Quantitative HBV entry assay based on the median entry endpoint dilution method

Internalized viral DNA was extracted as described above. A serial threefold dilution of the extracted DNA solution was performed. Then, a nested PCR targeting the P region was carried out with each attenuated DNA solution using a DNA thermocycler (GeneAmp PCR System 9600, Applied Biosystems) as follows. Two primer sets were utilized as described before [33] (first set: 5′-CTGAATCCCGCGGACGACCC-3′ and 5′-ACCCAAGGCACAGCTTGGAGG-3′, second set: 5′-GTCTGTGCCTTCTCATCTGCC-3′ and 5′-AGATGATTAGGCAGAGGTGAAAAA-3′). The target sequences obtained from the first set were amplified in a reaction mixture (50 μL) containing 1.0 U of Ex Taq polymerase (Takara Bio, Shiga, Japan), 200 μM dNTPs (Takara Bio), 25 pmol of each primer (Sigma Genosys, Japan), 10x Ex Taq buffer (Takara Bio) and 20 μL of template DNA. Conditions for the first PCR were: 35 cycles of denaturation for 1 min at 95 °C followed by extension for 3 min at 72 °C. After the last cycle, the samples were incubated for 6 min at 72 °C. Subsequently, 2 μL of amplified DNA product was used for nested PCR using the second primer set. The 50-μL reaction mixture contained the same reagents as described above. Conditions for the second PCR were: 28 cycles of denaturation for 1 min at 95 °C followed by extension for 3 min at 72 °C. The amplified product was separated on a 1.5% agarose gel (SeaKem LE agarose, FMC BioProducts, ME, USA) and the expected size of the PCR product was 294 bp. The dilution for recognizing HBV-DNA with 50% probability was calculated by the Reed–Munch method [34] based on the number of HBV-DNA-positive and negative samples. This dilution was defined as the median tissue culture dose for penetration (TC_{50}).

Detection of HBV covalently closed circular-DNA as evidence of infectivity

Covalently closed circular-DNA is shaped by the HBV genome in Dane particles, which appear to be circular, yet it is only partially double-stranded. Recognizing this specific DNA formed in the hepatocyte nucleus is widely considered to confirm previous evidence of active viral replication [15,16,33,35–37]. The HepG2 cells (1×10^6) were added to 2 mL of culture medium containing serially 10-fold diluted HBV and further incubated for 24 h in the presence or absence of NA. The experimental procedure for obtaining cellular DNA and intracellular viral DNA was described elsewhere. Total DNA was dissolved in 20 μL of distilled water and subjected to amplification by PCR with a primer pair as described by Köck et al. [36] (sense primer: 5′-CCTCTGCCGATCCATACTGCGGAAC-3′ and antisense primer: 5′-CTGCGAGGCGAGGGAGTTCTTCTTC-3′). The target sequences were amplified in a 50-μL reaction mixture containing the same reagents and conditions as in the first step of nested PCR, except for the last cycle, where the samples were incubated for 6 min at 72 °C. The amplified product was separated on a 1.5% agarose gel and showed a specific 1139 bp band of CCC-DNA. Subsequently, CCC-DNA was transferred onto a positively charged nylon membrane, Hybond-N^+ (Amersham Pharmacia Biotech, England), using 0.4 M NaOH as a transfer buffer. HBV-specific sequences were detected by hybridization according to the procedure described by Southern [38] with a digoxigenin (DIG)-labelled (DIG Oligonucleotide Tailing Kit, Boehringer Ingelheim GmbH, Germany) probe contains part of the HBV-DNA genome (5′-TGTCAACGACCGACCTTGAGGCATACTTCA-3′). The membrane was incubated with anti-DIG-alkaline phosphatase conjugates (DIG Luminescent Detection Kit, Boehringer Ingelheim GmbH) and subsequently incubated with chemiluminescent substrate CSPD® solution and exposed for 10 min at 42 °C to Hyperfilm™ ECL™ (Amersham Pharmacia Biotech) to visualize the hybridized probe. The dilution of HBV materials for recognizing CCC-DNA in HepG2 with 50% probability was calculated by the Reed–Munch method [34] based on the number of CCC-DNA-positive and negative samples. This dilution was defined as the median tissue culture infection dose ($TCID_{50}$).

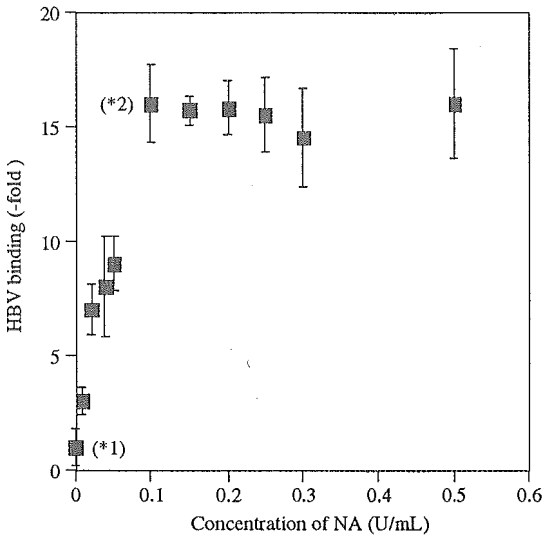

Fig. 1 Neuraminidase (NA) improves HBV binding. HBV (1×10^6 copies) was incubated with HepG2 (1×10^6 cells) in the presence of the given concentrations of NA for 3 h. The cells were then washed twice with PBS(−). The load of HBV binding on HepG2 was measured with the TaqMan PCR. The attachment of HBV was enhanced 16.5 times in the presence of 0.1 U/mL of NA compared with the control. This assay was performed four times. Error bars indicate standard deviations. The actual viral loads at (*1) and (*2) were 3.0×10^4 and 49.5×10^4 copies, respectively.

RESULTS

Effect of NA on HBV binding

First, we investigated the effect of NA on the binding of HBV to HepG2. The NA augmented the binding of this virus in a saturable and dose-dependent manner (Fig. 1). The attachment to HepG2 was enhanced 16.5 times in the presence of 0.1 U/mL of NA (49.5×10^4 copies) compared with the control (3.0×10^4 copies) (Fig. 1). No effect on cell growth was observed, even in the presence of 1.0 U/mL of NA (data not shown). In addition, almost the same enhancement of binding was obtained when HBV particles were pretreated with NA (0.1 U/mL) and then cultured with HepG2 (Fig. 5).

Effect of NA on HBV entry

Second, we studied the effect of 0.1 U/mL of NA on viral entry. HepG2 cells were harvested at various time points after the interaction as described in materials and methods. Then, we checked the number of copies of HBV-DNA within the cells by TaqMan PCR. It should be pointed out that the amount of internalized virus at 3 h incubation (1×10^4 copies; Fig. 2) obtained with the entry assay was clearly less than that obtained with the binding assay (49.5×10^4 copies; Fig. 1). Therefore, the trypsin treatment successfully removed the viruses present on the cell surface. The amount of internalized virus rose with the increase in the period of interaction. As a result, the entry of this virus (27.8×10^4 copies) at 24 h post-inoculation was increased 27.8 times compared with the control (1.0×10^4 copies; Fig. 2). To further confirm the effect of NA on HBV entry, the median entry endpoint dilution method was employed. We calculated the median tissue culture dose for penetration (TC_{50}) at 24 h post-inoculation by the Reed–Munch method [34], repeating the same experiment six times and obtaining reproducible results. Consequently, 1×10^6 copies of HBV in the presence of 0.1 U/mL of NA and in the absence of NA corresponded to a TC_{50} of $10^{1.93}$ and $10^{0.40}$ (Table 1), respectively. Therefore, NA-treatment accelerated the entry of HBV 34.7-fold relative to the control value (Table 1). This result is consistent with the value obtained with the TaqMan PCR methods (27.8 times; Fig. 2).

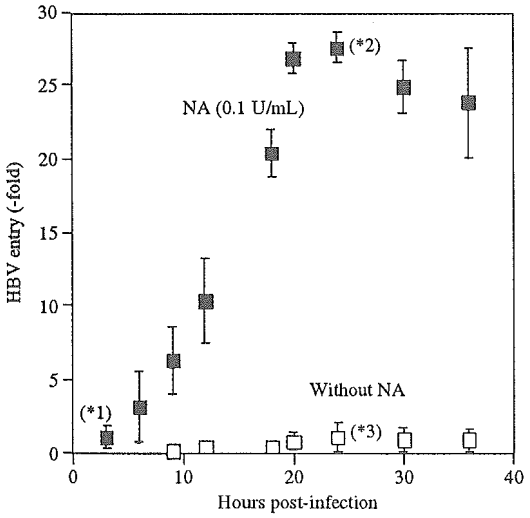

Fig. 2 Enhancement of HBV entry by NA. HBV (1×10^6 copies) interacted with HepG2 (1×10^6 cells) during a given period in the presence or absence of NA. The cells were treated with trypsin solution following two rinses with PBS(−), and were washed four times with culture medium. The number of viral particles penetrating the cells was evaluated with the TaqMan PCR. The number of internalized particles rose with time of interaction. The entry of HBV at 24 h post-inoculation was promoted 27.8 times compared with the control. This assay was performed four times. Error bars indicate standard deviations. The actual viral loads at (*1), (*2) and (*3) were 1.0×10^4, 27.8×10^4 and 1.0×10^4 copies, respectively.

Detection of CCC-DNA as evidence of HBV infectivity

When PCR and Southern-hybridization were performed using whole DNA samples extracted from 1×10^6 copies of HBV (1×10^6 copies) was incubated with Hep G2 (1×10^6 cells) in the presence or absence of NA. The cells were treated with trypsin solution following two rinses with PBS(−), and washed four times with culture medium after cultivation for 24 h. DNA obtained from HBV-associated HepG2 cells was diluted threefold. The HBV-DNA was recognized by nested PCR. The experiment was performed repeatedly, and the efficiency of viral penetration, described as the TC_{50}, was calculated according to the method of Reed–Muench based on the number of HBV-DNA-positive and negative samples. The HBV entry was accelerated 34.7 times in comparison with the control.

Table 1 Change in TC_{50} of HBV materials by the effect of NA

	Dilution of sample	HBV-DNA		TC_{50}
		Positive	Negative	
HBV without NA	1	6	0	
	3	2	4	
	9	0	6	$10^{0.40}$
	27	0	6	(2.50)
	81	0	6	
	243	0	6	
With 0.1 U/mL of NA	1	6	0	
	3	6	0	
	9	6	0	$10^{1.93}$
	27	5	1	(86.68)
	81	4	2	
	243	0	6	

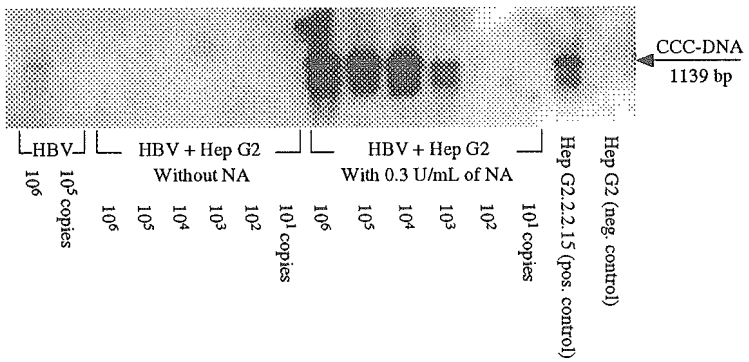

Fig. 3 Detection of CCC-DNA in HepG2 by PCR and Southern-hybridization. HepG2 cells (1×10^6) were inoculated with serially tenfold diluted HBV, and cultured for 24 h. The cells were treated with trypsin solution following two rinses with PBS(−), and were washed four times with culture medium. Whole DNA samples were dissolved in 20 μL of distilled water. CCC-DNA as evidence of infection was investigated by PCR and Southern-hybridization. When whole DNA samples extracted from 1×10^6 copies of HBV were utilized, a slight band could be seen at 1139 bp. This DNA product was not related to the actual infection. No CCC-DNA could be detected in the control, although the DNA product could be clearly seen when 1×10^3 DNA copies of HBV were inoculated in the presence of 0.3 U/mL of NA.

HBV, a slight band could be seen at 1139 bp (Fig. 3), however, this DNA product was not related to the actual infection, as previously stated [36]. An obvious intracellular CCC-DNA band was detected when we inoculated 1×10^3 copies of HBV into HepG2 cells in the presence of 0.3 U/mL of NA (Fig. 3). We repeated the experiment six times, and obtained reproducible results. We determined the median tissue culture infectious dose ($TCID_{50}$) according to the methods of Reed and Muench [34] and found that 1×10^6 copies of HBV corresponded to a $TCID_{50}$ of $10^{2.30}$. In other words, the viral burden needed for detecting infectivity with a probability of 50% is 4960 copies (Table 2). This solution corresponded to a $TCID_{50}$ of $10^{1.60}$ in the presence of 0.1 U/mL of NA. No CCC-DNA could be detected without NA (Fig. 3).

Table 2 Improvement of $TCID_{50}$ of HBV infectious materials by the effect of NA

	$TCID_{50}$ (orginal virus load: 10^6 copies)	HBV load for recognizing infectivity (CCC-DNA) with 50% probability
HBV without NA	n.d.	n.d.
With 0.1 U/mL of NA	$10^{1.60}$	25100
With 0.3 U/mL of NA	$10^{2.30}$	4960

Hep G2 cells (1×10^6) were infected with serially 10-fold diluted HBV, and cultured for 24 h in the presence or absence of NA. The cells were treated with trypsin solution following two rinses with PBS(−), and washed four times with culture medium. Whole DNA from HBV-associated HepG2 cells, was acquired. CCC-DNA as evidence of infection was investigated by PCR and Southern-hybridization. The infectivity, depicted as the $TCID_{50}$, was calculated according to the method of Reed–Muench based on the number of CCC-DNA-positive and negative samples. We found that 1×10^6 copies of HBV corresponded to a $TCID_{50}$ of $10^{2.30}$ in the presence of 0.3 U/mL of NA. From this data, 4960 copies of HBV are required for detecting infectivity with a probability of 50%. n.d., could not be determined.

Effect of asialo-fetuin on HBV binding

To confirm that the binding of HBV to HepG2 in the presence of NA is associated with the interaction between asialoglycoprotein on the virus and its receptor (ASGP-R) on HepG2 cells, asialo-fetuin was used to inhibit the binding of the virus in the presence of NA [11,25]. The attachment of HBV mixed with 0.1 U/mL of NA was clearly inhibited by asialo-fetuin in a dose-dependent manner (Fig. 4). This suggested that the binding is specific for ASGP-R and its ligand, asialoglycoprotein.

Effect of polyions on HBV binding

Desialylation itself must alter the electric charge of the HBV surface, because NA removed negatively charged sialic acid. To see whether the augmentation of binding was caused by an electrostatic effect, a binding experiment was performed in the presence of positively or negatively charged polymers. No change in attachment was seen at any concentration of the chemicals (data not shown).

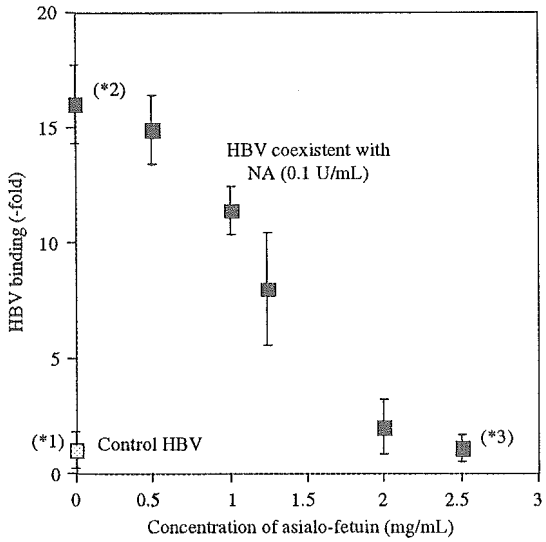

Fig. 4 Inhibition of HBV binding by asialo-fetuin. HBV (1×10^6 copies) was cultured with HepG2 (1×10^6 cells) in the presence of NA (0.1 U/mL) and various concentration of asialo-fetuin for 3 h. The cells were rinsed twice with PBS(−) after the culture. The number of virions binding to the cells was measured with the TaqMan PCR. The binding was blocked by asialo-fetuin in a dose-dependent manner. This assay was performed four times. Error bars indicate standard deviations. The actual viral loads at (*1), (*2) and (*3) were 3.0×10^4, 49.8×10^4 and 3.9×10^4 copies, respectively.

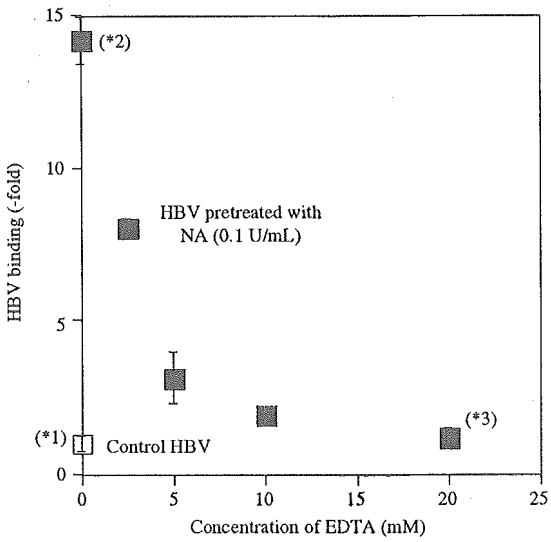

Fig. 5 Inhibition of HBV binding by ethylenediaminetetraacetic acid (EDTA). HBV (1×10^6 copies) was processed with 0.1 U/mL of NA and collected by the centrifugal method. Viral particles were cultured with HepG2 cells (1×10^6) for 1 h at 4 °C. Then, the cells were rinsed with various concentrations of EDTA for 15 min at RT. The number of virions binding to the cells was measured with the TaqMan PCR. EDTA promoted the dissociation of the virus from HepG2 in a dose-dependent manner. This assay was performed four times. Error bars indicate standard deviations. The actual viral loads at (*1), (*2) and (*3) were 2.1×10^4, 29.8×10^4 and 2.6×10^4 copies, respectively.

Effect of EDTA on HBV binding

To confirm that the desialylated viral particles interacted with the receptor, ASGP-R, on the host cell, EDTA was used to inhibit the binding [31]. The attachment of NA (0.1 U/mL)-pretreated HBV was obviously hampered by the calcium chelator in a dose-dependent manner (Fig. 5). This result also suggested that the interaction between the virus and cell was initiated by ASGP-R and asialoglycoprotein.

DISCUSSION

Over the past few decades, many studies have been performed to identify a cellular receptor or mediator for HBV infection. The area is truly important because the identification of receptor(s) is indispensable to elucidation of the mechanism of infection, and the fabrication of anti-HBV drugs or chemicals for inactivation of the virus lurking in blood products. Moreover, we have been able to expand on the idea of infectivity as a new index of viral load, allowing a detailed and accurate prognosis of HBV patients. As a result, various candidates have been proposed. However, none of them have appeared to be generally accepted as a true receptor for the virus. In this study, we refocused on ASGP-R because of its restricted expression on liver parenchymal cells and a previous report suggesting that ASGP-R is a cellular receptor [11], and addressed the effect of the desialylation of viral particles on binding, entry and infectivity in HepG2 cells.

The HBV binding was enhanced in the presence of 0.1 U/mL of NA compared with the control (Figs 1 & 4), and almost the same augmentation of the binding was found when HBV particles were pretreated with NA (0.1 U/mL) and then cultured with HepG2 (Fig. 5). This augmentation seemed to originate from a specific interaction between desialylated HBV particles and ASGP-R [23,31,39] on the cells, because we found that the binding of HBV in the presence of NA was clearly inhibited by asialo-fetuin (Fig. 4) and also the attachment of NA (0.1 U/mL)-pretreated HBV particles was hampered by EDTA, a calcium chelator [32] (Fig. 5). It is possible that the interaction between HBV and HepG2 is enhanced by the change in charge caused by desialylation. However, this can be excluded by the finding that binding efficacy was not altered in the presence of negatively charged or positively charged polymers (data not shown). Thus, the desialylation of viral particles is important for the enhancement of viral binding.

We also confirmed that viral entry was enhanced by NA treatment using two different assay methods (Fig. 2, Table 1). This indicated that once the viral particles bind to ASGP-R, they may be internalized through the receptor. According to Treichel et al. [11], the counterpart of ASGP-R is a pre-S1 region when it is an HBV infection. Therefore, it is possible that desialylation of a pre-S1 region on the viral envelope plays an important role in the enhancement of binding and entry, at least in our experimental conditions.

Next, we tried to detect CCC-DNA, which is a useful and reliable marker for the infectivity of HBV in an *in vitro* system [15,16,33,35–37]. We tried to detect CCC-DNA in DNA samples obtained 24 hrs after the start of culture, and succeeded in estimating the infectivity of HBV materials. CCC-DNA could be detected from the DNA samples obtained from the cells exposed to 1×10^3 DNA copies of HBV in the presence of 0.3 U/mL of NA, but never from the cells exposed to any number of viral copies in the absence of NA (Fig. 3). In addition, the $TCID_{50}$ at 0.1 U/mL of NA ($10^{1.60}$) increased to $10^{2.30}$ at 0.3 U/mL of NA (Table 2). A relatively higher concentration of enzyme was favourable in this assay. The change in concentration of NA may have occurred because HBV infection was initiated by multiple interactions between desialylated sites of the viral envelope and ASGP-R on HepG2 cells. Because a complete CCC-DNA formation only occurs inside the nucleus, these findings demonstrated that NA-treatment is effective for the enhancement of viral binding and entry. However, the level of both internalized DNA and CCC-DNA diminished gradually after the 24-hrs culture period and no viral DNA was detectable on the seventh day (data not shown).

On the basis of our data, the interaction between ASGP-R and desialylated glycoprotein or glycolipid on the surface of HBV particles appeared to be critical for the infection of HBV, at least in our experimental conditions. However, whether the interaction of these molecules is important or not for the establishment of a viral infection *in vivo* remains to be elucidated. In this sense, it was of interest that endothelial cells have desialylation activity [26,27] and the sinusoid in the liver was piled with endothelial cells [28]. In addition, it has recently been reported that duck HBV was first captured by liver sinusoidal endothelial cells and then penetrated liver parenchymal cells [40]. Thus, it is possible that liver sinusoidal endothelial cells are a source of endogenous sialidase *in vivo* and desialylated HBV particles enter liver cells through ASGP-R.

Finally, in order to carry out an HBV infection assay, the inoculation of animals such as chimpanzees or the other anthropoids with HBV [41,42], or the use of primary hepatocytes [43–45] is necessary at present. However, there are ethical problems and methodological difficulties with these assay systems. In contrast, our experimental system is a feasible and reproducible *in vitro* HBV infection assay, although no viral replication can be seen. Improvement of the sensitivity is needed in the future. In conclusion, (i) ASGP-R on HepG2 may be a specific receptor for HBV infection, and (ii) desialylation of HBV particles makes it possible to evaluate the infectivity of HBV in terms of CCC-DNA formation by enhancing the viral binding and entry.

ACKNOWLEDGEMENTS

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Hepatitis E Virus Transmission from Wild Boar Meat

Tian-Cheng Li,* Katsumi Chijiwa,† Nobuyuki Sera,† Tetsuya Ishibashi,† Yoshiki Etoh,† Yuji Shinohara,‡ Yasuo Kurata,‡ Miki Ishida,§ Shigeru Sakamoto,¶ Naokazu Takeda,* and Tatsuo Miyamura*

We investigated a case of hepatitis E acquired after persons ate wild boar meat. Genotype 3 hepatitis E virus (HEV) RNA was detected in both patient serum and wild boar meat. These findings provided direct evidence of zoonotic foodborne transmission of HEV from a wild boar to a human.

Hepatitis E virus (HEV), a causative agent of human hepatitis E, is a single-stranded positive-sense RNA virus recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (1,2). HEV is transmitted primarily by the fecal-oral route through contaminated drinking water. However, recent studies have demonstrated that various animal species have serum antibodies to HEV, suggesting that hepatitis E is a zoonotic disease (3). In Japan, 4 hepatitis E cases have been linked directly to eating raw deer meat (4), and several cases of acute hepatitis E have been epidemiologically linked to eating undercooked pork liver or wild boar meat (5,6). These cases provide convincing evidence of zoonotic food-borne HEV transmission. We report direct evidence of HEV transmission from a wild boar to a human.

The Study

A 57-year-old woman came to Iizuka Hospital on March 12, 2005, with malaise and anorexia. Although she was a healthy hepatitis B virus carrier and negative for serologic markers of hepatitis A and C, testing upon admission showed elevated levels of liver enzymes (alanine aminotranferase 752 IU/L, aspartate aminotransferase 507 IU/L, and γ-glutamyl transpeptidase 225U/L). A serum sample collected on March 16 was positive for both immunoglobulin M (IgM) and IgG antibodies to HEV when tested by an antibody enzyme-linked immunosorbent assay using recombinant viruslike particles (7). This led to the diagnosis of hepatitis E. The hepatitis was typical, acute, and self-limiting, and the patient recovered by the end of March.

The patient's husband traditionally hunted boar for food 3 or 4 times a year, and she had eaten boar meat on 2 occasions. With her husband, she ate the meat as part of a hot pot on December 28, 2004, 11 weeks before her illness, and again, grilled, on January 19, 2005, along with 10 other people (including her husband) 8 weeks before her illness. Disease did not develop in the other 10 people. Except for this wild boar meat, the patient had not eaten meat or liver from other wild animals. Since she had not traveled abroad in the past 30 years, transmission must have occurred in Japan. Two portions of meat from the wild boar (meats 1 and 2) eaten on December 28, 2004, and 1 portion from the other wild boar (meat 3) eaten on January 19, 2005, remained and were frozen.

Juice was obtained from the sliced meat by centrifugation at 10,000 × *g* for 15 min. The supernatant was used for RNA extraction. A nested reverse transcription–polymerase chain reaction (RT-PCR) was conducted to amplify part of open reading frame 2 (ORF2), which corresponds to nucleotides (nt) 5939–6297 of the genotype 1 HEV genome (GenBank D10330), with external sense primer HEV-F1 (5′-TAYCGHAAYCAAGGHTGGCG-3′) and antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-3′). A nested PCR was conducted with internal sense primer HEV-F2 (5′-GGBGTBGCNGAGGAGGAGGC-3′) and internal antisense primer HEV-R1 (5′-CGACGAAATYAATTCTGTCG-3′). This procedure allows amplification of HEV 1, 3, and 4 genotypes. A PCR product of 359 bp including the primer sequences was obtained from meat 3 by nested PCR. However, meats 1 and 2 were negative. HEV RNA was not detected in the patient's serum by the same amplification method. This may have resulted from an extremely small amount of RNA.

New primers for the nested RT-PCR were designed for a region within the 359 base region based on the meat 3 sequences, which corresponded to nt 5983–6243. The first PCR was performed with external sense primer HEV-WB-F1 (5′-ACCTCTGGCCTGGTAATGCT-3′) and antisense primer HEV-WB-R2 (5′-GAGAAGCGTATCAGCAAGGT-3′). The nested PCR was performed with internal sense primer HEV-WB-F2 (5′-TATTCATGGCTCTCCTGTCA-3′) and internal antisense primer HEV-WB-R1 (5′-ACAGTGTCAGAGTAATGCCT-3′). These primers allowed amplification of 281 nt, including the primer sequences from the patient serum collected on March 16, 2005. In contrast, meats 1 and 2 were negative with these new primers.

To further analyze the RNA in the patient serum and meat 3, RNA genomes encoding an entire ORF2 were

*National Institute of Infectious Diseases, Tokyo, Japan; †Fukuoka Institute of Health and Environmental Sciences, Fukuoka, Japan; ‡Tagawa Health, Welfare, and Environment Office, Fukuoka, Japan; §Fukuoka Prefectual Government, Fukuoka, Japan; and ¶Iizuka Hospital, Fukuoka, Japan

amplified as overlapping segments, nucleotide sequences were determined, and phylogenetic analysis was carried out with avian HEV as an outgroup. Avian HEV is a causative agent of chicken hepatitis-splenomegaly syndrome (8). Two sequences, 1 from the patient (DQ079629) and the other from meat 3 (DQ079630), were classified into genotype 3 (Figure). Only 1 nt difference was observed in the 1,980 nt of the entire ORF2; the nucleotide sequence identity was 99.95%. The difference was not accompanied by any amino acid changes. These data demonstrated that HEV infection was transmitted from the wild boar meat to the patient on January 19, 2005.

Conclusions

Currently, deer, pig, and wild boar are suspected sources of foodborne zoonotic transmission of HEV in Japan, and genotypes 3 and 4 of HEV are believed to be indigenous (4–6,9,10). Direct evidence for transmission of genotype 3 HEV from animals to humans was observed in acute hepatitis in 4 persons who had eaten uncooked deer meat that contained $\approx 10^7$ copies of HEV RNA (4). However, the rare finding of HEV antibody-positive deer in Japan suggest that deer are not the major zoonotic reservoir of HEV in this country (11). In contrast, high antibody-positive rates in domestic pig and wild boar, including HEV genotypes 3 and 4, have been frequently detected, suggesting that persons who eat uncooked meat are at risk for infection with HEV (12,13). This report is the first to provide direct evidence of zoonotic foodborne genotype 3 HEV transmission from wild boar to a human.

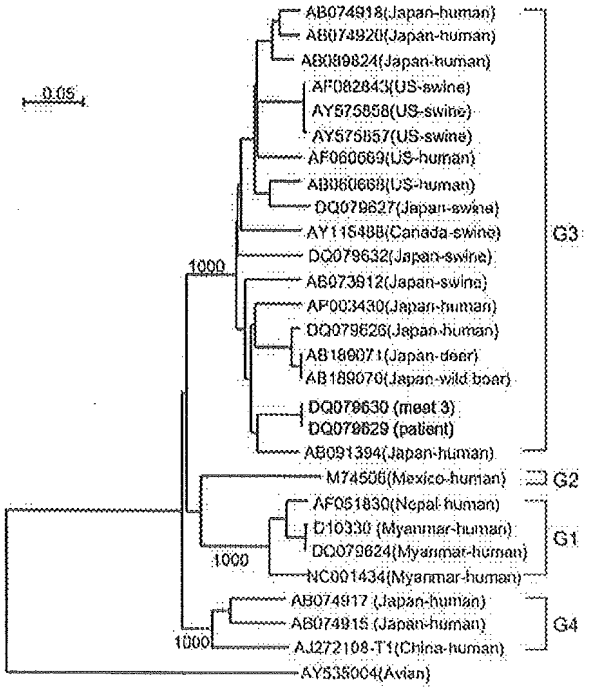

Figure. Phylogenetic tree of hepatitis E virus (HEV) reconstructed with avian HEV as an outgroup. Nucleotide sequences of the entire open reading frame 2 were analyzed by the neighbor-joining method. The bootstrap values correspond to 1,000 replications. The 2 nucleotide sequences characterized in this study are shown in **bold**. The horizontal scale bar at the top left indicates nucleotide substitutions per site.

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Dr Li is a senior researcher at National Institute of Infectious Diseases in Tokyo, Japan. His research focuses on epidemiology, expression of viral proteins, and the three-dimensional structure of hepatitis E virus.

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Address for correspondence: Tatsuo Miyamura, Department of Virology II, National Institute of Infectious Diseases. 4-7-1 Gakuen, Musashimurayama, Tokyo, 208-0011, Japan; fax: 81-42-565-4279; email: tmiyam@nih.go.jp