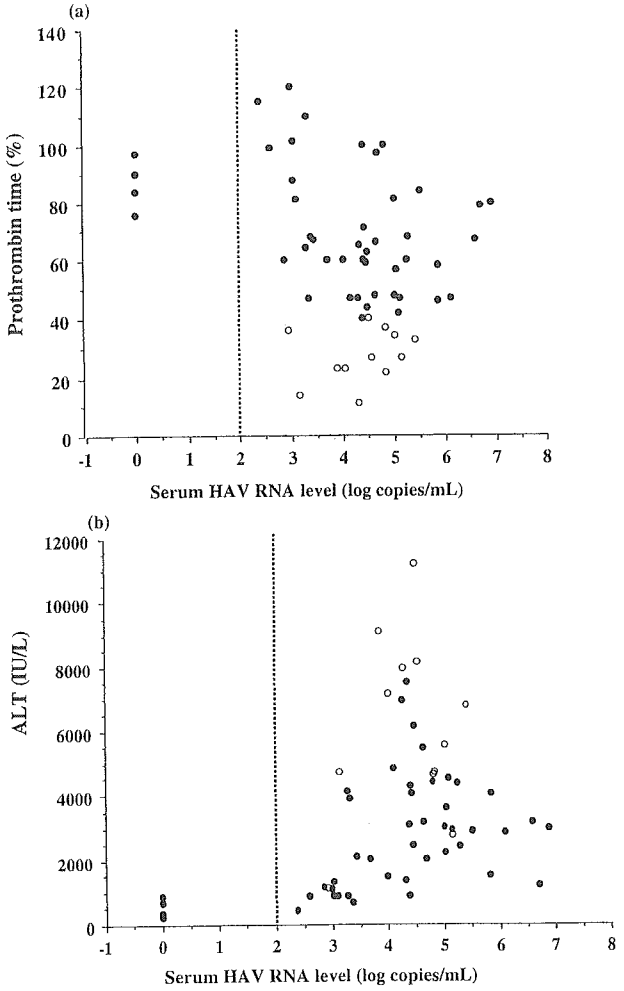

Figure 2 Scattered plots according to disease severity. (a) Correlation between the serum hepatitis A virus (HAV) RNA level and prothrombin time. Mild acute hepatitis (m-AH; ●): $r = -0.400$; $P = 0.0059$; $n = 46$. Severe acute hepatitis (s-AH; ○): $r = 0.285$; $P = 0.3693$; $n = 12$. (b) Correlation between the serum HAV RNA and alanine aminotransferase (ALT) levels. m-AH: $r = 0.462$; $P = 0.0012$; $n = 46$. s-AH: $r = 0.183$; $P = 0.5699$; $n = 12$. (⁞), quantification limit of serum HAV RNA level.

were significantly correlated with the peak viral load and divided the patients into two groups according to their peak viral load, that is, the low-viral-load group consisting of patients having less than 4.00 log copies/mL and the high-viral-load group consisting of patients having more than 4.00 log copies/mL. The high-viral-load group showed a significantly high ALT level (4255 ± 2262 vs 1849 ± 2085; $P = 0.0002$) and a significantly low PT level (54.7 ± 22.1 vs 73.4 ± 28.9; $P = 0.0054$) (Fig. 3). It also showed a significantly high C-reactive protein level (2.8 ± 2.5 vs 1.2 ± 1.3; $P = 0.0073$) and a significantly low platelet count (15.1 ± 5.6 vs 20.1 ± 9.3; $P = 0.0141$) (Fig. 4). However, there was no significant difference in the FDP level between the high-viral-load group and the low-viral-load group (19.8 ± 26.4 vs 8.3 ± 10.0; $P = 0.0667$). We also found no significant differences in variables associated with anemia and renal function between the two groups.

DISCUSSION

The study on how the load of the HAV influences disease severity in humans was difficult because many patients with severe AH-A including FH were treated with immunosuppressive agents such as corticosteroids or with plasma exchange and thus the viral load was modified by these therapies. Therefore, the serum samples obtained after receiving corticosteroid therapy or plasma exchange were excluded from the present study. As a result, we could obtain serum samples at only one time point, that is, on admission in a patient with FH, whereas in other patients, we could obtain serum samples at more than two time points.

In the analysis of serial change of the viral load, the mean period from the clinical onset to the time point of the peak viral load did not differ between the m-AH and s-AH groups. This result indicates that the immune response to viral replication might be at its maximum in the same time from the clinical onset regardless of the disease severity. To assess the influence of viral load on disease severity, we investigated the clinical findings according to disease severity determined on the basis of PT. The data showed that s-AH was more common in patients who were more than 40 years old and also in patients who had a history of alcohol intake that was more than 50 g of ethanol daily, which was compatible with a previous report.[2] Heavy alcohol drinkers might have underlying liver disease and appeared to be linked to increased severity in the present study. Recently, it has been reported that FH was related to female gender and acetaminophen intake.[18] There was no difference in gender or acetaminophen intake (data not shown) between the m-AH and s-AH groups. Therefore, these factors did not appeared to be related to severity in the present study. Furthermore, laboratory findings indicated that in the s-AH group, bilirubin, AST and ALT levels were significantly high.

Although there was no significant difference in the peak viral load between the two groups classified according to disease severity, we found a significant negative correlation with the minimal PT levels in the m-AH group and a significant positive correlation with the maximal ALT levels in the m-AH group. However, we did not find a correlation between the peak viral load and the maximal ALT level, or a correlation with the minimal PT in the s-AH group. These results indicate that the viral load of the HAV strongly affects hepatic injury, but it is necessary for severe AH including FH to have other factors such as older age or alcohol intake in addition to the viral load Recently, a conflicting observation has been reported, that is, a low or undetectable HAV viral load is associated with both low factor V level and FH.[18] However, it was difficult to investigate the peak viral load precisely because almost all of the patients had already reached the peak viral load on admission, as shown in Fig. 1. Therefore, the peak viral load might be underestimated in such patients, as in FH

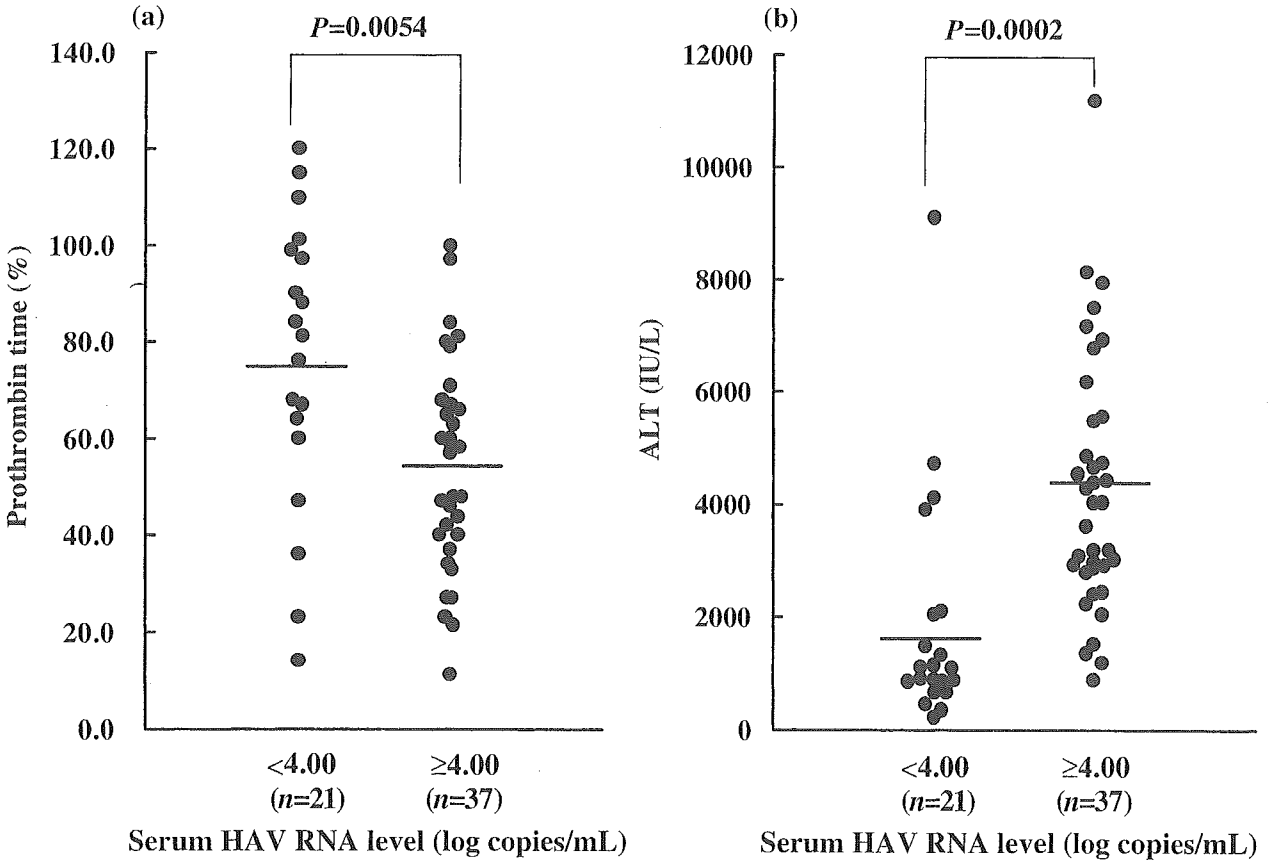

Figure 3 Comparison of (a) prothrombin time and (b) alanine aminotransferase (ALT) level between the serum hepatitis A virus (HAV) RNA levels <4.00 and ≥4.00. (—), mean value for each group.

patients assayed only on admission. Furthermore, it is difficult to evaluate the viral load at only one time point, even in the acute phase of AH, because there are two reasons for a low viral load. First, it might be due to a low infectious dose of HAV or a low viral replication, which results in mild hepatitis. Second, it might be caused by a host hyper immune response, which exacerbated hepatitis.

In a previous study of acute hepatitis B, a low level replication of hepatitis B virus (HBV) in patients with FH has been detected in the serum and liver tissue.[22,23] In addition, it was reported that the HBV DNA detected by two-step PCR was present before or after liver transplantation in seven out of 17 patients with FH lacking the serological evidence of HBV infection.[24] Thus, HBV-related FH is considered to be caused by an excessive host immune response associated with a marked decrease in viral load. To assess the direct influence of the immune response on viral load decrease, we analyzed changes in the viral load. In the present study, there were no significant differences in the decrease in the viral load between the m-AH and s-AH groups in the sequential analysis of the viral load. In contrast, in the multiple linear regression analysis the IgM level and ALT level were significantly associated with PT ($P = 0.0098$). These results show that the enhanced immunological reaction might affect the disease severity and that liver damage is closely associated with disease severity.

Furthermore, to compare the viral load results with humoral immunity, we analyzed the maximal IgM HA and IgM levels because the increase in IgM or IgM HA level reflects the interplay between the viral load and humoral immune response. As consistently described in the literature, the IgM HA level is high in the acute phase of the infection and decreases in the convalescence phase. In addition, the time course of the IgM level is the same as that of the IgM HA level. Interestingly, these two markers do not correlate with viral load. Thus, the results in the present study suggest that the development of severe hepatitis in AH-A is multifactorial, and the host immune reaction is not always strong in severe AH but might be responsible for disease severity to some extent. Recently, a novel CD4+ T-helper lymphocyte epitope in the VP3 region at position 102–121 has been identified *in vitro*,[25] whereas the sequence 110–121 of the VP3 protein of HAV is considered a B-cell epitope.[26] In a later report, antibodies generated by the VP3 (110–121) peptide bind to intact HAV and neutralize its infectivity and antipeptide antibodies inhibit serum binding to HAV in the convalescence phase. Thus, further studies of genetic factors affecting the immune response are required to analyze the mechanism underlying disease severity.

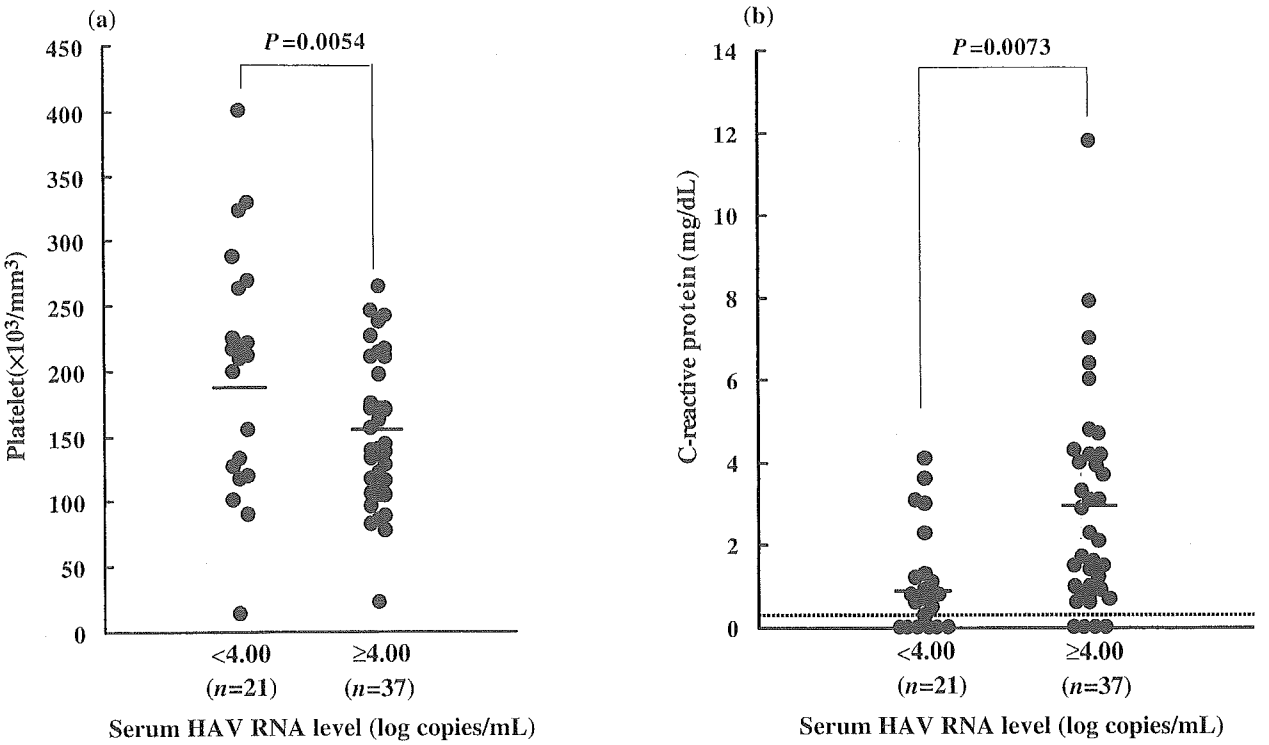

Figure 4 Comparison of (a) platelet count and (b) C-reactive protein level between the serum hepatitis A virus (HAV) RNA levels <4.00 and ≥4.00. (—), mean value for each group; (····), quantification limit of C-reactive protein level.

As for the HAV genotype, few studies have shown the influence of the HAV genotype on viral replication and very few data have been published on disease severity according to the genotype. In the analysis of 47 Japanese patients with AH-A, including eight FH, it was reported that there is no apparent association between disease severity and the sequence of the genotype-determining region.[27] However, a study of 11 German patients suggested that the longest duration of viremia was found in patients infected with genotype IA.[19] In contrast, conflicting data from a French study group showed that the proportions of genotypes other than genotype IA were significantly high in FH.[18] The question on whether the peak viral load depends on the genotype, or whether disease severity depends on genotype, must be clarified in further studies because there was only a small number of patients with genotypes other than IA in the present study.

As for extrahepatic manifestations according to disease severity, laboratory findings such as increases in the white blood cell count, levels of BUN, creatinine and C-reactive protein, or decreases in the red blood cell count, hemoglobin level, hematocrit, platelet count and AT-III level, were more common in the s-AH group. The results are reasonable because severe hepatitis is characterized by severe coagulopathy and can rapidly deteriorate to coma and death due to cerebral edema and multiorgan dysfunction. Interestingly, in the analysis of the correlation of the peak viral load with the peak values of variables a significant negative correlation with the minimal platelet count and a significant positive correlation with the maximal C-reactive protein level regardless of the disease severity were also noted. Thrombocytopenic purpura was reported as a manifestation of AH-A.[5,28–32] As for the mechanism underlying thrombocytopenia in AH-A, it was reported that thrombocytopenic purpura might occur due to an autoimmune mechanism accompanied by antiphospholipid antibodies and a good response to steroid therapy or might be the initial finding of virus-associated hemophagocytosis diagnosed by bone marrow aspiration.[5,28–32] However, previous experimental data indicated that inoculation of the human bone marrow with the HAV results in a dose- and duration-of-incubation-dependent suppression of hematopoietic progenitor growth *in vitro*.[33] In this previous report, hematopoietic suppression seemed to be independent of human interferon-alpha, -beta, -gamma and tumor necrosis factor. Although none of the patients had associated thrombocytopenic purpura in the present study, the result indicates that thrombocytopenia as a manifestation of AH-A depends on the load of the HAV, which is consistent with previous data. These findings support the concept that the direct infection of progenitor cells by HAV in the bone marrow might be responsible for thrombocytopenia.

An increased C-reactive protein level was previously reported to be useful for identifying AH-A among acute hepatitis.[34,35] In a previous report, C-reactive protein was stained in the cytoplasm of hepatocytes under light microscopy and immunoreactive products were observed in the rough endoplasmic reticulum by electron microscopy. In the acute phase, the intensity of staining was slightly greater in AH-A than in any other

forms of acute hepatitis, decreasing during the convalescence phase in AH-A.[34] Furthermore, the increase in C-reactive protein level was observed consistently during the first week of AH-A similar to other acute inflammatory proteins, such as alpha 1-antitrypsin, orosomucoid, haptoglobin and alpha 1-antichymotrypsin.[35] Therefore, it is noted that the increase in C-reactive protein level is not always accompanied by bacterial infection or sepsis in AH-A. An analysis of our data shows a fairly good correlation between the peak viral load and C-reactive protein level, which was not influenced by disease severity. Although the increase in C-reactive protein level closely correlated with AH-A, the mechanical basis for the relationship between HAV and C-reactive protein level is not well understood. However, it was considered that the viral load is responsible for the increase in the C-reactive protein level in the acute phase and thrombocytopenia, which are the extrahepatic manifestation of AH-A.

Interestingly, although acute renal failure and aplastic anemia or pure red cell aplasia are common extrahepatic manifestations, we found no significant association between the viral load and renal dysfunction or anemia. On the basis of our results, we considered that these complications emerged in accordance with disease severity. Further studies are required to determine why the viral load affects different hematological disorders.

In summary, we showed that the load of HAV affects both hepatic damage and disease severity determined on the basis of ALT and PT in m-AH, but not in s-AH. Furthermore, viral load is closely correlated with an increase in the C-reactive protein level and enhancement of thrombocytopenia, which is the extrahepatic manifestation. Agents that inhibit viral replication would probably have an effect on this extrahepatic manifestation. Hence, it was considered that the assay of the viral load of HAV could help elucidate clinical manifestations and management of AH-A

ACKNOWLEDGMENTS

The authors would like to thank Ms Fumiyo Endo (Iwate Prefectural University) for technical assistance.

REFERENCES

1 Robertson BH, Jansen RW, Khanna B *et al.* Genetic relatedness of hepatitis A virus strains recovered from different geographic regions. *J. General Virol.* 1992; **73**: 1365–77.
2 Keeffe EB. Is hepatitis A more severe in patients with chronic hepatitis B and other chronic liver diseases? *Am. J. Gastroenterol.* 1995; **90**: 201–5.
3 Huo TI, Wu JC, Chiu CF, Lee SD. Severe hyperbilirubinemia due to acute hepatitis A superimposed on a chronic hepatitis B carrier with glucose-6-phosphate dehydrogenase deficiency. *Am. J. Gastroenterol.* 1996; **91**: 158–9.
4 Domenech P, Palomeque A, Martinez-Gutierrez A, Vinolas N, Vela E, Jimenez R. Severe aplastic anemia following hepatitis A. *Acta Haematol.* 1986; **76**: 227–9.
5 Cohen O, Mevorach D, Ackerman Z, Oren R. Thrombocytopenic purpura as a manifestation of acute hepatitis A. *J. Clin. Gastroenterol.* 1993; **17**: 166–7.
6 Simmons J, Stein L, Kaufman A. Pure red cell aplasia and hepatitis A. *South Med. J.* 1993; **86**: 1274–6.
7 Mourani S, Dobbs SM, Genta RM, Tandon AK, Yoffe B. Hepatitis A virus-associated cholecystitis. *Ann. Intern. Med.* 1994; **120**: 398–400.
8 Davis TV, Keeffe EB. Acute pancreatitis associated with acute hepatitis A. *Am. J. Gastroenterol.* 1992; **87**: 1648–50.
9 Chio F Jr, & Bakir AA. Acute renal failure in hepatitis A. *Int. J. Artif. Organs* 1992; **15**: 413–16.
10 Ilan Y, Hillman M, Oren R, Zlotogorski A, Shouval D. Vasculitis and cryoglobulinemia associated with persisting cholestatic hepatitis A virus infection. *Am. J. Gastroenterol.* 1990; **85**: 586–7.
11 Pelletier G, Elghozi D, Trepo C, Laverdant C, Benhamou JP. Mononeuritis in acute viral hepatitis. *Digestion* 1985; **32**: 53–6.
12 Safadi R, Ben-Hur T, Shouval D. Mononeuritis multiplex: a rare complication of acute hepatitis A. *Liver* 1996; **16**: 288–9.
13 Tabor E. Guillain–Barre syndrome and other neurologic syndromes in hepatitis A, B, and non-A, non-B. *J. Med. Virol.* 1987; **21**: 207–16.
14 Davis LE, Brown JE, Robertson BH, Khanna B, Polish LB. Hepatitis A post-viral encephalitis. *Acta Neurol. Scand.* 1993; **87**: 67–9.
15 Fujiwara K, Yokosuka O, Fukai K, Imazeki F, Saisho H, Omata M. Analysis of full-length hepatitis A virus genome in sera from patients with fulminant and self-limited acute type A hepatitis. *J. Hepatol.* 2001; **35**: 112–19.
16 Fujiwara K, Yokosuka O, Ehata T *et al.* Association between severity of type A hepatitis and nucleotide variations in the 5′ non-translated region of hepatitis A virus RNA: strains from fulminant hepatitis have fewer nucleotide substitutions. *Gut* 2002; **51**: 82–8.
17 Fujiwara K, Yokosuka O, Ehata T, Imazeki F, Saisho H. PCR-SSCP analysis of 5′-nontranslated region of hepatitis A viral RNA. comparison with clinicopathological features of hepatitis A. *Dig. Dis. Sci.* 2000; **45**: 2422–7.
18 Rezende G, Roque-Afonso AM, Samuel D *et al.* Viral and clinical factors associated with the fulminant course of hepatitis A infection. *Hepatology* 2003; **38**: 613–18.
19 Normann A, Jung C, Vallbracht A, Flehmig B. Time course of hepatitis A viremia and viral load in the blood of human hepatitis A patients. *J. Med. Virol.* 2004; **72**: 10–16.
20 Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature* 1989; **339**: 237–8.
21 Robertson BH, Jansen RW, Khanna B *et al.* Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. *J. General Virol.* 1992; **73**: 1365–77.
22 Brechot C, Bernuau J, Thiers V *et al.* Multiplication of hepatitis B virus in fulminant hepatitis B. *Br. Med. J. (Clin. Res. Ed.)* 1984; **288**: 270–71.
23 Schaefer E, Koeppen H, Wirth S. Low level virus replication in infants with vertically transmitted fulminant hepatitis and their anti-HBe positive mothers. *Eur. J. Pediatr.* 1993; **152**: 581–4.
24 Wright TL, Mamish D, Combs C *et al.* Hepatitis B virus and apparent fulminant non-A, non-B hepatitis. *Lancet* 1992; **339**: 952–5.

25 Sánchez G, Pintó RM, Bosch A. A novel CD4+ T-helper lymphocyte epitope in the VP3 protein of hepatitis A virus. *J. Med. Virol.* 2004; **72**: 525–32.
26 Bosch A, Gonzalez-Dankaart JF, Haro I, Gajardo R, Perez JA, Pinto RM. A new continuous epitope of hepatitis A virus. *J. Med. Virol.* 1998; **54**: 95–102.
27 Fujiwara K, Yokosuka O, Imazeki F *et al.* Analysis of the genotype-determining region of hepatitis A viral RNA in relation to disease severities. *Hepatol. Res.* 2003; **25**: 124–34.
28 Scott JX, Gnananayagam EJ, Gupta S, Simon A, Mukhopadhya A. Thrombocytopenic purpura as initial presentation of acute hepatitis A. *Indian J. Gastroenterol.* 2003; **22**: 192–3.
29 Avci Z, Turul T, Catal F *et al.* Thrombocytopenia and emperipolesis in a patient with hepatitis A infection. *Pediatr. Hematol. Oncol.* 2002; **19**: 67–70.
30 Ertem D, Acar Y, Pehlivanoglu E. Autoimmune complications associated with hepatitis A virus infection in children. *Pediatr. Infect. Dis. J.* 2001; **20**: 809–11.
31 Ertem D, Acar Y, Arat C, Pehlivanoglu E. Thrombotic and thrombocytopenic complications secondary to hepatitis A infection in children. *Am. J. Gastroenterol.* 1999; **94**: 3653–5.
32 Watanabe M, Shibuya A, Okuno J, Maeda T, Tamama S, Saigenji K. Hepatitis A virus infection associated with hemophagocytic syndrome: report of two cases. *Intern. Med.* 2002; **41**: 1188–92.
33 Busch FW, de Vos S, Flehmig B, Herrmann F, Sandler C, Vallbracht A. Inhibition of in vitro hematopoiesis by hepatitis A virus. *Exp. Hematol.* 1987; **15**: 978–82.
34 Atono Y, Sata M, Tanikawa K. Kinetics of C-reactive protein in acute viral hepatitis. *Gastroenterol. Jpn* 1989; **24**: 655–62.
35 Kindmark CO, Laurell CB, Iwarson S, Persson K. Sequential changes of the plasma-protein pattern in cases of hepatitis A. *Ups. J. Med. Sci.* 1988; **93**: 233–44.

JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 49–56
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.49–56.2005

Vol. 43, No. 1

Simultaneous Detection of Immunoglobulin A (IgA) and IgM Antibodies against Hepatitis E Virus (HEV) Is Highly Specific for Diagnosis of Acute HEV Infection

Masaharu Takahashi,[1] Shigeyuki Kusakai,[2] Hitoshi Mizuo,[3] Kazuyuki Suzuki,[4] Kuniko Fujimura,[5] Kazuo Masuko,[6] Yoshiki Sugai,[7] Tatsuya Aikawa,[8] Tsutomu Nishizawa,[1] and Hiroaki Okamoto[1]*

Division of Virology, Department of Infection and Immunity, Jichi Medical School, Tochigi-Ken,[1] Institute of Immunology, Tokyo,[2] Department of Internal Medicine, Kin-ikyo Chuo Hospital, Hokkaido,[3] First Department of Internal Medicine, Iwate Medical University, Iwate-Ken,[4] Japanese Red Cross Yamaguchi Blood Center, Yamaguchi-Ken,[5] Masuko Memorial Hospital and Masuko Institute for Medical Research, Aichi-Ken,[6] Department of Internal Medicine, Iwaki Kyoritsu General Hospital, Fukushima-Ken,[7] and Aikawa Internal Medicine Hospital, Ibaraki-Ken,[8] Japan

Received 3 July 2004/Returned for modification 21 September 2004/Accepted 29 September 2004

Serum samples collected from 68 patients (age, mean ± the standard deviation [SD], 56.3 ± 12.8 years) at admission who were subsequently molecularly diagnosed as having hepatitis E and from 2,781 individuals who were assumed not to have been recently infected with hepatitis E virus (HEV; negative controls; 52.9 ± 18.9 years), were tested for immunoglobulin M (IgM) and IgA classes of antibodies to HEV (anti-HEV) by in-house solid-phase enzyme immunoassay with recombinant open reading frame 2 protein expressed in the pupae of silkworm as the antigen probe. The 68 patients with hepatitis E had both anti-HEV IgM and anti-HEV IgA. Among the 2,781 controls, 16 (0.6%) had anti-HEV IgM alone and 4 (0.1%) had anti-HEV IgA alone: these IgA/IgM anti-HEV-positive individuals were not only negative for HEV RNA but lack IgG anti-HEV antibody as well (at least in most of the cases). Periodic serum samples obtained from 15 patients with hepatitis E were tested for HEV RNA, anti-HEV IgM, and anti-HEV IgA. Although HEV RNA was detectable in the serum until 7 to 40 (21.4 ± 9.7) days after disease onset, both IgM and IgA anti-HEV antibodies were detectable until 37, 55, or 62 days after disease onset in three patients and up through the end of the observation period (50 to 144 days) in 12 patients. These results indicate that detection of anti-HEV IgA alone or along with anti-HEV IgM is useful for serological diagnosis of hepatitis E with increased specificity and longer duration of positivity than that by RNA detection.

Hepatitis E, the major form of enterically transmitted non-A, non-B hepatitis, is caused by hepatitis E virus (HEV). HEV is transmitted primarily by the fecal-oral route. Waterborne epidemics are characteristic of hepatitis E in developing regions of Africa, the Middle East, and Southeast and Central Asia, where sanitation conditions are suboptimal; one epidemic has also been documented in North America (Mexico) (32). HEV-associated hepatitis also occurs among individuals in industrialized countries with no history of travel to areas where HEV is endemic (6, 9, 18, 25, 36, 37, 39, 41, 52, 54). Recently, accumulating lines of evidence indicate that hepatitis E is a zoonosis, and pigs or other animals may act as reservoirs for HEV infection in humans (9, 15, 20–24, 27, 39, 42, 45, 56). A significant proportion of healthy individuals in industrialized countries where hepatitis E is not endemic are seropositive for HEV antibodies (8, 19, 46). Therefore, several epidemiological questions remain unanswered. The success of future studies on clinical and subclinical HEV infection not only in developing countries but also in industrialized countries will greatly depend on the availability of assays that are sensitive and specific.

HEV was recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae*. The genome of HEV is a 7.2-kb, positive-sense, single-stranded RNA. It contains a short 5′ untranslated region, three open reading frames (ORFs; ORF1, ORF2 and ORF3), and a short 3′ untranslated region terminated by a poly(A) tract (12, 34, 44, 53). ORF1 encodes nonstructural proteins, ORF2 encodes the capsid protein, and ORF3 encodes a cytoskeleton-associated phosphoprotein. Extensive diversity has been noted among HEV isolates, and HEV sequences have been classified into four major genotypes (genotypes 1 to 4) (37). In Japan, polyphyletic HEV strains of genotype 3 or 4 or both have been isolated from patients with sporadic acute or fulminant hepatitis E who had no history of travel to countries where this virus is endemic (1, 25, 30, 40, 41, 56).

The immunoglobulin M (IgM) class of antibody against HEV (anti-HEV IgM) is used as a reliable and sensitive marker of recent HEV infection (2–4, 38). However, the specificity of the solid-phase assay for anti-HEV IgM has been questioned in some cases, particularly in patients with IgM-rheumatoid factors in the serum, which have activity against the Fc portion of IgG directed to HEV antigen and may elicit

* Corresponding author. Mailing address: Division of Virology, Department of Infection and Immunity, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-Machi, Tochigi-Ken 329-0498, Japan. Phone: 81-285-58-7404. Fax: 81-285-44-1557. E-mail: hokamoto@jichi.ac.jp.

a false-positive result (10). Virus-specific IgA class antibodies have been detected during the acute stage of infection with hepatitis A virus (HAV) (57) or hepatitis B virus (HBV) (28). The IgA class of antibodies has also been detected in sera from patients with hepatitis E (2, 14, 47). Although a few previous studies reported that anti-HEV IgA could be utilized as an additional confirmatory antibody for recent HEV infection (2, 47), the clinical and epidemiological implications of positivity for anti-HEV IgA remain to be clarified.

Therefore, in the present study, we compared the sensitivity and specificity of the anti-HEV IgA and anti-HEV IgM assays and evaluated their ability to diagnose hepatitis E by using serum samples from 68 patients who were subsequently molecularly diagnosed as having hepatitis E and from 2,781 individuals who were assumed to not have been recently infected with HEV as negative controls in an attempt to improve the serological diagnosis of recent HEV infection that is occurring epidemically in developing countries and more frequently than previously thought in industrialized countries, including Japan.

MATERIALS AND METHODS

Serum samples. The present study included serum samples obtained from 68 patients (56 males and 12 females; age, mean ± the standard deviation [SD], 56.3 ± 12.8 [range, 25 to 86] years) at admission who had detectable HEV RNA and who were subsequently diagnosed as having sporadic acute or fulminant hepatitis E. Thirty of the 68 patients had been included in our previous studies for detection of HEV RNA and phylogenetic analysis of HEV isolates (1, 17, 25, 40, 41, 55, 56). The present study also included periodic serum samples collected from 15 of the HEV-infected patients, from whom one or more serum samples had been obtained during each of the following periods: between 0 and 10 days, between 20 and 40 days, and between 50 and 70 days after the onset of the illness. In addition, serum samples obtained from 2,781 individuals who were assumed to not have been recently infected with HEV (1,282 males and 1,499 females; 52.9 ± 18.9 [0 to 97] years) were used as negative controls, and they included 675 samples from voluntary blood donors with normal alanine aminotransferase level (328 males and 347 females; 39.0 ± 16.0 [16 to 64] years), 127 samples from patients with type A, type B, or type C acute hepatitis (77 males and 50 females; 35.7 ± 12.4 [16 to 78] years), 274 samples from patients with type B or type C chronic liver disease (158 males and 116 females; 55.0 ± 13.6 [23 to 83] years), 472 samples from patients on maintenance hemodialysis (262 males and 210 females; 59.0 ± 12.4 [24 to 94] years), 147 samples from patients with primary biliary cirrhosis (27 males and 120 females; 59.7 ± 10.7 [31 to 79] years), 186 samples from patients with rheumatoid arthritis (21 males and 165 females; 63.8 ± 13.2 [26 to 91] years), and 900 samples from patients (409 males and 491 females; 58.5 ± 20.7 [0 to 97] years) who received a routine health examination or care for various disorders at one of our hospitals.

The presence of IgM class antibodies to HAV (anti-HAV IgM), antibodies to HBV core IgM, hepatitis B surface antigen (HBsAg), and antibodies to hepatitis C virus (HCV) (anti-HCV) was determined by commercially available kits (HAVAB-M and CORZYME-M [Abbott Laboratories, Abbott Park, Ill.], Mycell II HBsAg [Institute of Immunology Co., Ltd., Tokyo, Japan], and Abbott HCV PHA Second Generation [Abbott Japan, Tokyo, Japan]). The presence of HBV DNA and HCV RNA was determined by the methods described previously (29, 43). The study protocol conformed to the ethical guidelines and was approved by the ethics committees of the institutions. Informed consent was obtained from each patient.

ELISAs for detecting anti-HEV antibodies. Our previously described in-house enzyme-linked immunosorbent assays (ELISAs) methods for detection of IgM and IgA anti-HEV antibodies using purified recombinant ORF2 protein (25) were performed with the following modifications. Wells of microplates (part no. 762071; Greiner Bio-One GmbH, Frickenhausen, Germany) were coated with 50 μl of the recombinant ORF2 protein (5 μg/ml in phosphate-buffered saline [pH 7.5]) and incubated at room temperature overnight. After removal of the coating buffer, 100 μl of 10 mM Tris-buffered saline (pH 7.5) containing 2.5% (vol/vol) Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and 0.18% Tween 20 was added. The microplates were incubated at room temperature for 4 h. The blocking buffer was discarded, and each well was washed five times with saline containing 2% lactose (Kanto Chemical Co., Inc., Tokyo, Japan) and then freeze-dried. To test for anti-HEV IgM, 50 μl of each sample was added to each well at a dilution of 1:100 in 10 mM Tris-buffered saline containing 40% Block Ace, 0.18% Tween 20, and a mock protein (optical density [OD] at 280 nm = 0.1) that had been obtained from the pupae of silkworm infected with nonrecombinant baculovirus. The microplates were incubated at room temperature for 1 h and were then washed five times with washing buffer (saline with 0.05% Tween 20). A total of 50 μl of phosphate-buffered saline containing 25% (vol/vol) fetal bovine serum (Sigma Chemical, St. Louis, Mo.) and peroxidase-conjugated mouse monoclonal anti-human IgM (M-49; Institute of Immunology Co., Ltd.) (50) was added to each well. The microplates were incubated at room temperature for 1 h and then washed five times with washing buffer. Then, 50 μl of tetramethylbenzidine-soluble reagent (BioFX Laboratories, Inc., Owings Mills, Md.) as a substrate was added to each well. The plate was incubated at room temperature for 30 min in the dark, and then 50 μl of tetramethylbenzidine stop buffer (BioFX Laboratories, Inc.) was added to each well. The OD value of each sample was read at 450 nm. For the anti-HEV IgA assay, peroxidase-labeled mouse monoclonal anti-human IgA (A-13; Institute of Immunology Co., Ltd.) (28) was used in place of the enzyme-labeled anti-human IgM. Test samples with OD values equal to or greater than the cutoff value were considered to be positive for anti-HEV IgM or anti-HEV IgA.

In addition, anti-HEV IgG was assayed according to the method described previously, and the cutoff value used for the anti-HEV IgG assay was 0.152 (25).

The specificity of the anti-HEV assays was verified by absorption with the same recombinant ORF2 protein (50 μg/ml at the final concentration for anti-HEV IgG or anti-HEV IgA assay; 150 μg/ml at the final concentration for anti-HEV IgM assay) that was used as the antigen probe. Briefly, prior to testing, the serum sample was diluted 1:100, 1:300, 1:1,000, 1:3,000, 1:10,000, or 1:30,000 to adjust its OD value to <1.5. If the OD value of the tested sample was reduced by ≥50% in the anti-HEV IgM assay or ≥70% in the anti-HEV IgA or IgG assay after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV.

Detection of HEV RNA. Reverse transcription-PCR (RT-PCR) was performed for detection of HEV RNA in serum. Total RNA was extracted from 100 μl of serum, reverse transcribed, and then subjected to nested PCR with ORF2 primers as described previously (25, 42). The size of the amplification product of the first-round PCR was 506 bp and that of the second-round PCR was 457 bp. The nested RT-PCR assay used had the capability of amplifying all four known genotypes of HEV strains reported thus far (25, 42, 56). The RT-PCR assay was performed in duplicate, and the reproducibility was confirmed. The specificity and sensitivity of the RT-PCR assay were assessed as described previously (25, 42).

RESULTS

Determination of the cutoff values for the anti-HEV IgM and anti-HEV IgA assays. Since the prevalence of HEV infection in the southern part of Japan is low (8, 30), it was assumed that voluntary blood donors in Yamaguchi Prefecture, which is located in the southern part of mainland Honshu of Japan, were highly unlikely to have been infected with HEV in the period just prior to their donating blood. Therefore, to determine the cutoff values for the anti-HEV IgM and anti-HEV IgA assays, serum samples from 675 donors with a normal alanine aminotransferase level who donated blood at the Japanese Red Cross Blood Center in Yamaguchi Prefecture were used as a panel in the present study. In the anti-HEV IgM assay, the OD values ranged from 0.001 to 0.542, and the value of 0.440, which was calculated as 7 SD above the mean value (0.072), was used as the tentative cutoff value. In the anti-HEV IgA assay, OD values ranging from 0.000 to 1.754 were obtained from the 675 control sera; the OD value of 0.642 (mean + 7 SD) was used as the cutoff value for anti-HEV IgA.

Although 16 (2.4%) of the 675 serum samples were positive for anti-HEV IgG (Table 1), the 16 samples tested negative for HEV RNA, and their OD values ranged from 0.036 to 0.161 (below the cutoff value) in the anti-HEV IgM assay and from 0.019 to 0.180 (below the cutoff value) in the anti-HEV IgA

TABLE 1. Prevalence of anti-HEV IgM and anti-HEV IgA among various groups of subjects

Group	No. of subjects studied	Age (yr) (mean ± SD)	No. (%) of subjects with:			
			Anti-HEV IgG[a]	Anti-HEV IgM	Anti-HEV IgA	Both anti-HEV IgM and anti-HEV IgA
Blood donors with normal ALT	675	39.0 ± 16.0	16 (2.4)	1 (0.1)	1 (0.1)	0
Patients with acute hepatitis	127	35.7 ± 12.4	11 (8.7)	4 (3.1)	2 (1.6)	0
Type A	57	35.1 ± 9.3	7 (12.3)	4 (7.0)	1 (1.8)	0
Type B	61	34.6 ± 12.9	3 (4.9)	0	1 (1.6)	0
Type C	9	47.6 ± 20.4	1 (11.1)	0	0	0
Patients with chronic liver diseases	274	55.0 ± 13.6	26 (9.5)	2 (0.7)	0	0
Chronic hepatitis	182	51.2 ± 13.4	15 (8.2)	1 (0.5)	0	0
Liver cirrhosis	57	62.9 ± 10.2	7 (12.3)	1 (1.8)	0	0
Hepatocellular carcinoma	35	61.7 ± 11.2	4 (11.4)	0	0	0
Hemodialysis patients	472	59.0 ± 12.4	60 (12.7)	2 (0.4)	0	0
Patients with primary biliary cirrhosis	147	59.7 ± 10.7	15 (10.2)	4 (2.7)	0	0
Patients with rheumatoid arthritis	186	63.8 ± 13.2	6 (3.2)	3 (1.6)	0	0
Hospital patients[b]	900	58.5 ± 20.7	24 (2.7)	0	1 (0.1)	0
Total of control subjects[c]	2,781	52.9 ± 18.9	158 (5.7)	16 (0.6)	4 (0.1)	0
Patients with hepatitis E	68	56.3 ± 12.8	68 (100)	68 (100)	68 (100)	68 (100)

[a] Positivity for anti-HEV IgG was confirmed in all 226 samples by the absorption test (see Materials and Methods).
[b] They received a routine health examination or care for various disorders at one of our hospitals.
[c] They were assumed not to have been recently infected with HEV.

assay, suggesting the absence of present HEV infection in the studied population.

Detection of anti-HEV IgM and anti-HEV IgA in individuals who were assumed not to have been infected recently with HEV. Among the serum samples obtained from the above-mentioned 675 donors, only one sample was positive for anti-HEV IgM with an OD value of 0.542, and a different sample was positive for anti-HEV IgA alone with an OD value of 1.754 (Table 2). However, these two serum samples were negative for anti-HEV IgG and HEV RNA, suggesting that the anti-HEV IgM or IgA was falsely detected in these two samples.

Using the cutoff values described above, the remaining 2,106 serum samples obtained from 127 patients with type A, type B, or type C acute hepatitis, 274 patients with type B or type C chronic liver disease, 472 patients on maintenance hemodialysis, 147 patients with primary biliary cirrhosis, 186 patients with rheumatoid arthritis, and 900 patients who received routine health examination or medical care for various disorders

TABLE 2. Serum samples that were falsely positive for anti-HEV IgM or IgA in the in-house ELISAs used in the present study

Sample ID no.	Diagnosis	Age (yr)/sex[a]	OD at 450 nm[b]			HEV RNA
			Anti-HEV IgM	Anti-HEV IgA	Anti-HEV IgG	
389	Blood donor	59/M	**0.542 (8)**	0.038	0.075	—[c]
674	Blood donor	55/F	0.024	**1.754 (5)**	0.025	–
868	Health check-up	62/M	0.078	**0.946 (9)**	0.034	–
1614	Hemodialysis	55/F	**2.541 (8)**	0.056	0.089	–
1761	Hemodialysis	84/M	**1.018 (23)**	0.194	0.259 (83) (+)	–
2110	Acute hepatitis (type A)	21/F	**1.986 (−7)**	0.173	1.824 (92) (+)	–
2136	Acute hepatitis (type A)	28/F	**1.509 (−6)**	0.042	0.036	–
2113	Acute hepatitis (type A)	43/F	**0.559 (25)**	0.046	0.089	–
2102	Acute hepatitis (type A)	31/F	**0.445 (5)**	0.079	**0.388 (−1)**	–
2099	Acute hepatitis (type A)	34/M	0.286	**0.731 (6)**	0.037	–
2061	Acute hepatitis (type B)	55/M	0.055	**0.692 (−6)**	0.028	–
2201	Primary biliary cirrhosis	48/F	**1.215 (20)**	0.068	0.049	–
2229	Primary biliary cirrhosis	61/F	**0.545 (−6)**	0.072	0.054	–
2232	Primary biliary cirrhosis	60/F	**0.470 (9)**	0.083	0.068	–
2257	Primary biliary cirrhosis	52/F	**0.462 (17)**	0.081	0.023	–
2463	Rheumatoid arthritis	70/M	**0.933 (8)**	0.106	0.071	–
2496	Rheumatoid arthritis	83/F	**0.785 (22)**	0.038	0.022	–
2545	Rheumatoid arthritis	71/F	**0.522 (18)**	0.103	0.024	–
2919	Liver cirrhosis (type C)	61/M	**0.594 (28)**	0.325	0.265 (86) (+)	–
3007	Chronic hepatitis (type B)	44/M	**0.488 (2)**	0.020	0.008	–

[a] M, male; F, female.
[b] If the OD value of the tested sample was reduced by only <50% in the anti-HEV IgM assay or < 70% in the anti-HEV IgA or IgG assay after absorption with the recombinant ORF2 protein, the result was considered to be false positive, and such samples are indicated in boldface. Numbers in parentheses are percent values.
[c] —, no HEV RNA was detected.

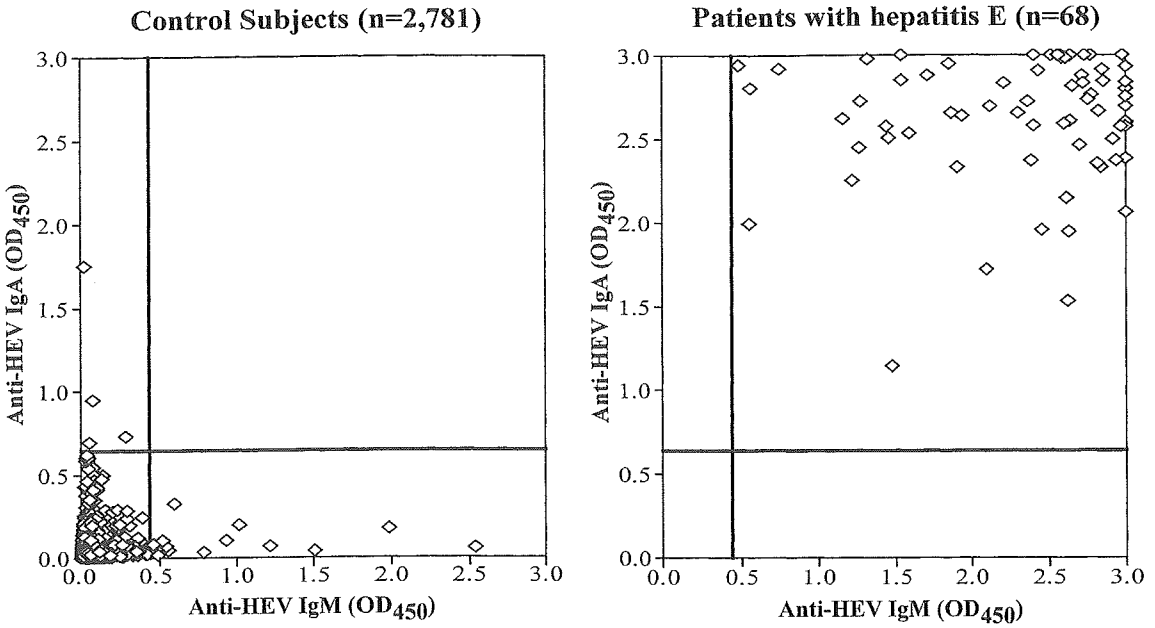

FIG. 1. Distribution of OD values from two ELISAs for anti-HEV IgM and anti-HEV IgA among patients with hepatitis E and among the control subjects. Serum samples from 2,781 subjects who were assumed not to have been infected recently with HEV and from 68 patients with hepatitis E were concurrently tested by the solid-phase ELISAs for anti-HEV IgM and anti-HEV IgA. Horizontal and vertical lines represent the cutoff values for anti-HEV IgA and anti-HEV IgM, respectively.

at one of our hospitals, were tested for anti-HEV IgM and anti-HEV IgA (Table 1). Among the 2,781 subjects who were assumed not to have been infected recently with HEV, including the 675 blood donors described above, anti-HEV IgM was detected in the serum samples from 16 subjects (0.6% or 16 of 2,781), including 4 patients with acute hepatitis A (7.0% or 4 of 57). Anti-HEV IgA was detected in the serum samples from four other patients (0.1% or 4 of 2,781) (Table 1), the difference being statistically significant ($P = 0.0139$ [χ^2-test]). Although the 16 samples had OD values of anti-HEV IgM greater than the cutoff value, with the OD value ranging from 0.445 to 2.541, and the other four samples had OD values of anti-HEV IgA greater than the cutoff value, with the OD value ranging from 0.692 to 1.754, positivity for HEV antibodies could not be confirmed by the absorption test in any of the 20 samples (Table 2). Furthermore, none of these 20 serum samples with anti-HEV IgM or anti-HEV IgA alone had detectable HEV RNA, indicating that these serum samples were falsely positive for anti-HEV IgM or anti-HEV IgA in the ELISAs used.

Of note, among the 2,781 samples from subjects who were assumed not to have been recently infected with HEV in the present study, no serum sample was positive for both IgM and IgA anti-HEV antibodies (Fig. 1).

Detection of anti-HEV IgM and anti-HEV IgA in patients with hepatitis E. Serum samples obtained from 68 patients with sporadic acute or fulminant hepatitis E were tested for the presence of IgM and IgA anti-HEV antibodies. All 68 patients had anti-HEV IgM with OD values ranging from 0.486 to >3.0 and anti-HEV IgA with OD values ranging from 1.146 to >3.0 (Fig. 1). The presence of anti-HEV IgM and anti-HEV IgA was confirmed by the absorption test in the serum samples from all 68 patients, indicating that patients with virologic evidence of the early phase of HEV infection are positive for both anti-HEV IgM and anti-HEV IgA, in sharp contrast to the 20 patients in the control group who had anti-HEV IgM or IgA alone. This finding suggests that the combinatorial detection of both classes of antibodies (IgM and IgA) is efficient for serological diagnosis of hepatitis E with increased accuracy. Among the 68 patients with hepatitis E, four patients (5.9%) had an OD value of <1.000 in the anti-HEV IgM assay, and only one patient had an OD value of <1.500 in the anti-HEV IgA assay. All 68 patients had high levels (1.235 to >3.000) of anti-HEV IgG, 59 (86.8%), of whom had an OD value of >2.000.

Detection of anti-HEV IgM, anti-HEV IgA, and HEV RNA in follow-up serum samples from infected patients. Figure 2 shows the HEV RNA, anti-HEV IgM, and anti-HEV IgA profiles associated with the HEV infection in 15 patients (patients 1 to 15). From these 15 patients, in addition to the serum sample obtained at admission, 3 to 30 other serum samples, including those obtained between 20 and 40 days and between 50 and 70 days after the disease onset, were available. HEV RNA remained detectable in the serum until 7 to 40 (21.4 ± 9.7) days but disappeared 15 to 59 (32.7 ± 13.4) days after the onset of the disease. Anti-HEV IgM and IgA antibodies were both detectable up through the end of the observation period (50 to 144 [72.8 ± 28.5] days after disease onset) in 12 of the 15 patients. In the remaining three patients (patients 1, 3, and 5), both IgM and IgA anti-HEV antibodies were detectable until 37, 55, and 62 days, respectively, after disease onset, but either the IgM or IgA class of anti-HEV antibodies disappeared at 44, 62, and 104 days, respectively, after the disease onset. The presence of anti-HEV IgM and anti-HEV IgA was

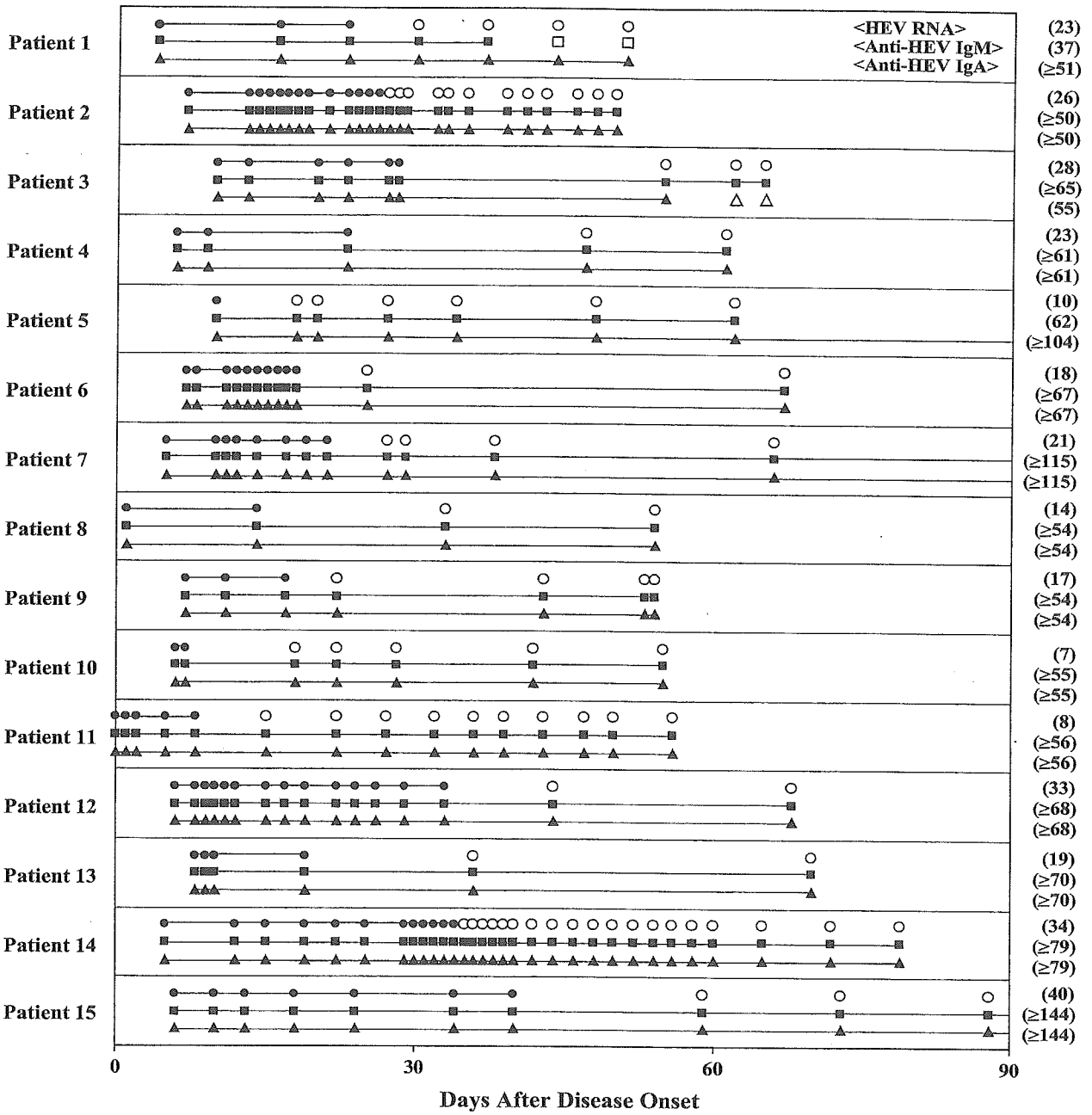

FIG. 2. Detection of HEV RNA, anti-HEV IgM and anti-HEV IgA in initial and follow-up serum samples from 15 patients (patients 1 to 15) with hepatitis E. For each patient, closed and open circles in the top row represent positivity or negativity for HEV RNA, respectively; closed and open boxes in the middle row represent positivity or negativity for anti-HEV IgM, respectively; and closed and open triangles in the bottom row represent positivity or negativity for anti-HEV IgA, respectively. The number in parentheses at the end of each row indicates the final day on which HEV RNA, anti-HEV IgM, or anti-HEV IgA was detectable. Patients 1 and 9 contracted fulminant hepatitis E and died 56 and 54 days, respectively, after onset of the illness.

also confirmed by the absorption test in the follow-up serum samples from all 15 patients, including the last two specimens from patients 1 and 3, who became positive for only anti-HEV IgA and IgM, respectively. The IgG antibody level was as high as 3.0 OD at admission in all 15 patients and persisted at a high level. There was no discernible reduction in the IgG antibody level up through the end of the observation period.

DISCUSSION

The diagnosis of acute or fulminant hepatitis E is based on detection of the HEV genome in serum or feces by RT-PCR (3, 5, 13, 25) or detection of newly elicited antibodies to HEV (3, 4, 14, 19, 25, 38, 49, 59). The presence of a specific antibody of the IgM class is diagnostic of recent or ongoing infection. As

is our in-house ELISA, a solid-phase (sandwich or indirect) ELISA method for detecting anti-HEV IgM is simple and is currently used in the majority of reported in-house ELISAs (3, 4, 14, 19, 25, 38, 49, 59), as well as in a commercial kit marketed in Asia by Genelabs Diagnostics (Singapore). One of the weaknesses of the solid-phase ELISA format is reduced sensitivity due to competition among virus-specific IgM, IgA, and IgG for antigen-binding sites. It has been pointed out that sensitivity is compromised when corresponding IgG titers are disproportionately higher than those of the IgM antibodies (16). Another potential weakness of the solid-phase test for IgM antibody is that IgM-rheumatoid factor in sera from patients with rheumatoid arthritis may elicit a false-positive result (10). Recently, to overcome these weaknesses in the solid-phase ELISAs, an IgM class capture system was introduced by Yu et al. (58). In the class capture system, competing IgG antibodies (also IgA antibodies) in the sample are eliminated at the beginning of the assay, thus enhancing the reaction between anti-HEV IgM and the HEV antigen, although its efficiency depends on the capacity of solidified antibodies against total human IgM molecules containing anti-HEV IgM to capture the HEV antigen. The class capture assay developed by Yu et al. (58) provided a reliable method for detecting anti-HEV IgM and had specificities comparable to those determined by the solid-phase assay when acute-phase sera with high anti-HEV IgM levels were tested and had higher sensitivity for samples with a low anti-HEV IgM concentration or with a high anti-HEV IgG concentration. However, as described by Seriwatana et al. (38), we had to stop developing an IgM class capture ELISA after initial experiments demonstrated poor sensitivity despite the use of substantially greater amounts of the recombinant HEV antigen and several monoclonal antibodies raised against recombinant HEV antigen with distinct specificities to detect the recombinant HEV antigen captured by anti-HEV IgM (unpublished observations). Therefore, in the present study, we chose the solid-phase ELISA format for detecting anti-HEV antibodies.

It has been reported that anti-HEV IgA can be utilized as an additional confirmatory antibody for recent HEV infection (2). Although the presence of a specific antibody of the IgA class is diagnostic for recent infection in several viral or nonviral diseases, including type A, type B, or type C acute hepatitis (28, 35, 57), as well as *Chlamydia trachomatis* infection, *Chlamydia pneumoniae* infection, and cholera (26, 33, 51), the clinical and epidemiological significance of positivity for anti-HEV IgA remains to be fully verified. In the present study, we used the IgM and IgA anti-HEV tests together to characterize serum specimens from 68 patients with acute or fulminant hepatitis E and from 2,781 subjects who were assumed to not have been recently infected with HEV as negative controls. With this dual testing, we obtained the following results. (i) Both anti-HEV IgM and anti-HEV IgA were detectable in serum samples obtained at admission from all 68 patients tested who were subsequently diagnosed molecularly as having hepatitis E (estimated sensitivity rate of the assay: 100% and 100%, respectively). (ii) Among the 2,781 serum samples collected from subjects who were assumed to not have been recently infected with HEV as negative controls, 16 samples (0.6%) were falsely positive for anti-HEV IgM alone and four samples (0.1%) were falsely positive for anti-HEV IgA alone, indicating that the false-positive rate was significantly lower in the anti-HEV IgA assay than in the anti-HEV IgM assay used ($P = 0.0139$) (the estimated specificity rates of the assays were 99.4 and 99.9%, respectively). (iii) Of the 2,781 serum samples collected from the subjects who were assumed to not have been recently infected with HEV, none was positive for both anti-HEV IgM and anti-HEV IgA (estimated specificity rate of the dual assay: 100%), indicating that an erroneous diagnosis of hepatitis E based on serological assay can be minimized by performing the anti-HEV IgM assay on samples that show positive results by the anti-HEV IgA assay or by performing combinatorial assay for anti-HEV IgA and anti-HEV IgM.

Regarding the duration of seropositivity for anti-HEV IgM, it has been reported that sera collected from patients during various hepatitis E outbreaks 3 to 4 months and 6 to 12 months after the onset of jaundice, 50 and 40%, respectively, were positive for anti-HEV IgM (7). In three cases of imported hepatitis E in Japan, the duration of seropositivity for anti-HEV IgM was 66, 112, and 154 days, respectively, from disease onset (19). Little is known about the duration of seropositivity for anti-HEV IgA in HEV-infected patients. Although the duration of observation was limited in the present study, anti-HEV IgA was detectable up through the end of the observation period (50 to 144 days after disease onset) in 14 of the 15 patients with hepatitis E and until 55 days after disease onset in the remaining one patient, suggesting that the durations of seropositivity for anti-HEV IgA and anti-HEV IgM, as determined by the assays that were used, are similar (Fig. 2).

In the circulation, IgA occurs in both monomeric and polymeric forms. Antibodies of the IgA class are unique in that they are produced in response to antigenic stimuli applied locally (48) and have distinct molecular forms. As for anti-HEV IgA, it is unclear whether our assay is detecting both dimeric secretory IgA and monomeric IgA, since the monoclonal antibody to IgA (A-13) that is used as an enzyme-labeled antibody in the present study can bind to various IgA species, such as secretory IgA and two subclasses of IgA (IgA1 and IgA2) (11, 28). However, it seems likely that only polymeric IgA antibody of either the IgA1 or the IgA2 subclass against HEV can be detected as described for IgA antibodies to hepatitis B core in type B acute hepatitis (11). Although an individual may have IgA deficiency, which may elicit a false-negative result in the anti-HEV IgA test, it has been reported that absence or deficiency (<1/100 of the average of normal controls) of total IgA was observed in only 4 (0.004%) of 93,020 apparently healthy blood donors and in 1 (0.01%) of 6,800 hospital patients in Japan: the absence of IgA was found at a frequency of 0.001% (31), indicating that false-negative results in the anti-HEV IgA assay due to the absence or deficiency of IgA in the circulation may be negligible.

Based on the results obtained in the present study, we conclude that, in solid-phase ELISA, the anti-HEV IgA assay is significantly more specific than the anti-HEV IgM assay with regard to ability to diagnose hepatitis E; that anti-HEV IgA could be the first-choice marker as a diagnostic indicator of recent HEV infection when the solid-phase ELISA method is used; and that the diagnostic accuracy increases when positive

results obtained by the anti-HEV IgA assay are confirmed by additional or simultaneous detection of anti-HEV IgM. However, due to the limited number of patients with hepatitis E enrolled in the present study, further studies are needed to verify our conclusions in larger cohorts.

ACKNOWLEDGMENTS

We are grateful to Kazuko Tamura and Toshihiko Nakashima for technical assistance during this study.

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and from the Ministry of Health, Labor, and Welfare of Japan.

REFERENCES

1. **Aikawa, T., M. Kojima, M. Takahashi, T. Nishizawa, and H. Okamoto.** 2002. Identification of indigenous hepatitis E virus from a Japanese patient who contracted sporadic acute hepatitis in 1982. J. Infect. Dis. **186:**1535–1536.
2. **Chau, K. H., G. J. Dawson, K. M. Bile, L. O. Magnius, M. H. Sjogren, and I. K. Mushahwar.** 1993. Detection of IgA class antibody to hepatitis E virus in serum samples from patients with hepatitis E virus infection. J. Med. Virol. **40:**334–338.
3. **Clayson, E. T., K. S. A. Myint, R. Snitbhan, D. W. Vaughn, B. L. Innis, L. Chan, P. Cheung, and M. P. Shrestha.** 1995. Viremia, fecal shedding, and IgM and IgG responses in patients with hepatitis E. J. Infect. Dis. **172:**927–933.
4. **Dawson, G. J., K. H. Chau, C. M. Cabal, P. O. Yarbough, G. R. Reyes, and I. K. Mushahwar.** 1992. Solid-phase enzyme-linked immunosorbent assay for hepatitis E virus IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides. J. Virol. Methods **38:**175–186.
5. **Erker, J. C., S. M. Desai, and I. K. Mushahwar.** 1999. Rapid detection of hepatitis E virus RNA by reverse transcription-polymerase chain reaction using universal oligonucleotide primers. J. Virol. Methods **81:**109–113.
6. **Erker, J. C., S. M. Desai, G. G. Schlauder, G. J. Dawson, and I. K. Mushahwar.** 1999. A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques. J. Gen. Virol. **80:**681–690.
7. **Favorov, M. O., Y. E. Khudyakov, E. E. Mast, T. L. Yashina, C. N. Shapiro, N. S. Khudyakova, D. L. Jue, G. G. Onischenko, H. S. Margolis, and H. A. Fields.** 1996. IgM and IgG antibodies to hepatitis E virus (HEV) detected by an enzyme immunoassay based on an HEV-specific artificial recombinant mosaic protein. J. Med. Virol. **50:**50–58.
8. **Fukuda, S., J. Sunaga, N. Saito, K. Fujimura, Y. Itoh, M. Sasaki, F. Tsuda, M. Takahashi, T. Nishizawa, and H. Okamoto.** 2004. Prevalence of antibodies to hepatitis E virus among Japanese blood donors: identification of three blood donors infected with a genotype 3 hepatitis E virus. J. Med. Virol. **73:**554–561.
9. **Harrison, T. J.** 1999. Hepatitis E virus-an update. Liver **19:**171–176.
10. **Hermann, K., and D. Erdman.** 1995. Diagnosis by serologic assays, p. 121–138. *In* E. Lennette, D. D. Lennette, and E. Lennette (ed.), Diagnostic procedures for viral, rickettsial, and chlamydial infections, 7th ed. American Public Health Association, Washington, D.C.
11. **Hikata, M., K. Tachibana, M. Imai, S. Naito, A. Oinuma, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1986. Immunoglobulin A antibody against hepatitis B core antigen of polymeric and monomeric forms, as well as of IgA1 and IgA2 subclasses, in acute and chronic infection with hepatitis B virus. Hepatology **6:**652–657.
12. **Huang, C.-C., D. Nguyen, J. Fernandez, K. Y. Yun, K. E. Fry, D. W. Bradley, A. W. Tam, and G. R. Reyes.** 1992. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology **191:**550–558.
13. **Huang, F. F., G. Haqshenas, D. K. Guenette, P. G. Halbur, S. K. Schommer, F. W. Pierson, T. E. Toth, and X. J. Meng.** 2002. Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. J. Clin. Microbiol. **40:**1326–1332.
14. **Joshi, M. S., A. M. Walimbe, V. A. Arankalle, M. S. Chadha, and S. D. Chitambar.** 2002. Hepatitis E antibody profiles in serum and urine. J. Clin. Lab. Anal. **16:**137–142.
15. **Kabrane-Lazizi, Y., J. B. Fine, J. Elm, G. E. Glass, H. Higa, A. Diwan, C. J. Gibbs, Jr., X.-J. Meng, S. U. Emerson, and R. H. Purcell.** 1999. Evidence for widespread infection of wild rats with hepatitis E virus in the United States. Am. J. Trop. Med. Hyg. **61:**331–335.
16. **Kemeny, D. M.** 1992. Titration of antibodies. J. Immunol. Methods **150:**57–76.
17. **Kuno, A., K. Ido, N. Isoda, Y. Satoh, K. Ono, S. Satoh, H. Inamori, K. Sugano, N. Kanai, T. Nishizawa, and H. Okamoto.** 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.
18. **Kwo, P. Y., G. G. Schlauder, H. A. Carpenter, P. J. Murphy, J. E. Rosenblatt, G. J. Dawson, E. E. Mast, K. Krawczynski, and V. Balan.** 1997. Acute hepatitis E by a new isolate acquired in the United States. Mayo Clin. Proc. **72:**1133–1136.
19. **Li, T.-C., J. Zhang, H. Shinzawa, M. Ishibashi, M. Sata, E. E. Mast, K. Kim, T. Miyamura, and N. Takeda.** 2000. Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. J. Med. Virol. **62:**327–333.
20. **Meng, X.-J., R. H. Purcell, P. G. Haubur, J. R. Lehman, D. M. Webb, T. S. Tsareva, J. S. Haynes, B. J. Thacker, and S. U. Emerson.** 1997. A novel virus in swine is closely related to the human hepatitis E virus. Proc. Natl. Acad. Sci. USA **94:**9860–9865.
21. **Meng, X.-J., P. G. Halbur, M. S. Shapiro, S. Govindarajan, J. D. Bruna, I. K. Mushahwar, R. H. Purcell, and S. U. Emerson.** 1998. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J. Virol. **72:**9714–9721.
22. **Meng, X. J.** 2000. Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? J. Hepatol. **33:**842–845.
23. **Meng, X. J., B. Wiseman, F. Elvinger, D. K. Guenette, T. E. Toth, R. E. Engle, S. U. Emerson, and R. H. Purcell.** 2002. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. J. Clin. Microbiol. **40:**117–122.
24. **Meng, X. J.** 2003. Swine hepatitis E virus: cross-species infection and risk in xenotransplantation. Curr. Top. Microbiol. Immunol. **278:**185–216.
25. **Mizuo, H., K. Suzuki, Y. Takikawa, Y. Sugai, H. Tokita, Y. Akahane, K. Itoh, Y. Gotanda, M. Takahashi, T. Nishizawa, and H. Okamoto.** 2002. Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. J. Clin. Microbiol. **40:**3209–3218.
26. **Ngeh, J., S. Gupta, and C. Goodbourn.** 2004. The reproducibility of an enzyme-linked immunosorbent assay for detection of *Chlamydia pneumoniae*-specific antibodies. Clin. Microbiol. Infect. **10:**171–174.
27. **Nishizawa, T., M. Takahashi, H. Mizuo, H. Miyajima, Y. Gotanda, and H. Okamoto.** 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.
28. **Nomura, N., M. Imai, F. Tsuda, S. Furuta, Y. Akahane, K. Tachibana, S. Usuda, Y. Miyakawa, and M. Mayumi.** 1985. Immunoglobulin A antibody against hepatitis B core antigen in the acute and persistent infection with hepatitis B virus. Gastroenterology **89:**1109–1113.
29. **Okamoto, H., S. Mishiro, H. Tokita, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1994. Superinfection of chimpanzees carrying hepatitis C virus of genotype II/1b with that of genotype III/2a or I/1a. Hepatology **20:**1131–1136.
30. **Okamoto, H., M. Takahashi, and T. Nishizawa.** 2003. Features of hepatitis E virus infection in Japan. Intern. Med. **42:**1065–1071.
31. **Ozawa, N., M. Shimizu, M. Imai, Y. Miyakawa, and M. Mayumi.** 1986. Selective absence of immunoglobulin A1 or A2 among blood donors and hospital patients. Transfusion **26:**73–76.
32. **Purcell, R. H., and S. U. Emerson.** 2001. Hepatitis E virus, p. 3051–3061. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, M. A. Martin, R. A. Lamb, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott/The Williams & Wilkins Co., Philadelphia, Pa.
33. **Qadri, F., E. T. Ryan, A. S. Faruque, F. Ahmed, A. I. Khan, M. M. Islam, S. M. Akramuzzaman, D. A. Sack, and S. B. Calderwood.** 2003. Antigen-specific immunoglobulin A antibodies secreted from circulating B cells are an effective marker for recent local immune responses in patients with chorela: comparison to antibody-secreting cell responses and other immunological markers. Infect. Immun. **71:**4808–4814.
34. **Reyes, G. R., M. A. Purdy, J. P. Kim, K. C. Luk, L. M. Young, K. E. Fry, and D. W. Bradley.** 1990. Isolation of cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science **247:**1336–1339.
35. **Sato, S., S. Fujiyama, M. Tanaka, M. Goto, Y. Taura, S. Kawano, T. Sato, and H. Yasuo.** 1994. IgM and IgA antibodies generated against hepatitis C virus core antigen in patients with acute and chronic HCV infection. Dig. Dis. Sci. **39:**2022–2031.
36. **Schlauder, G. G., G. J. Dawson, J. C. Erker, P. Y. Kwo, M. F. Knigge, D. L. Smalley, J. E. Rosenblatt, S. M. Desai, and I. K. Mushahwar.** 1998. The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. J. Gen. Virol. **79:**447–456.
37. **Schlauder, G. G., and I. K. Mushahwar.** 2001. Genetic heterogeneity of hepatitis E virus. J. Med. Virol. **65:**282–292.
38. **Seriwatana, J., M. P. Shrestha, R. M. Scott, S. A. Tsarev, D. W. Vaughn, K. S. A. Myint, and B. L. Innis.** 2002. Clinical and epidemiological relevance of quantitating hepatitis E virus-specific immunoglobulin M. Clin. Diagn. Lab. Immunol. **9:**1072–1078.
39. **Smith, J. L.** 2001. A review of hepatitis E virus. J. Food Prot. **64:**572–586.
40. **Suzuki, K., T. Aikawa, and H. Okamoto.** 2002. Fulminant hepatitis E in Japan. N. Engl. J. Med. **347:**1456.
41. **Takahashi, M., T. Nishizawa, A. Yoshikawa, S. Sato, N. Isoda, K. Ido, K. Sugano, and H. Okamoto.** 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.
42. **Takahashi, M., T. Nishizawa, H. Miyajima, Y. Gotanda, T. Iita, F. Tsuda, and H. Okamoto.** 2003. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J. Gen. Virol. **84:**851–862.
43. **Takahashi, M., T. Nishizawa, Y. Gotanda, F. Tsuda, F. Komatsu, T. Kawabata, K. Hasegawa, M. Altankhuu, U. Chimedregzen, L. Narantuya, H. Hoshino, K. Hino, Y. Kagawa, and H. Okamoto.** 2004. High prevalence of antibodies to hepatitis A and E viruses and viremia of hepatitis B, C, and D viruses among apparently healthy populations in Mongolia. Clin. Diagn. Lab. Immunol. **11:**392–398.
44. **Tam, A. W., M. M. Smith, M. E. Guerra, C. Huang, D. W. Bradley, K. E. Fry, and G. R. Reyes.** 1991. Hepatitis E virus (HEV): molecular cloning and sequence of the full-length viral genome. Virology **185:**120–130.
45. **Tei, S., N. Kitajima, K. Takahashi, and S. Mishiro.** 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet **362:**371–373.
46. **Thomas, D. L., P. O. Yarnough, D. Vlahov, S. A. Tsarev, K. E. Nelson, A. J. Saah, and R. H. Purcell.** 1997. Seroreactivity to hepatitis E virus in areas where the disease is not endemic. J. Clin. Microbiol. **35:**1244–1247.
47. **Tokita, H., H. Harada, Y. Gotanda, M. Takahashi, T. Nishizawa, and H. Okamoto.** 2003. Molecular and serological characterization of sporadic acute hepatitis E in a Japanese patient infected with a genotype III hepatitis E virus in 1993. J. Gen. Virol. **84:**421–427.
48. **Tomasi, T. B., and J. Bienenstock.** 1968. Secretory immunoglobulins, p. 1–96. *In* F. J. Dixon and H. G. Kunkel (ed.), Advances in immunology, vol. 9. Academic Press, Inc., New York, N.Y.
49. **Tsarev, S. A., T. S. Tsareva, S. U. Emerson, A. Z. Kapikian, J. Ticehurst, W. London, and R. H. Purcell.** 1993. ELISA for antibody to hepatitis E virus (HEV) based on complete open-reading frame-2 protein expressed in insect cells: identification of HEV infection in primates. J. Infect. Dis. **168:**369–378.
50. **Tsuda, F., S. Naito, E. Takai, Y. Akahane, S. Furuta, Y. Miyakawa, and M. Mayumi.** 1984. Low molecular weight (7s) immunoglobulin M antibody against hepatitis B virus core antigen in the serum for differentiating acute from persistent hepatitis B virus infection. Gastroenterology **87:**159–164.
51. **Verkooyen, R. P., M. F. Peeters, J. H. Van Rijsoort-Vos, W. I. Van der Meijden, and J. W. Mouton.** 2002. Sensitivity and specificity of three new commercially available *Chlamydia trachomatis* tests. Int. J. STD AIDS **13**(Suppl. 2)**:**23–25.
52. **Wang, Y., R. Ling, J. C. Erker, H. Zhang, H. Li, S. Desai, I. K. Mushahwar, and T. J. Harrison.** 1999. A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. J. Gen. Virol. **80:**169–177.
53. **Wang, Y., H. Zhang, R. Ling, H. Li, and T. J. Harrison.** 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.
54. **Wang, Y., D. F. Levine, R. P. Bendall, C. G. Teo, and T. J. Harrison.** 2001. Partial sequence analysis of indigenous hepatitis E virus isolated in the United Kingdom. J. Med. Virol. **65:**706–709.
55. **Yamamoto, T., H. Suzuki, T. Toyota, M. Takahashi, and H. Okamoto.** 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.
56. **Yazaki, Y., H. Mizuo, M. Takahashi, T. Nishizawa, N. Sasaki, Y. Gotanda, and H. Okamoto.** 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.
57. **Yoshizawa, H., Y. Itoh, S. Iwakiri, F. Tsuda, S. Nakano, Y. Miyakawa, and M. Mayumi.** 1980. Diagnosis of type A hepatitis by fecal IgA antibody against hepatitis A antigen. Gastroenterology **78:**114–118.
58. **Yu, C., R. E. Engle, J. P. Bryan, S. U. Emerson, and R. H. Purcell.** 2003. Detection of immunoglobulin M antibodies to hepatitis E virus by class capture enzyme immunoassay. Clin. Diagn. Lab. Immunol. **10:**579–586.
59. **Zhang, J.-Z., S. W. K. Im, S. H. Lau, T. N. Chau, S. T. Lai, S. P. Ng, M. Peiris, C. Tse, T. K. Ng, and M. H. Ng.** 2002. Occurrence of hepatitis E virus IgM, low avidity IgG serum antibodies, and viremia in sporadic cases of non-A, -B, and -C acute hepatitis. J. Med. Virol. **66:**40–48.

J Iwate Med Assoc Vol. 57, No. 4 (Oct 2005) pp. 419-426.

Genotypes of hepatitis B virus (HBV) and those clinical characteristics in HBV carrier residents in Iwate, Japan: Results from health-screening program

Yukiho KASAI[1], Kazuyuki SUZUKI[1], Koichi ABE[1], Tomiko KOYAMA[2] and Hiroaki OKAMOTO[3]

[1] Department of Medicine I, School of Medicine, Iwate Medical University, Morioka, Japan

[2] Health Science Association, Iwate, Japan

[3] Division of Virology, Department of Infection and Immunity, Jichi Medical School, Tochigi, Japan

岩手県におけるB型肝炎ウイルス（HBV）キャリア住民のHBV遺伝子型とその臨床的特徴

葛西幸穂[1]，鈴木一幸[1]，阿部弘一[1]，小山富子[2]，岡本宏明[3]

[1] 岩手医科大学医学部，内科学第1講座

[2] 岩手県予防医学協会

[3] 自治医科大学感染免疫学講座ウイルス部門

(Received on March 2, 2005 & Accepted on March 10, 2005)

Abstract

Recent studies indicate that genotypes of hepatitis B virus (HBV) are closely associated with pathogenesis and clinical outcome in HBV related liver diseases. However, the prevalence of these HBV genotypes and clinical features in HBV carriers remain unclear. From 1977 to 2004, a health-screening program in Iwate Prefecture identified 6,711 HBV carriers. A sample of 661 cases (male, 343; female, 318) was extracted using a stratified random sampling method. In addition, further 30 HBV carriers complicated with HCC during long-term follow-up were identified from the registration system for cancer patients of Iwate Prefecture, and they were enrolled in the study. HBV genotypes were determined using an enzyme linked immunosorbent assay kit. Distribution of HBV genotypes showed 19 (2.9%) with genotype A, 297 (44.9%) with genotype B, and 345 (52.2%) with genotype C. In 30 cases with HCC, genotype B and genotype C were 30% and 70%, respectively. HBV genotypes are closely associated with age, positive rate of HB e antigen (HBeAg), and fluctuation of serum alanine aminotransferase (ALT) levels during a follow-up. Cases that developed HCC with genotype B were found to be significantly older compared to genotype C. In conclusion, the prevalence of genotypes B and C were equal in HBV carriers residing in Iwate Prefecture. Differences between HBV genotypes, in particular genotypes B and C, were closely associated with positive rate of HBeAg, fluctuating serum ALT levels, and clinical outcomes of these carriers.

Key words : health-screening, HBV carrier residents, HBV genotype, HB e antigen, hepatocellular carcinoma

I. Introduction

In Asian and African countries, Hepatitis B virus (HBV) infection is still a major cause of acute hepatitis (AH) and various forms of chronic infection, including asymptomatic HBV carrier state, chronic hepatitis (CH),

liver cirrhosis (LC), and hepatocellular carcinoma (HCC), because infection occurs predominantly through perinatal transmission to babies from carrier mothers who are positive for hepatitis B e antigen (HBeAg) in serum or through horizontal spread during infancy. Eight genotypes of HBV from A to H are currently recognized[1, 2] and have distinct geographical distributions[3-7]. In general, while genotypes B and C frequently occur in Asia[4-6], genotypes A and D are common in Western countries[7]. In Japanese patients with chronic HBV infection, the geographic distribution of genotypes B and C also differs among regions of Japan[6]. In particular, genotype B is relatively frequent in the Tohoku region (the northern part of mainland Japan), which includes Iwate Prefecture, compared to other regions except the Okinawa region. Many recent studies have shown that HBV genotypes are closely associated with clinical manifestations and outcome in patients with acute or chronic liver diseases[8-10]. Furthermore, recent reports have also demonstrated that the differences between genotypes B and C, and subtypes of genotype B are considered as causative agents of HCC[5, 10-13]. However, these data were primarily obtained from inpatients and outpatients who visited one hospital.

Approximately one million people are estimated to be HBV carriers in Japan. The majority of these HBV carriers are receiving no medical treatment or follow-up care. A small number of these HBV carriers who are HBeAg positive or have abnormal levels of serum alanine aminotransferase ALT are recognized as a high-risk group for developing CH and LC or HCC[5, 14-16]. However, to ensure precise health management for all HBV carriers, it is important to clarify the differences between clinical characteristics of all HBV carriers with different genotypes.

In the present study, we clarify: 1) the prevalence of HBV genotypes in HBV carriers identified during a health-screening program in Iwate Prefecture; 2) the relationship between HBV genotypes and age; 3) the relationship between HBV genotypes and rate of positive HBeAg and changes of serum transaminase levels during long-term follow up; and 4) the prevalence of HBV genotypes and the clinical profiles of HBV carriers complicated with hepatocellular carcinoma during a long-term follow-up.

II. Subjects and Methods

1. Subjects

From 1977 to 2004, a total of 381,601 Japanese residents (male: 172,961; female: 208,640) of Iwate Prefecture received a health screen at the institute of Iwate Healthy Science Association. Among these individuals, 6,711 residents (male: 3,576; female: 3,135) were diagnosed as HBV carriers. Using the stratified random sampling method, 661 HBV carriers (male: 343; female: 318) were recruited into samples of approximately equal age and number of individuals for a follow-up every six months or annually by the Iwate Healthy Science Association until 2004. Further 60 HBV carriers complicated with HCC during a follow-up were also recruited from the Registration System for Cancer Patients of Iwate Prefecture. However, only 30 cases with HCC were examined in this study because blood samples could not be obtained from all cases.

Demographic information for HBV carriers

Table 1. Demographics of HBV carriers with or without HCC

	Cases without HCC	Cases with HCC
Total number of HBV carriers	661	30
Sex (male:female)	343:318	21:9
Median age (years, range)	41.9 (16~80)	47.4 (35~67)
Male	41.3 (16~75)	47.0 (35~65)
Female	42.9 (19~80)	48.6 (35~67)
Median follow-up period (years, range)	10 (2~26)	8 (2~15)
Male	9 (2~26)	8 (2~15)
Female	10 (2~26)	9 (4~13)
Serum transaminase levels (mean ± SD)		
AST (IU/L)	24.2 ± 23.0*	54.3 ± 41.2
ALT (IU/L)	29.9 ± 23.6*	38.3 ± 33.3

* $p<0.05$ (compared to cases with HCC)

with or without HCC are shown in Table 1.

The study protocol was approved by the Human Ethics Review Committee of Iwate Medical University, and was permitted by the Committee of Iwate Health Service Association and Iwate Medical Association.

2. Methods

We used the initial serum samples for determination of HBV genotypes which were stored at −20℃ in the institute of Iwate Health Service Association.

HBV genotypes were determined using an enzyme linked immunosorbent assay (ELISA) kit (Institute of Immunology Co., Ltd., Tokyo, Japan) according to the method previously reported by Usuda, et al.[17]. Briefly, 10 μl of a serum sample was placed on a plate fixed with monoclonal antibodies against epitope *b* (located in the pre-S antigen of HBV, and common to all genotypes), epitope m (specific to genotype B), and epitopes *k*, *s* and *u* (associated with several genotypes). A reactive enzyme was then added for color development, and absorbency was measured to determine HBV genotype.

HBsAg was determined using commercial hemagglutination assay kits (MyCell, Institute of Immunology Co. Ltd., Tokyo, Japan) HBeAg and anti-HB e antibody (anti-HBe) were also determined using commercial enzyme immunoassay kits.

Serum levels of ALT, asparate aminotransferase (AST) and γ-glutamyltransferase (γ-GTP) were examined using a routine automatic analyzer.

3. Statistical analysis

Data are expressed as mean±standard deviation (SD) or median (range). Comparisons between the groups were performed by Chi-square test of Fisher's exact test. Probabilities of less than 0.05 were considered statistically significant.

III. Results

1. Distribution of HBV genotypes and their relationship with age

Of 680 cases, 19 (2.9%) were genotype A, 297 (44.9%) were genotype B, and 345 (52.2%) were genotype C. The 30 cases with HCC showed 9 (30%) cases of genotype B and 21 (70%) cases of genotype C. Genotype A was not detected in HCC cases.

Table 2. The relationship between positive rate of HBeAg in each genotype and age at first examination

	Genotype A	Genotype B	Genotype C
Total numbers	19	297	345
Sex (male:female)	13:6	157:140	173:172
Numbers of HBeAg positive (%) #	0 (0)	11 (3.7)	115 (33.3) $^{++}$, **
Sex (male:female)	0:0	8:3	66:49
>29 years	0 (0)	7 (24.1)	29 (43.9)
30 ~39	0 (0)	2 (3.3)	37 (33.0)
40 ~49	0 (0)	0 (0)	28 (28.9)
50 ~59	0 (0)	1 (1.2)	13 (26.5)
60 ~69	0 (0)	1 (2.9)	7 (36.8)
70<	0 (0)	0 (0)	1 (50.0)
Liver function tests			
AST (IU/L)	22.4 ± 11.1	25.8 ± 12.7	33.8 ± 29.9^{+}, *
ALT (IU/L)	29.3 ± 22.1	27.7 ± 17.5	40.8 ± 50.0
g-GTP (IU/L)	29.4 ± 22.3	28.9 ± 40.9	36.2 ± 47.2

$^{+}$, * $p<0.01$ (compared to genotypes A and B, respectively)
$^{++}$, ** $p<0.001$ (compared to genotypes A and B, respectively)
Percentage is the rate of HBeAg positive cases among the total number of HBV carriers in each age group

The relationship between each HBV genotype and age is shown in Figure 1. When the age of HBV carriers was compared based on age in 2003, genotype A was found only in the 20~30-year-old carriers. The rate of genotype B gradually increased as age increased and was the highest in carriers over 70 years old. The rate of genotype C was higher in 40 to 60 years old, but lower in carriers over 70 years old.

2. Positive rate of HBeAg in each HBV genotype

The positive rate of HBeAg for each genotype of HBV was 0% in genotype A, 3.7% in genotype B and 33.3% in genotype C. The positive rate of HBeAg in genotype C was significantly higher ($p<0.001$) than that in genotypes A and B. The relationship between

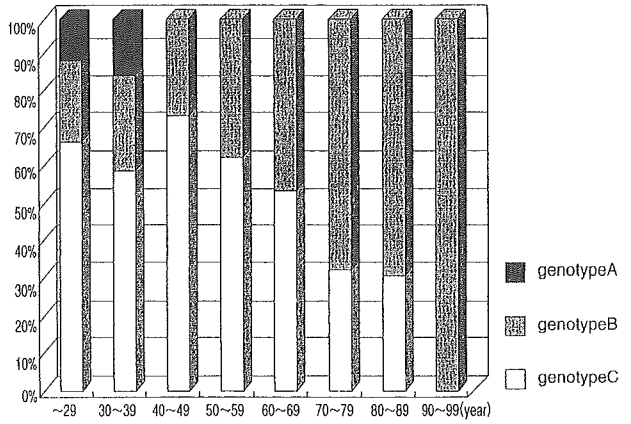

Fig. 1. The relationship between each HBV genotype and age

Table 3. Profiles of HCC cases with genotype B or C

	Genotype B (9)	Genotype C (21)
Age developed to HCC (years)	64 ± 13*	52.7 ± 9
Numbers of HBeAg positive (%)	0 (0%)	6 (27.3%)
Serum ALT levels during follow-up	39.4 ± 22.8	65.5 ± 52.8

* $p<0.05$ (compared to genotype C)

the positive rate of HBeAg in genotypes B and C and age is shown in Table 2. In 20- to 30-year-old genotype B carriers, 11 carriers exhibited a relatively higher positive rate of HBeAg. On the other hand, in genotype C, the percentages of HBeAg positive cases were higher than genotype B at all ages, in particular, in the carriers in their 20s.

3. Changes in the HBeAg/anti-HBe system in genotype B and C carriers during follow-up

Six (54.5%) of 11 genotype B carriers who were positive for HBeAg at the first examination seroconverted to anti-HBe during follow-up, while 5 (45.5%) carriers remained HBeAg positive during follow-up. Of the 115 genotype C carriers who were positive for HBeAg at the first examination, 24 (20.9%) carriers seroconverted to anti-HBe and 12 (10.4%) carriers were positive for alternately between HBeAg and anti-HBe, while 79 (68.7%) carriers remained HBeAg positive during follow-up. Duration until the seroconversion from HBeAg to anti-HBe was 4.5±2.3 years (range; 2 ~ 8 years) in genotype B, and 6.0±4.2 years (1 ~15 years) in genotype C.

4. Relationship between HBV genotypes and serum ALT levels during follow-up

Serum ALT levels during a long-term follow-up were compared among the three HBV genotypes. Serum ALT levels of each HBV genotype were 29.3± 22.1 IU/L in genotype A, 27.7±17.5 IU/L in genotype B, 40.8±50.0 IU/L in genotype C. The levels of serum ALT in the genotype C were significantly higher ($p<0.05$) than those of genotype B, but no significant differences were observed between genotypes A and B.

5. Analysis of HBV carriers complicated with HCC

Comparison between HCC cases with genotype B or C is shown in Table 3. Cases with genotype B were significantly older compared to the cases with genotype C. All cases with genotype B exhibited a negative rate of HBeAg at the start of follow-up, while HBeAg was positive in 27.3% of cases with genotype C. Serum ALT levels during follow-up were significantly higher in genotype C than in genotype B.

IV. Discussion

In Iwate Prefecture, the immunoprophylaxis of perinatal transmission of HBV was started in 1981 and covered more than 60% of all babies by 1986 when it became a mandatory national program[18]. Briefly, 20.7% of HBV carrier mothers were positive for HBsAg and their babies received immunoprophylaxis. As a result of this program, the prevalence of HBsAg decreased from 0.75% in children born between 1978 and 1980 to 0.23% in those born between 1981 and 1985, and further to 0.04% in those born between 1986 and 1990. Therefore, it is believed that the prevention of

perinatal HBV transmission influences not only mother-to-baby transmission but also horizontal transmission from HBV carrier children who might be infected during infancy. However, there are still many HBV carriers who have not received immunoprophylaxis under the national program for prevention of HBV infection.

Among the HBV carriers residing in Japan, a small number of HBeAg positive carriers or carriers with abnormal levels of serum ALT are recognized as a high-risk group for developing CH and LC or HCC[5, 14-16]. However, clinical features and outcome in the majority of HBV carriers with different HBV subtypes remain unclarified.

In this large-scale survey three genotypes were found among HBV carriers residing in Iwate Prefecture who were identified during a health-screening program between 1977 and 2004. HBV genotypes B and C were the most common, followed by genotype A. In a previously published report by Orito and colleagues[6], concerning the geographic distribution of HBV genotypes among patients with chronic liver disease in Japan, the prevalence of genotype B in the Tohoku area including Iwate Prefecture was reported to be higher (22.9%) than that of the other mainland areas. However, the rate of HBV genotype B in this study was higher than expected (44.5%), even if a high rate of HBV genotype B was considered to be endemic in the Tohoku area. A possible reason for this is that our subjects lived without any medical management.

Generally, in HBV carrier residents with genotype B, seroconversion from HBeAg to anti-HBe occurs at a young age (10-20 years after birth), resulting in stabilization of liver function. Therefore, the majority of HBV genotype B carriers live as asymptomatic carriers who do not require medical management or follow-up[16]. Actually, the positive rate of HBeAg in carrier residents with genotype B was extremely low compared to carrier residents with genotype C at the start of follow-up. On the other hand, it has been found that seroconversion from HBeAg to anti-HBe at a young age is not frequent in genotype C carriers, and in most patients abnormal serum ALT levels remain[5, 6, 16]. Therefore, the HBV carriers with genotype C have many opportunities to visit the hospital and receive the medical management, resulting in high prevalence of genotype C. In the present study, the prevalence in every age group of genotype C carriers were significantly lower than rates of anti-HBe of genotype B carriers, while the serum AST and ALT levels were higher in genotype C. Ishikawa, et al.[16] previously showed that the seroconversion from HBeAg to anti-HBe was less likely to occur in genotype C carriers, especially in carriers in their 40s. These subjects were also more likely to develop chronic liver disease, because their serum transaminase levels fluctuated and their HBV-DNA levels were high.

Interestingly, in the present study we found that the prevalence rate of genotype A was relatively high among young people from 20 to 30 years old. Genotype A is the predominant genotype in Europe and the United States[2]. A previous report concerning the geographic distribution of HBV genotypes in Japan showed a low prevalence rate (1.7%) of HBV genotype A[6]. A recent report has suggested that acute hepatitis patients infected with HBV genotype A often transfer to a persistent

HBV carrier state[19]. Also, in Europe, most HBV infections are genotypes A and D, and significantly more genotype A carriers developed chronic liver disease when compared with genotype D carriers[20]. The reason for the increased prevalence rate of genotype A in Iwate Prefecture among the young generation is unclear, and it is therefore necessary to follow these carriers over the long term.

HBV is one of the major causative agents of HCC in Japan. In particular, HBV genotypes B and C are frequently seen in patients with HCC. Previous reports in Japan showed that the mean age is higher in HCC patients with genotype B than in those with genotype C, although results in Taiwan and another Asian countries are controversial [5, 11-13]. In general, genotype B is less prevalent than genotype C among patients with liver cirrhosis, because HBV genotype B is associated with earlier seroconversion from HBeAg to the corresponding anti-HBe and with lower histological activity scores. In the present study, we also demonstrated that HBV carriers with genotype B and HCC were significantly older than cases with genotype C. In addition, genotype B carriers showed lower serum ALT levels during follow-up than genotype C carriers. Therefore, these results suggest that genotype C carriers might have a tendency for persistent fluctuation of abnormal serum ALT levels over the long-term, accelerating the development of HCC.

Recently, lamivudine, an oral cytosine nucleoside analogue, which potently inhibits HBV replication by interfering with HBV reverse transcriptase activity, has been used clinically for the treatment of chronic HBV infection[21-23]. This therapy for chronic HBV infection induced a marked decrease in HBV-DNA and ALT levels, resulting in histological improvement, although lamivudine-resistant HBV strains have appeared in long-term lamivudine therapy[24, 25]. Therefore, this therapy is expected to change the natural course of HBV carriers with persistent abnormal liver function.

In conclusion, the prevalence of genotypes B and C were equal in HBV carriers residing in Iwate Prefecture. Differences between HBV genotypes, in particular genotypes B and C, were closely associated with positive rate of HBeAg, fluctuating serum ALT levels, and clinical outcomes of these carriers.

The authors would like to thank Prof. S. Horiuchi (Department of Biochemistry, Iwate Medical University, School of Medicine) for her helpful advice and comments.

内容自抄

B型肝炎ウイルス（HBV）には8つの遺伝子型（A～H）が存在しているが，HBVキャリア住民の遺伝子型頻度や自然歴は十分に検討されていない．そこで，検診受診者を対象にHBVキャリアの遺伝子型とその臨床的特徴を検討した．岩手県予防医学協会でHBVキャリアと診断された661例と岩手県癌登録事業より肝細胞癌で死亡が確認された30例を対象とした．遺伝子型の測定はELISA法を用いた．岩手県のHBVキャリア住民の遺伝子型の頻度は，各遺伝子型と年齢との関係をみると，Aは20～30歳代でのみ認められ，Bは70歳以上で高く，Cは40～60歳代では半数以上を占めた．各遺伝子型のHBe抗原陽性率の頻度は，CがA，Bより高率であった．経過観察期間中の血清ALT値は，Cが，A，Bに比較して有意に高値であった．肝癌例ではBでの発癌年齢はCに比較して有意に高齢であり，経過観察期間中の血清ALT値も低値であった．