[1] Masaharu Takahashi,[1] Tatsuya Aikawa,[3] Hitoshi Mizuo,[4] Kazuyuki Suzuki,[5] Tooru Shimosegawa,[2] and Hiroaki Okamoto[1]*

[1]*Division of Virology, Department of Infection and Immunity, Jichi Medical School, Tochigi-Ken, Japan*
[2]*Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan*
[3]*Aikawa Internal Medicine Hospital, Ibaraki-Ken, Japan*
[4]*Department of Internal Medicine, Kin-ikyo Chuo Hospital, Sapporo, Hokkaido, Japan*
[5]*First Department of Internal Medicine, Iwate Medical University, Iwate-Ken, Japan*

It was suggested that hepatitis E virus (HEV) genotype 4 is associated more closely with the severity of hepatitis E than genotype 3, although the virological basis remains unknown. The aim of this study was to examine whether genomic differences among genotype 4 HEVs are responsible for the development of fulminant hepatitis. Full-length sequences of genotype 4 HEVs from three patients with fulminant hepatitis and six patients with acute self-limited hepatitis were determined. The sequences were analyzed with those of 13 genotype 4 HEV isolates whose entire nucleotide sequence is known. Analysis of 22 full-length sequences (fulminant hepatitis, 5; acute hepatitis, 17) revealed that C at nt 1816 and U at nt 3148 (U3148), both of which do not change the amino acid sequences, were significantly associated with fulminant hepatitis ($P=0.0489$, respectively). When partial nucleotide sequences containing nt 1816 or nt 3148 were determined in 16 additional HEV isolates of genotype 4, a closer association between U3148 and fulminant hepatitis ($P=0.0018$) was observed. The comparison of 86 HEV isolates of all four genotypes showed that U3148 had a stronger association with fulminant hepatitis than other nucleotides at nt 3148 ($P=0.0006$). Patients infected with HEV with U3148 had a significantly lower value of the lowest prothrombin activity ($P=0.0293$). Nt 3148 is located within the RNA helicase domain, and 22-nt sequence including nt 3148 was well conserved among all genotypes. A silent substitution of U3148 in HEV may be associated with the development of fulminant hepatitis. Further studies are needed to clarify the underlying mechanism. ***J. Med. Virol. 78:476–484, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: hepatitis E virus; fulminant hepatitis; full-length genome; genotype; silent mutation

INTRODUCTION

Hepatitis E virus (HEV) is a major cause of epidemic and sporadic hepatitis in many developing countries [Purcell and Emerson, 2001]. Recently, sporadic cases of HEV infections have been reported in industrialized countries [Harrison, 1999; Schlauder and Mushahwar, 2001; Smith, 2001; Okamoto et al., 2003], where zoonotic transmission of HEV has been suggested; animals such as swine serve as reservoirs for HEV [Meng, 2003; Tei et al., 2003; Yazaki et al., 2003]. HEV is currently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [Emerson et al., 2004a; http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_hepev.htm]. The genome of HEV is a single-stranded, positive-sense RNA of approximately 7.2 kb and consists of a short 5′-untranslated region, three partially overlapping open reading frames (ORF1, ORF2, and ORF3), and a short

The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB220971–AB220979 (full-length sequences) and AB221706–AB221758 (partial ORF1 sequences).

Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant sponsor: Ministry of Health, Labor, and Welfare of Japan.

*Correspondence to: Dr. Hiroaki Okamoto, Division of Virology, Department of Infection and Immunity, Jichi Medical School, 3311-1 Yakushiji, Shimotsuke-Shi, Tochigi-Ken 329-0498, Japan. E-mail: hokamoto@jichi.ac.jp

Accepted 15 December 2005

DOI 10.1002/jmv.20565

Published online in Wiley InterScience (www.interscience.wiley.com)

3′-untranslated region terminated by a poly(A) tract [Tam et al., 1991; Wang et al., 2000]. Based on sequence analysis, HEV sequences have been classified into four major genotypes (1–4). Genotype 1 is the main cause of hepatitis E in developing countries in Asia and Africa, and genotype 2 has been documented in Mexico and Nigeria. Genotype 3 or 4 has been described in the United States, European countries, China, Taiwan, and Japan [Schlauder and Mushahwar, 2001; Mizuo et al., 2002].

Infection of HEV induces self-resolving hepatitis or a subclinical state in most cases, but it can cause fulminant hepatitis. It was reported that HEV infection is a major cause of fulminant hepatitis in endemic areas for HEV [Nanda et al., 1994; Coursaget et al., 1998; Sheikh et al., 2002]. Patients with fulminant hepatitis have been reported in an industrialized country [Suzuki et al., 2002]. Recent observations suggest that the HEV genotype influences the severity of hepatitis E, and that genotype 4 is associated more strongly with the severe form of hepatitis E than genotype 3 [Mizuo et al., 2005]. To date, nine HEV-associated fulminant hepatitis cases have been reported in Japan, and seven of them were infected with genotype 4 HEV [Suzuki et al., 2002; Ohnishi et al., 2003; Takahashi et al., 2003; Yazaki et al., 2003; Kato et al., 2004; Takahashi et al., 2005], although a larger number of patients are infected with genotype 3 than genotype 4 in Japan [Okamoto et al., 2003]. These observations suggest that there is a close relationship between infection with genotype 4 HEV and progression to fulminant hepatitis, which prompted us to investigate whether genomic differences among HEVs are responsible for the development of fulminant hepatitis. The full-length genome of genotype 4 HEV in patients with fulminant hepatitis and in those with acute self-resolving hepatitis was determined.

MATERIALS AND METHODS

Serum Samples

Sera collected between 1998 and 2004 in Japan from three patients with type E fulminant hepatitis and six patients with the mild form of type E acute hepatitis, who had a lowest prothrombin activity of ≥80%, were used for full-length sequencing of HEV. Clinical characteristics and laboratory data of the three patients with fulminant hepatitis (isolate names: HE-JF3, HE-JF4, and HE-JF5) [Suzuki et al., 2002; Yazaki et al., 2003] and six acute hepatitis patients (isolate names: HE-JA2, HE-JA19, HE-JA28, HE-JA36, HE-JA37, and HE-JA41) [Mizuo et al., 2002, 2005; Yazaki et al., 2003] were reported previously.

Serum samples collected between 1993 and 2004 from 20 patients with genotype 3 HEV and 16 patients with genotype 4 HEV were used for partial sequencing of ORF1 of HEV. One each of the patients with genotype 3 or 4 was diagnosed with type E fulminant hepatitis whose isolate was designated as HE-JF2 and HE-JF1, respectively [Suzuki et al., 2002]. The remaining 34 patients were diagnosed as having type E acute hepatitis [Mizuo et al., 2002, 2005; Kuno et al., 2003; Yajima et al., 2003; Yazaki et al., 2003; Sainokami et al., 2004; Saitoh et al., 2004; Yamamoto et al., 2004; Hijioka et al., 2005].

Amplification of Full-Length HEV Genome

Total RNA was extracted from 120 to 300 μl of serum, and subjected to cDNA synthesis and nested polymerase chain reaction (PCR) for six overlapping regions excluding the extreme 5′- and 3′-terminal regions; the amplified regions were nt 37–1199 (1163 nt) (primer sequences excluded), nt 991–3148 (2158 nt), nt 3029–4603 (1575 nt), nt 4401–5325 (925 nt), nt 5240–5998 (759 nt), and nt 5985–7142 (1158 nt).

The 5′-end sequence (nt 1–60) was determined by a modified rapid amplification of cDNA ends (RACE) technique called RNA ligase-mediated RACE (RLM-RACE) with the First Choice RLM-RACE kit (Ambion, Austin, TX), as described previously [Okamoto et al., 2001]. Amplification of the 3′-end sequence (nt 7071–7240: excluding poly [A] tail) was attempted by the RACE method as described previously [Okamoto et al., 2001].

Amplification of Partial Nucleotide Sequences of ORF1 Including Nt 1816 and 3148

Total RNA extracted from 50 μl of serum was reverse-transcribed and subjected to PCR with genotype-specific primers. To amplify the nt 1543–2086 sequence (primer sequences excluded) of genotype 4 HEV, first round PCR was performed with primers HE296 and HE269, and second round PCR was performed with primers HE297 and HE298 (Table I). To amplify the nt 2827–3286 sequence of genotype 4 HEV, HE299 and HE302 were used for the first round PCR and HE300 and HE301 for the second round PCR. Genotype 3-specific PCR was performed to amplify the nt 1546–2074 sequence with HE303 and HE306 in the first round and HE304 and HE282 in the second round. To amplify the nt 2995–3469 sequence of genotype 3 HEV, HE307 and HE310 were used in the first round and HE308 and HE309 in the second round.

Semi-Quantitation of HEV RNA

Semi-quantitation of HEV RNA was performed by the end-point dilution method, with primers targeting the ORF2 region as described previously [Mizuo et al., 2002]. The highest dilution (10^N) of extracted RNA that was found to be positive was estimated and it was converted to the relative titer per 1 ml of serum.

Sequence Analysis

The amplification products were sequenced on both strands either directly or after cloning into pT7Blue T-Vector (Novagen, Inc., Madison, WI), using the BigDye Terminator Cycle Sequencing Ready Reaction Kit. Sequence analysis was performed using Genetyx-Mac (version 12.6.6; Genetyx Corp., Tokyo, Japan) and

TABLE I. Positions and Nucleotide Sequences of Primers Used for Polymerase Chain Reaction (PCR) Amplification of Partial Nucleotide Sequences of Hepatitis E Virus (HEV) ORF1

Primer	Polarity	Nucleotide position[a]	Specificity	Nucleotide sequence[b]
HE269	Antisense	2135–2154	Genotype 4	5′ ARSCCYGAMACCGACCAGGT 3′
HE282	Antisense	2075–2094	Genotype 3	5′ GACTCCCARVYRTGSCCRGG 3′
HE296	Sense	1509–1528	Genotype 4	5′ AGGGTTAYGAYAAYGAGGCA 3′
HE297	Sense	1523–1542	Genotype 4	5′ GAGGCATTTGARGGGTCGGA 3′
HE298	Antisense	2087–2106	Genotype 4	5′ AARGGRTTRGYWGACTCCCA 3′
HE299	Sense	2765–2784	Genotype 4	5′ GAYGCYTGGGARCGYAACCA 3′
HE300	Sense	2807–2826	Genotype 4	5′ CTKACYGAGCCRGCBATAGC 3′
HE301	Antisense	3287–3306	Genotype 4	5′ TTTGTWGGDACMAGCTCAGG 3′
HE302	Antisense	3303–3322	Genotype 4	5′ RGTAASRTGCCACCACTTTG 3′
HE303	Sense	1448–1467	Genotype 3	5′ CRGTGGYTVGGSCAGGAGTG 3′
HE304	Sense	1526–1545	Genotype 3	5′ GCYTAYGAGGRBTCYGAGGT 3′
HE306	Antisense	2087–2106	Genotype 3	5′ AADGGRTTVGCAGRCTCCCA 3′
HE307	Sense	2933–2952	Genotype 3	5′ CGTGCYTGYGCYGGYTGCAC 3′
HE308	Sense	2975–2994	Genotype 3	5′ TAYCAGTTYACYGCYGGGGT 3′
HE309	Antisense	3470–3489	Genotype 3	5′ TGRACHGTRATYGCACCAGG 3′
HE310	Antisense	3518–3537	Genotype 3	5′ GCYGTRGCTATRATTGTGGT 3′

[a]The nucleotide numbers are in accordance with HE-JA1 (AB097812).
[b]R = A or G; S = C or G; Y = C or T; M = A or C; V = A, C or G; W = A or T; K = G or T; B = C, G or T; D = A, G or T; H = A, C or T.

ODEN (version 1.1.1) from the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan) [Ina, 1994]. Sequences were aligned by CLUSTAL W (version 1.8) [Thompson et al., 1994]. A phylogenetic tree was constructed by the neighbor-joining method [Saitou and Nei, 1987]. Bootstrap values were determined with 1,000 resamplings of the data sets [Felsenstein, 1985]. The final tree was obtained using the TreeView program (version 1.6.6) [Page, 1996].

Statistical Analysis

Statistical analyses were performed using Fisher's exact probability test for comparison of proportions between two groups and the Mann–Whitney U test for comparison of continuous variables between two groups. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Analysis of Full-Length Genome of Genotype 4 HEV Isolates

Seven of the nine HEV isolates, that is, HE-JF4, HE-JF5, HE-JA19, HE-JA28, HE-JA36, HE-JA37, and HE-JA41, had the same genomic length of 7239 nt, excluding the poly(A) tract at the 3′-terminus. HE-JF3 and HE-JA2 had a genomic length of 7240 nt and 7243 nt, respectively; the differences in genomic length were attributed to an insertion of 1 nt or 4 nt in HE-JF3 and HE-JA2, respectively, in the 3′-untranslated region. When the full genome sequences were compared with those of reported HEV isolates, HE-JF3 was closely related to HE-JA1 [Nishizawa et al., 2003] with a nucleotide identity of 99.6%. HE-JF4 shared an identity of 99.7% with HE-JF5, and had the highest identity of 99.9% with both JYW-Sap02 and JTS-Sap02 [Takahashi et al., 2004]. HE-JA19 and HE-JA37, which shared an identity of 99.3%, were 99.2% and 98.9% similar to JKK-Sap00 [Takahashi et al., 2003], respectively. HE-JA28, HE-JA36, and HE-JA41 were 99.5–99.6% identical to each other and had an identity of 98.4–98.7% with JSM-Sap95 [Takahashi et al., 2004]. HE-JA2 was only 84.3–86.8% similar to the other genotype 4 isolates. A phylogenetic tree was constructed based on the full genome sequence of genotype 1–4 HEV (Fig. 1). HE-JF3 segregated into a cluster consisting of JSN-Sap-FH02 [Takahashi et al., 2003] and JSF-Tot03 [Takahashi et al., 2005], both of which had been obtained from fulminant hepatitis patients. HE-JF4, HE-JF5, HE-JA19, and HE-JA37 were grouped into another cluster consisting of JKK-Sap00, JYW-Sap02, and JTS-Sap02. HE-JA28, HE-JA36, and HE-JA41 formed a mini-cluster, separate from the latter cluster.

To investigate nucleotide differences that may be related to fulminant hepatitis, the nine full-length sequences of HEV determined in this study were examined along with 13 previously reported, entire or nearly entire HEV sequences [Wang et al., 2000; Kuno et al., 2003; Liu et al., 2003; Nishizawa et al., 2003; Takahashi et al., 2003, 2004, 2005]. No nucleotide substitutions within the 5′-and 3′-untranslated regions were specific for the HEV isolates from patients with fulminant hepatitis, although some of the reported isolates lacked the extreme 5′-terminal part of the 5′-untranslated region. A total of 7,145 nucleotides within the coding region of the 5 genomes (HE-JF3, HE-JF4, HE-JF5, JSN-Sap-FH02, and JSF-Tot03) recovered from patients with fulminant hepatitis were compared with those of the 17 genomes recovered from patients with acute hepatitis. At each nucleotide position, we examined whether a particular nucleotide was significantly more prevalent among the HEV genomes from patients with fulminant hepatitis than among those from patients with acute hepatitis. A P-value was determined for each nucleotide position.

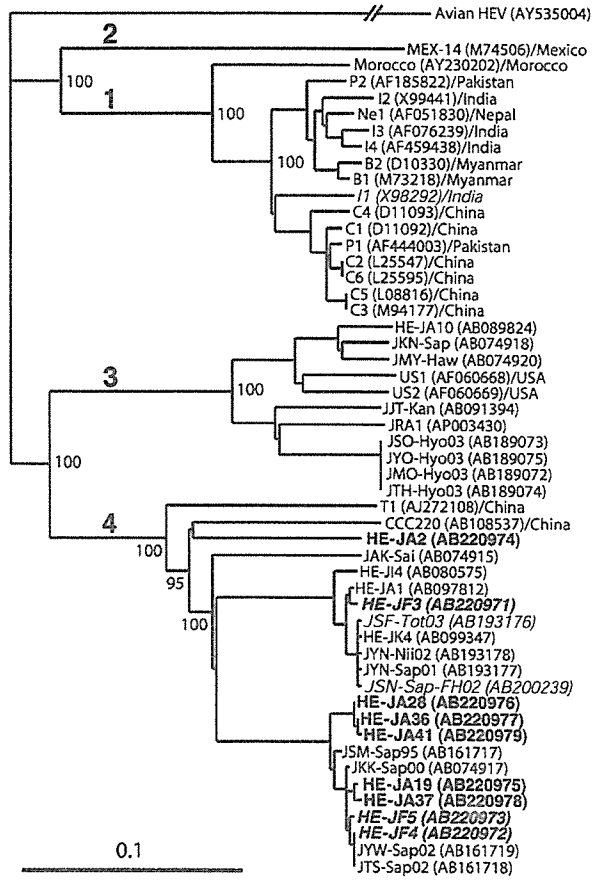


Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the full-length nucleotide sequences of 51 human hepatitis E virus (HEV) isolates, using a chicken HEV isolate (AY535004) as an outgroup. The nine HEV isolates whose full-length sequence was determined in the present study are indicated in bold type for visual clarity. Forty-two isolates whose entire or nearly entire sequence has been reported were included for comparison, with the accession number in parentheses. After the slash, the name of the country other than Japan where the HEV isolate was isolated is shown. The six isolates that were obtained from patients with fulminant hepatitis are indicated in italic type. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings.

All 21 nucleotide substitutions with $P < 0.2$ did not change the amino acids (Table II). Among them, C at nt 1816 (C1816) and U at nt 3148 (U3148) were seen significantly more frequently among the HEV isolates from patients with fulminant hepatitis than among those from patients with acute hepatitis (100% vs. 47%, $P = 0.0489$; 100% vs. 47%, $P = 0.0489$).

Comparison of Nucleotides at Nt 1816 and 3148 in Genotype 4 HEV Isolates

To determine the nucleotides at nt 1816 and 3148 in an additional 16 HEV isolates from patients with genotype 4 HEV, including a patient with fulminant hepatitis (HE-JF1), the partial nucleotide sequences of two different regions within ORF1 (nt 1543–2086 and nt 2827–3286) were determined. The nucleotides at nt 1816 and 3148 in the obtained sequences and those in the 22 full-length genomes were compared between the fulminant and acute hepatitis patients (Table III). C at nt 1816 and U at nt 3148 remained significantly more prevalent among the patients with fulminant hepatitis than among those with acute hepatitis ($P = 0.0268$ and $P = 0.0018$, respectively). Of note, U3148 was found to have a closer relationship with fulminant hepatitis than C1816.

Comparison of the Nucleotides at Nt 3148 in HEV Isolates of All Four Genotypes

Figure 2A depicts the genomic organization of the four genotypes of HEV and the location of nt 3148. Nt 3148 is located within the RNA helicase domain of ORF1, and is the third base of a triplet codon encoding valine. The consensus sequence of each of the four genotypes of HEVs whose entire or nearly entire sequence was known was determined. When the consensus sequences of the 4 genotypes were aligned, a 22-nt sequence including nt 3148 was found to be well conserved (Fig. 2B). To compare the 22-nt sequence among additional isolates, the nucleotide sequence of nt 2995–3469 was determined for 20 additional isolates from patients infected with genotype 3 HEV, including that from a fulminant hepatitis patient (HE-JF2). Comparison of the 22-nt sequence among 86 isolates including the 20 genotype 3 and 16 genotype 4 isolates, whose partial nucleotide sequence was determined in the present study, disclosed that there are some minor substitutions in this conserved area (Fig. 2C). Notably, genotype 4 isolates had a much higher prevalence of U3148 than isolates of the other three genotypes (39.5% [15/38] vs. 4.2% [2/48], $P < 0.0001$), and U3148 was associated significantly more frequently with fulminant hepatitis than other nucleotides at nt 3148 (C or G) (35.3% vs. 2.9%, $P = 0.0006$). On the other hand, at nt 1816, no particular nucleotide was seen significantly more frequently among the HEV isolates from patients with fulminant hepatitis than among those from patients with acute hepatitis when the 86 isolates of four distinct genotypes were compared (data not shown).

Comparison of Demographic Characteristics and Laboratory Parameters Among the Hepatitis E Patients According to the Presence of U3148

The demographic features and laboratory parameters were compared in relation to the presence or absence of U3148 among 48 patients with type E acute or fulminant hepatitis for whom such data were available [Mizuo et al., 2002, 2005; Suzuki et al., 2002; Kuno et al., 2003; Yajima et al., 2003; Yazaki et al., 2003; Sainokami et al., 2004; Saitoh et al., 2004; Yamamoto et al., 2004; Hijioka et al., 2005]. Twenty-seven patients were infected with genotype 4 HEV and the remaining 21 patients with genotype 3 HEV. A patient who was infected with both genotypes 3 and 4 HEV isolates [Takahashi et al., 2002]

TABLE II. Differences in the Nucleotide Sequence of Genotype 4 HEV Genomes Obtained From Patients With Fulminant or Acute Hepatitis E

		Nucleotide no.[b]																				
Isolate name[a]	Diagnosis	37	370	421	1345	1816	1963	2101	2128	2224	2725	3148	3185	3796	3856	4579	4888	5071	5907	5943	6126	6216
HE-JF3	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JF4	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JF5	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JSN-Sap-FH02	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JSF-Tot03	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JA2	AH	U	U	G	C	C	A	C	C	A	U	C	U	U	C	C	A	U	A	U	U	U
HE-JA19	AH	C	C	G	U	U	C	U	U	U	U	C	U	C	U	U	G	U	C	C	C	C
HE-JA28	AH	C	U	C	C	U	U	C	C	C	C	C	C	U	C	C	A	C	U	U	U	G
HE-JA36	AH	C	U	C	C	U	U	C	C	C	C	C	C	U	C	C	A	C	U	U	U	G
HE-JA37	AH	C	C	A	U	U	C	U	U	U	U	C	U	C	U	U	G	U	C	C	C	C
HE-JA41	AH	C	U	C	C	U	U	C	C	C	C	C	C	U	C	C	A	C	U	U	U	G
T1	AH	C	U	G	C	G	C	C	C	U	C	C	C	C	C	C	C	C	A	U	U	U
CCC220	AH	U	C	U	C	C	U	C	C	U	C	U	C	C	C	C	A	C	A	C	U	G
HE-JA1	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JK4	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JAK-Sai	AH	U	U	A	U	U	A	C	U	C	C	U	C	U	C	C	G	C	U	U	U	C
JSM-Sap95	AH	C	C	G	U	U	C	U	U	C	C	C	C	U	U	U	G	C	U	U	C	C
JKK-Sap00	AH	C	C	G	U	U	C	U	U	U	U	C	U	C	U	U	G	U	C	U	C	C
JYN-Sap01	AH	U	C	G	U	C	C	U	U	C	U	U	U	C	U	U	G	U	C	C	C	C
JYW-Sap02	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JTS-Sap02	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JYN-Nii02	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
		U/C	C/U	G/H	U/C	C/K	C/W	U/C	U/C	U/M	U/C	U/C	U/C	C/U	U/C	U/C	G/M	U/C	C/W	C/U	C/U	C/K
	FH	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0
	AH	9/8	11/6	11/6	11/6	8/9	11/6	10/7	11/6	10/7	10/7	8/9	10/7	11/6	10/7	10/7	11/6	10/7	9/8	9/8	10/7	11/6
	P value[c]	0.076	0.166	0.166	0.166	**0.049**	0.166	0.114	0.166	0.114	0.114	**0.049**	0.114	0.166	0.114	0.114	0.166	0.114	0.076	0.076	0.114	0.166

FH, fulminant hepatitis; AH, acute hepatitis; H = A or C or U; K = G or U; W = A or U; M = A or C.
Nucleotide substitutions in which the prevalence of a particular nucleotide at a nt position was higher among the HEV genomes from FH patients than among those from AH patients with $P < 0.2$ are shown.
[a]The isolate names whose entire sequences have been determined in this study are indicated in bold face.
[b]Nucleotide numbers are in accordance with HE-JA1 (AB097812).
[c]*P* values (Fisher's exact probability test) that are significant are indicated in bold face.

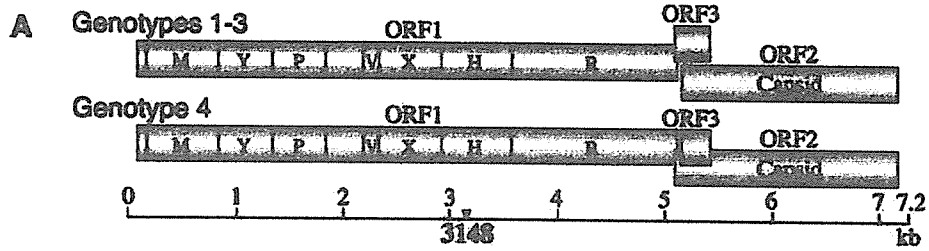
TABLE III. Comparison of Nucleotides at Nt 1816 and 3148 Among 38 Isolates of Genotype 4 HEV Recovered From Patients With Fulminant or Acute Hepatitis E

	Nt 1816		Nt 3148	
Patients with	C	G or U	U	C
Fulminant hepatitis	5	1	6	0
Acute hepatitis	10	22	9	23
P value	0.0268		0.0018	

was excluded. The patients infected with HEV with U3148 tended to have a higher peak level of total bilirubin than those with C3148 or G3148 ($P = 0.0721$) (Table IV). Of note, the value of the lowest prothrombin activity was significantly lower in patients with U3148 than in those with C3148 or G3148 ($P = 0.0293$).

DISCUSSION

It is generally considered that the severity of hepatitis E depends on host factors of the infected patients such as



B

```
Genotype 1  UGGUCCCGACCCGGGAGUUGCGUAAUGCCUGGCGCCGCCGCGGCUUCGCUGCUUUCACCCCGCACAC
Genotype 2  -U--G--C--U--C---C-U--G--C--U-----G-----G-----U--G--A-----U--------
Genotype 3  -U--G--C---------C-U------AGU-----U-----G--U--U--G--C-----A--U-----
Genotype 4  ----A--A--U--U---C-------CU-G-----A--------G--U--A--C-A---A--C-----

Genotype 1  UGCCGCUAGAGUCACCCAGGGGCGCCGGGUUGUCAUUGAUGAGGCCCCAUCCCUUCCCCCUCACCUG
Genotype 2  ---G--CC-U-----UAGC--C--UA----------------------U--G--C-----A---U--
Genotype 3  G--G--CC-U--U--UAUA--C----C-----G---------------C--A--G-----
Genotype 4  C--G--CC-U--U----GU--C--UA----------------------G--A-----A--A---U--

Genotype 1  CUGCUGCUCCACAUGCAGCGGGCCGCCACCGUCCACCUUCUUGGCGACCCGAAUCAGAUCCCAGCCA
Genotype 2  -----UU-A--U--------U--U--AU-U--G-----C-----G----------------C----
Genotype 3  ------U-A--------------U--U-G-----U--C--C--U-----A----------U--U-
Genotype 4  --UU----G--------------U-GU-G--------C----------C--C--A-----U----
```

C

22-nt sequence with nt 3148	HEV genotype 1	2	3	4
GGGUUGUCAUUGAUGAGGCCCC	7	1	0	20
----------U--	6	0	0	1
----------A--U--	1(1)	0	0	0
----------C-----U--	1	0	0	0
-U--C-----C-----A-----	1	0	0	0
-C-----G----------U--	0	0	14	0
-U-----G-----C--------	0	0	7(1)	0
-C-----G----------	0	0	3	0
-U-----G----------	0	0	3	0
-U-----U-----C--------	0	0	2	0
-C-----G-----C--------	0	0	1	0
-U-----G--------A--A--	0	0	1	0
-------U-----C--------	0	0	0	6(2)
-------U----------	0	0	0	5(2)
--A-----U-----C--------	0	0	0	2(1)
-------U--C--C--------	0	0	0	1(1)
----C--U--C--C-----U--	0	0	0	1
----C----------	0	0	0	1
-AA-------C--C--------	0	0	0	1
Total	16(1)	1(0)	31(1)	38(6)

Fig. 2. Characteristics of nt 3148 in HEV and its surrounding sequence. **A**: Organization of the HEV genome of genotypes 1–3 and that of genotype 4 showing the location of nt 3148. M, methyltransferase; Y, Y domain; P, papain-like protease; V, proline-rich hinge domain; X, X domain; H, RNA helicase; R, RNA polymerase. **B**: Comparison of consensus sequences of the region including nt 3148 (201 nt) in each of the four distinct genotypes of HEV. The inverted triangle indicates the position of nt 3148, and the box indicates a conserved area including nt 3148. The consensus sequence of each genotype was deduced from nucleotides common to each of the four genotypes (genotype 1, 16 isolates; genotype 2, 1 isolate; genotype 3, 11 isolates; and genotype 4, 22 isolates). **C**: Comparison of a 22-nt sequence including nt 3148 among 86 HEV isolates of four distinct genotypes. The inverted triangle indicates nt 3148. The numbers on the right of the sequences indicate the number of isolates with the indicated sequences, and the number in parentheses indicate the number of isolates obtained from patients with fulminant hepatitis. The genotype 1 isolate from a patient with fulminant hepatitis (I1, X98292 [Donati et al., 1997]), and the genotype 3 isolate from a patient with fulminant hepatitis (HE-JF2 [Suzuki et al., 2002]) are shown.

TABLE IV. Comparison of Demographic Characteristics and Laboratory Parameters Between Patients Infected With HEV With U at Nt 3148 or Those With Another Nucleotide at Nt 3148

	Nt 3148		
	U (n = 8)	C or G (n = 40)	*P* value[a]
Age (years)	58.5 ± 8.9	57.7 ± 12.0	0.7085
Male [number (%)]	8 (100)	33 (82.5)	0.2532
Peak ALT (IU/l)	2,814 ± 1,009	2,459 ± 1,237	0.2685
Peak total bilirubin (mg/dl)	16.5 ± 10.3	9.6 ± 8.3	0.0721
Lowest prothrombin activity (%)	51.8 ± 29.1	76.8 ± 25.9	**0.0293**
High HEV load[b] [number (%)]	3 (37.5)	10 (25.0)	0.3704

The demographic characteristics and laboratory parameters of patients with type E acute or fulminant hepatitis were studied [Mizuo et al., 2002, 2005; Suzuki et al., 2002; Kuno et al., 2003; Yajima et al., 2003; Yazaki et al., 2003; Sainokami et al., 2004; Saitoh et al., 2004; Yamamoto et al., 2004; Hijioka et al., 2005]. Twenty-seven patients were infected with genotype 4 HEV and 21 patients were infected with genotype 3 HEV.

[a]*P* value that is statistically significant is indicated in bold face.

[b]With HEV RNA titer of $\geq 10^5$ copies/ml at the first examination.

pregnancy [Harrison, 1999; Purcell and Emerson, 2001; Smith, 2001] or aging [Harrison, 1999]. The mortality rate among pregnant women who acquired hepatitis E is as high as 20%. In addition, the presence of an underlying disease may influence the severity of hepatitis E [Mizuo et al., 2005]. However, similar to other known hepatitis viruses, viral factors may play a role in the pathogenesis of type E fulminant hepatitis. Hepatitis B virus variants with mutations in the precore region [Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Terazawa et al., 1991; Yotsumoto et al., 1992] and/or the core promoter [Sato et al., 1995] have been implicated in fulminant hepatitis. Possible associations were also suggested between the severity of hepatitis A and significant numbers of nucleotide substitutions in the 5′-untranslated region of the hepatitis A virus genome [Fujiwara et al., 2001, 2002]. Recently, it was suggested that the severity of hepatitis E is influenced by the genotype of HEV, based on the finding that patients infected with genotype 4 HEV tend to have more severe disease than those with genotype 3 HEV in Japan [Mizuo et al., 2005]. The mortality rate of hepatitis E in developing countries where genotype 1 HEV prevails is reported to be about 1% [Purcell and Emerson, 2001]. As for genotype 2 HEV, no fulminant cases have been reported thus far. In Japan where HEV isolates of genotypes 3 and 4 circulate, 4 (14.8%) of 27 patients with genotype 4 HEV and 1 (4.8%) of 21 patients with genotype 3 HEV died due to fulminant hepatitis, indicating that genotype 4 HEV may be more closely associated with the development of fulminant hepatitis than HEV of other genotypes. Genotype 4 HEV is unique in that there is an insertion of a single nucleotide (U) at nt 5159, which affects both ORF2 and ORF3 (Fig. 2A). The ORF2 of genotype 4 HEV overlaps ORF1 by one nt, whereas ORF2 in all reported isolates of genotypes 1–3 begins 41 nt downstream of ORF1. The first initiation codon of ORF3 in genotype 4 HEV isolates is 28 nt downstream of ORF1, in contrast with ORF3 in reported isolates of genotypes 1–3 which overlaps ORF1 by one nt. Consequently, genotype 4 HEV has an additional 14 codons in ORF2. The predicted size of ORF3 of genotype 4 HEV at the 5′-terminal portion is nine codons shorter than that of reported isolates of genotypes 1–3 [Wang et al., 2000; Takahashi et al., 2003]. The uniqueness of the genotype 4 HEV genome may explain, at least in part, its association with the severe form of hepatitis E.

Amino acid substitutions in viral proteins that are related with altered pathogenesis have been well documented [Brack et al., 1998; Raychaudhuri et al., 1998; Lum et al., 2003; Glenn and Novembre, 2004]. The viral RNA 5′- and 3′-untranslated region sequences can also affect the expression of disease symptom [Slobodskaya et al., 1996; Brack et al., 1998; Bryant et al., 2005]. In the present study, amino acid changes in the coding regions or nucleotide substitutions in the 5′- and 3′-untranslated regions, that may be associated with the development of fulminant hepatitis, were not observed among patients who were infected with genotype 4 HEV and diagnosed with fulminant hepatitis. However, a silent substitution of U at nt 3148, that is located within the RNA helicase domain of ORF1, was observed significantly more frequently among genotype 4 HEV isolates than among isolates of the other genotypes ($P < 0.0001$), and among HEV isolates obtained from patients with fulminant hepatitis than among those obtained from patients with acute hepatitis ($P = 0.0006$). The results suggest that U3148 in the HEV genome is associated with progression to fulminant hepatitis or the severe form of hepatitis E. The underlying reason for the association of a silent substitution at nt 3148 in the HEV genome with the progression to fulminant hepatitis remains unknown. Two possible explanations are as follows. One explanation is that the silent substitution at nt 3148 may influence the efficiency of replication of HEV. Nt 3148 is located at the RNA helicase domain [Koonin et al., 1992] and the particular nucleotide at nt 3148 may alter the secondary structure of the genome, thereby affecting the expression of RNA helicase. The secondary structure of the RNA genome with U3148 may be favorable for translation of the RNA helicase and RNA polymerase whose coding region is located downstream of the RNA

helicase domain, as discussed previously [Hirata et al., 2003]. Another explanation is that the nucleotide sequence containing nt 3148 may regulate the transcription of the subgenomic mRNA of the HEV genome. It was reported that in the liver of cynomolgus macaques infected with HEV, there was a subgenomic mRNA that was shorter than the entire genome and had a common 3′-end with genomic RNA [Tam et al., 1991; Yarbough et al., 1991]. The 22-nt sequence including nt 3148 was conserved among all four genotypes, supporting the latter explanation. As the 5′-end of the subgenomic mRNA has not been determined as yet, it is uncertain whether nt 3148 can affect the transcription of the mRNA. As to whether the sequence including nt 3148 plays a role at the genomic level, the 15-nt sequence including U3148 is homologous to the sequence 5′ UGCYAUUGAGCAGGC 3′ (nt 67–81) in the methyltransferase domain of ORF1, which is well conserved among the genotypes, and further investigation may be warranted.

Of interest, a plant virus, *Apple stem grooving virus* (ASGV), that contained a single silent substitution in the coding region, did not induce the symptoms in host plants that are characteristic of the wild-type virus [Hirata et al., 2003]. As the substitution did not affect the abundance of mRNA transcribed from the downstream, the mechanism of symptom attenuation is under discussion. Its genome consists of a single-stranded, positive-sense RNA of 6.5 kb that is 5′-capped and 3′-polyadenylated. Some features of the ASGV genome resemble those of the HEV genome, suggesting that a silent substitution in the HEV genome may also affect the symptoms in the host. HEV RNA replication occurred in primate cell cultures transfected with in vitro transcripts of an infectious cDNA clone of the HEV genome [Emerson et al., 2004b]. Studies using a mutagenized genotype 4 HEV with U3148 that is constructed in vitro, may elucidate the mechanism by which the silent substitution of U3148 leads to progression to fulminant hepatitis.

In conclusion, the results of this study suggest that a silent substitution of U at nt 3148 in genotype 4 HEV is associated closely with the occurrence of fulminant hepatitis. As the number of patients with type E fulminant hepatitis is limited, accumulation of patients with type E fulminant hepatitis not only in Japan but also in other countries and extensive clinical and virological analyses of a large number of such cases are needed in future studies to evaluate our proposal. Studies on the mechanism by which the silent substitution of U3148 leads to progression to fulminant hepatitis may elucidate a novel determinant of disease severity of HEV infection.

ACKNOWLEDGMENTS

We thank Yasuyuki Yazaki (Kobayashi Hospital, Hokkaido, Japan), Takeshi Yamamoto (Tohoku Rosai Hospital, Miyagi, Japan), Yoshiaki Yajima (Sendai City Hospital, Miyagi, Japan), Yoshiki Sugai (Iwaki Kyoritsu General Hospital, Fukushima, Japan), Akiko Kuno (Jichi Medical School, Tochigi, Japan), Hajime Tokita (National Tokyo Hospital, Tokyo, Japan), Natsuko Saitoh (Ishikawa Prefectural Central Hospital, Ishikawa, Japan), Yoichi Koizumi (Shinonoi General Hospital, Nagano, Japan), Yoshihiro Akahane (Kofu Municipal Hospital, Yamanashi, Japan), and Susumu Hijioka (Kumagun Municipal Taragi Hospital, Kumamoto, Japan) for supplying serum samples.

REFERENCES

Brack K, Frings W, Dotzauer A, Vallbracht A. 1998. A cytopathogenic, apoptosis-inducing variant of hepatitis A virus. J Virol 72:3370–3376.

Bryant JE, Vasconcelos PF, Rijnbrand RC, Mutebi JP, Higgs S, Barrett AD. 2005. Size heterogeneity in the 3′ noncoding region of South American isolates of yellow fever virus. J Virol 79:3807–3821.

Coursaget P, Buisson Y, N'Gawara MN, Van Cuyck-Gandre H, Roue R. 1998. Role of hepatitis E virus in sporadic cases of acute and fulminant hepatitis in an endemic area (Chad). Am J Trop Med Hyg 58:330–334.

Donati MC, Fagan EA, Harrison TJ. 1997. Sequence analysis of full length HEV clones derived directly from human liver in fulminant hepatitis E. In: Rizzetto M, Purcell RH, Gerin JL, Verme G, editors. Viral hepatitis and liver disease. Torino: Edizioni Minerva Medica. pp 313–316.

Emerson SU, Anderson D, Arankalle A, Meng XJ, Purdy M, Schlauder GG, Tsarev SA. 2004a. *Hepevirus*. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Virus taxonomy: VIIIth report of the ICTV. London: Elsevier/Academic Press. pp 853–857.

Emerson SU, Nguyen H, Graff J, Stephany DA, Brockington A, Purcell RH. 2004b. In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. J Virol 78:4838–4846.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791.

Fujiwara K, Yokosuka O, Fukai K, Imazeki F, Saisho H, Omata M. 2001. Analysis of full-length hepatitis A virus genome in sera from patients with fulminant and self-limited acute type A hepatitis. J Hepatol 35:112–119.

Fujiwara K, Yokosuka O, Ehata T, Saisho H, Saotome N, Suzuki K, Okita K, Kiyosawa K, Omata M. 2002. Association between severity of type A hepatitis and nucleotide variations in the 5′ nontranslated region of hepatitis A virus RNA: Strains from fulminant hepatitis have fewer nucleotide substitutions. Gut 51:82–88.

Glenn AA, Novembre FJ. 2004. A single amino acid change in gp41 is linked to the macrophage-only replication phenotype of a molecular clone of simian immunodeficiency virus derived from the brain of a macaque with neuropathogenic infection. Virology 325:297–307.

Harrison TJ. 1999. Hepatitis E virus—An update. Liver 19:171–176.

Hijioka S, Sato Y, Iwashita Y, Indou Y. 2005. A case of acute hepatitis E who had a history of frequent ingestion of raw meat and viscera from wild deer and boars (in Japanese). Jpn J Gastroenterol 102:723–728.

Hirata H, Lu X, Yamaji Y, Kagiwada S, Ugaki M, Namba S. 2003. A single silent substitution in the genome of *Apple stem grooving virus* causes symptom attenuation. J Gen Virol 84:2579–2583.

Ina Y. 1994. ODEN: A program package for molecular evolutionary analysis and database search of DNA and amino acid sequences. Comput Appl Biosci 10:11–12.

Kato M, Taneichi K, Matsubayashi K. 2004. A mini-outbreak of HEV infection in those who enjoyed *Yakiniku* party: One died of fulminant hepatitis (in Japanese). Kanzo 45:688.

Koonin EV, Gorbalenya AE, Purdy MA, Rozanov MN, Reyes GR, Bradley DW. 1992. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: Delineation of an additional group of positive-strand RNA plant and animal viruses. Proc Natl Acad Sci USA 89:8259–8263.

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.

Kuno A, Ido K, Isoda N, Satoh Y, Ono K, Satoh S, Inamori H, Sugano K, Kanai N, Nishizawa T, Okamoto H. 2003. Sporadic acute hepatitis E of a 47-year-old man whose pet cat was positive for antibody to hepatitis E virus. Hepatol Res 26:237–242.

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.

Liu Z, Chi B, Takahashi K, Mishiro S. 2003. A genotype IV hepatitis E virus strain that may be indigenous to Changchun, China. Intervirology 46:252–256.

Lum JJ, Cohen OJ, Nie Z, Weaver JG, Gomez TS, Yao XJ, Lynch D, Pilon AA, Hawley N, Kim JE, Chen Z, Montpetit M, Sanchez-Dardon J, Cohen EA, Badley AD. 2003. Vpr R77Q is associated with long-term nonprogressive HIV infection and impaired induction of apoptosis. J Clin Invest 111:1547–1554.

Meng XJ. 2003. Swine hepatitis E virus: Cross-species infection and risk in xenotransplantation. Curr Top Microbiol Immunol 278:185–216.

Mizuo H, Suzuki K, Takikawa Y, Sugai Y, Tokita H, Akahane Y, Itoh K, Gotanda Y, Takahashi M, Nishizawa T, Okamoto H. 2002. Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. J Clin Microbiol 40:3209–3218.

Mizuo H, Yazaki Y, Sugawara K, Tsuda F, Takahashi M, Nishizawa T, Okamoto H. 2005. Possible risk factors for the transmission of hepatitis E virus and for the severe form of hepatitis E acquired locally in Hokkaido, Japan. J Med Virol 76:341–349.

Nanda SK, Yalcinkaya K, Panigrahi AK, Acharya SK, Jameel S, Panda SK. 1994. Etiological role of hepatitis E virus in sporadic fulminant hepatitis. J Med Virol 42:133–137.

Nishizawa T, Takahashi M, Mizuo H, Miyajima H, Gotanda Y, Okamoto H. 2003. Characterization of Japanese swine and human hepatitis E virus isolates of genotype IV with 99% identity over the entire genome. J Gen Virol 84:1245–1251.

Ohnishi S, Kang JH, Maekubo H, Takahashi K, Mishiro S. 2003. A case report: Two patients with fulminant hepatitis E in Hokkaido, Japan. Hepatol Res 25:213–218.

Okamoto H, Takahashi M, Nishizawa T, Fukai K, Muramatsu U, Yoshikawa A. 2001. Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. Biochem Biophys Res Commun 289:929–936.

Okamoto H, Takahashi M, Nishizawa T. 2003. Features of hepatitis E virus infection in Japan. Intern Med 42:1065–1071.

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.

Page RD. 1996. TreeView: An application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358.

Purcell RH, Emerson SU. 2001. Hepatitis E virus. In: Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE, editors. Fields virology, 4th edition. Philadelphia, PA: Lippincott Williams and Wilkins. pp 3051–3061.

Raychaudhuri G, Govindarajan S, Shapiro M, Purcell RH, Emerson SU. 1998. Utilization of chimeras between human (HM-175) and simian (AGM-27) strains of hepatitis A virus to study the molecular basis of virulence. J Virol 72:7467–7475.

Sainokami S, Abe K, Kumagai I, Miyasaka A, Endo R, Takikawa Y, Suzuki K, Mizuo H, Sugai Y, Akahane Y, Koizumi Y, Yajima Y, Okamoto H. 2004. Epidemiological and clinical study of sporadic acute hepatitis E caused by indigenous strains of hepatitis E virus in Japan compared with acute hepatitis A. J Gastroenterol 39:640–648.

Saitoh N, Doyama H, Omori T, Yamada S, Shimazaki H. 2004. A domestic case of acute sporadic hepatitis E identified in Hokuriku district (in Japanese). Kanzo 45:268–273.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

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

Schlauder GG, Mushahwar IK. 2001. Genetic heterogeneity of hepatitis E virus. J Med Virol 65:282–292.

Sheikh A, Sugitani M, Kinukawa N, Moriyama M, Arakawa Y, Komiyama K, Li TC, Takeda N, Ishaque SM, Hasan M, Suzuki K. 2002. Hepatitis E virus infection in fulminant hepatitis patients and an apparently healthy population in Bangladesh. Am J Trop Med Hyg 66:721–724.

Slobodskaya OR, Gmyl AP, Maslova SV, Tolskaya EA, Viktorova EG, Agol VI. 1996. Poliovirus neurovirulence correlates with the presence of a cryptic AUG upstream of the initiator codon. Virology 221:141–150.

Smith JL. 2001. A review of hepatitis E virus. J Food Prot 64:572–586.

Suzuki K, Aikawa T, Okamoto H. 2002. Fulminant hepatitis E in Japan. N Engl J Med 347:1456.

Takahashi M, Nishizawa T, Yoshikawa A, Sato S, Isoda N, Ido K, Sugano K, Okamoto H. 2002. Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad. J Gen Virol 83:1931–1940.

Takahashi K, Kang JH, Ohnishi S, Hino K, Miyakawa H, Miyakawa Y, Maekubo H, Mishiro S. 2003. Full-length sequences of six hepatitis E virus isolates of genotypes III and IV from patients with sporadic acute or fulminant hepatitis in Japan. Intervirology 46:308–318.

Takahashi K, Toyota J, Karino Y, Kang JH, Maekubo H, Abe N, Mishiro S. 2004. Estimation of the mutation rate of hepatitis E virus based on a set of closely related 7.5-year-apart isolates from Sapporo, Japan. Hepatol Res 29:212–215.

Takahashi K, Okada K, Kang JH, Karino Y, Ichida T, Matsuda H, Ohnishi S, Toyota J, Yamagiwa S, Maekubo H, Abe N, Mishiro S. 2005. A lineage of hepatitis E virus within genotype IV, associated with severe forms of hepatitis (in Japanese). Kanzo 46:389–390.

Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, Reyes GR. 1991. Hepatitis E virus (HEV): Molecular cloning and sequencing of the full-length viral genome. Virology 185:120–131.

Tei S, Kitajima N, Takahashi K, Mishiro S. 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 362:371–373.

Terazawa S, Kojima M, Yamanaka T, Yotsumoto S, Okamoto H, Tsuda F, Miyakawa Y, Mayumi M. 1991. Hepatitis B virus mutants with precore-region defects in two babies with fulminant hepatitis and their mothers positive for antibody to hepatitis B e antigen. Pediatr Res 29:5–9.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Wang Y, Zhang H, Ling R, Li H, Harrison TJ. 2000. The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. J Gen Virol 81:1675–1686.

Yajima Y, Takahashi N, Yamagishi H, Kodama A, Miyazaki A, Sugiyama K, Eda Y, Okamoto H. 2003. An acute hepatitis case domestically infected with a hepatitis E virus whose nucleotide sequence showed a high similarity to that from a domestic swine in Japan (in Japanese). Jpn J Gastroenterol 100:454–458.

Yamamoto T, Suzuki H, Toyota T, Takahashi M, Okamoto H. 2004. Three male patients with sporadic acute hepatitis E in Sendai, Japan, who were domestically infected with hepatitis E virus of genotype III or IV. J Gastroenterol 39:292–298.

Yarbough PO, Tam AW, Fry KE, Krawczynski K, McCaustland KA, Bradley DW, Reyes GR. 1991. Hepatitis E virus: Identification of type-common epitopes. J Virol 65:5790–5797.

Yazaki Y, Mizuo H, Takahashi M, Nishizawa T, Sasaki N, Gotanda Y, Okamoto H. 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol 84:2351–2357.

Yotsumoto S, Kojima M, Shoji I, Yamamoto K, Okamoto H, Mishiro S. 1992. Fulminant hepatitis related to transmission of hepatitis B variants with precore mutations between spouses. Hepatology 16:31–35.

肝臓　47巻8号　384-391（2006）

＜原　著＞

本邦に於けるE型肝炎ウイルス感染の統計学的・疫学的・ウイルス学的特徴：全国集計254例に基づく解析

阿部　敏紀[1)]　相川　達也[2)]　赤羽　賢浩[3)]　新井　雅裕[4)]　朝比奈靖浩[5)]
新敷　吉成[6)]　茶山　一彰[7)]　原田　英治[8)]　橋本　直明[9)]　堀　亜希子[10)]
市田　隆文[11)]　池田　広記[12)]　石川　晶久[13)]　伊藤　敬義[14)]　姜　貞憲[15)]
狩野　吉康[16)]　加藤　秀章[17)]　加藤　将[18)]　川上　万里[19)]　北嶋　直人[20)]
北村　庸雄[21)]　正木　尚彦[22)]　松林　圭二[23)]　松田　裕之[24)]　松井　淳[25)]
道堯浩二郎[26)]　三原　弘[27)]　宮地　克彦[28)]　宮川　浩[29)]　水尾　仁志[30)]
持田　智[31)]　森山　光彦[32)]　西口　修平[33)]　岡田　克夫[34)]　齋藤　英胤[35)]
佐久川　廣[36)]　柴田　実[37)]　鈴木　一幸[38)]　高橋　和明[4)]　山田剛太郎[39)]
山本　和秀[40)]　山中　太郎[41)]　大和　弘明[42)]　矢野　公士[43)]　三代　俊治[4)*]

要旨：極く最近まで殆んど不明状態にあった我国のE型肝炎の実態を明らかにする目的で，我々は全国から総数254例のE型肝炎ウイルス（HEV）感染例を集め，統計学的・疫学的・ウイルス学的特徴を求めてこれを解析した．その結果，[i] HEV感染は北海道から沖縄まで全国津々浦々に浸透していること；[ii] 感染者の多くは中高年（平均年齢約50歳）で，且つ男性優位（男女比約3.5対1）であること；[iii] 我国に土着しているHEVはgenotype 3とgenotype 4であるが，後者は主に北海道に偏在していること；[iv] 年齢と肝炎重症度との間に相関があること；[v] Genotype 3よりはgenotype 4による感染の方が顕性化率も重症化率も高いこと；[vi] 発生時期が無季節性であること；[vii] 集積症例全体の約30%は動物由来食感染，8%は輸入感染，2%は輸血を介する感染に帰せしめ得たものの，過半の症例（約60%）に於いては感染経路が不明のままであること；等の知見を得た．

索引用語：　E型肝炎　E型肝炎ウイルス　疫学　日本

1）一心病院，2）相川内科病院，3）甲府市立甲府病院，4）東芝病院，5）武蔵野赤十字病院，6）富山大学三内，7）広島大学分子病態制御内科，8）国立療養所東京病院，9）東京逓信病院，10）国保中央病院，11）順天堂大学静岡病院，12）関西医大三内，13）日立総合病院，14）昭和大学二内，15）手稲渓仁会病院，16）札幌厚生病院，17）豊川市民病院，18）北海道大学二内，19）鳥取大学二内，20）市立加西病院，21）順天堂大学浦安病院，22）国立国際医療センター，23）北海道赤十字血液センター，24）松田内科医院，25）至聖病院，26）愛媛大学三内，27）高岡市民病院，28）大阪医大二内，29）帝京大学溝口，30）勤医協中央病院，31）埼玉医大消化器肝臓内科，32）日本大学消化器肝臓内科，33）兵庫医大肝胆膵科，34）鳥取県立中央病院，35）慶應大学消化器内科，36）ハートライフ病院，37）NTT東日本関東病院，38）岩手医大一内，39）川崎医大川崎病院，40）岡山済生会総合病院，41）板橋中央総合病院，42）北見赤十字病院，43）国立病院機構長崎医療センター
*Corresponding author: shunji.mishiro@po.toshiba.co.jp

＜受付日2006年5月10日＞＜採択日2006年6月26日＞

Table 1 Remarkable predominance of male over female, irrespective of severity of the disease.

Gender	Total n = 243	Disease categories			
		Subclinical n = 71	AH[a] n = 135	ASH[a] n = 21	FH[a] n = 16
Female	55(23%)	18(34%)	29(21%)	4(19%)	4(25%)
Male	188(77%)	53(66%)	106(79%)	17(81%)	12(75%)
F/M ratio	1 / 3.4	1 / 2.9	1 / 3.7	1 / 4.3	1 / 3

[a]Abbreviations : AH, acute hepatitis ; ASH, acute severe hepatitis (defined by prolonged prothrombin time, i.e., PT value < 40%) ; FH, fulminant hepatitis.

Table 2 Age of the subjects, possibly influencing clinical manifestations.

Age in yrs	Total n = 242	Disease categories			
		Subclinical n = 70	AH n = 135	ASH n = 21	FH n = 16
Less than 40	63(26%)	38(54%)[a]	21(16%)	3(14%)	1(6%)
40 to 59	105(43%)	20(29%)	70(52%)	11(53%)	4(25%)
60 or more	74(31%)	12(17%)	44(32%)	7(33%)	11(69%)[b]
Mean ± SD	50.1 ± 15.6	42.3 ± 15.9[c]	52.8 ± 14.4	52.8 ± 15.6	58.9 ± 10.1[d]

[a] $P < 0.001$, 0.003, < 0.001 against "AH", "ASH", "FH" respectively; [b] $P = 0.010$, < 0.001 against "AH", "Subclinical" (Chi square test). [c] $P < 0.001$, 0.009, < 0.001 against "AH", "ASH", "FH" (t test) ; [d] $P = 0.047$ against "AH" (Welch test).

緒　言

我国や西欧諸国は，アジア・アフリカの熱帯亜熱帯地域諸国と異なり，E型肝炎が頻発する地域ではないから，相当数の症例を集積するには時間と手間がかかる故，100例以上の症例を纏めて解析した報告は，我々の知る限り英文であれ和文であれ一報だに存在しない．我々は，約3年の歳月をかけて，共著者の夫々が過去およびリアルタイムに経験した症例の情報と検体を持ち寄り，更にはこれに我国から学会や論文で発表された症例の情報をも追加し，2006年1月末までに総数254例の，国内で経験されたHEVヒト感染例を集積することを得た．かほどの多数例を纏めて解析した仕事は未見であるし，聊か興味深い知見も得られたので，以下にそれを報告する．

方　法

症例の任意登録

共著者の夫々が，過去及び現在進行形で経験したHEV感染例について，地域，年齢，性，発病年，発病月，病型診断(Subclinical, Acute Hepatitis, Acute Severe Hepatitis, Fulminant Hepatitisのいずれか)，経過中最高ALT値，経過中最高総ビリルビン値，経過中最延長プロトロンビン時間値，ウイルス学的診断根拠（HEV RNA陽性，あるいはIgM抗体・IgG抗体共陽性)，HEV genotype，推定あるいは確定された感染経路，海外渡航歴の有無，等の情報を任意登録した．2006年1月末の時点で，この『任意登録』によって集積し得た症例数はn＝206である．尚，HEV RNAが陽性でありながらgenotypingが未施行であった症例については，可能な限り検体の入手に努力し，sequencingを行った（方法後出)．

既報告例の引用登録

国内学会での過去の報告例については，抄録から上記調査項目に相当するデータを拾い集めた．論文発表例[1)~16)]については，一部は，当該論文著者自身から上記調査項目に相当するデータを任意登録して貰ったが，それが不可能であった場合には論文中の記載から該当データを引用登録した．この『引用登録』によって集積し得た症例数はn＝48であり，そのうち最古の症例