Molecular and serological characterization of sporadic acute hepatitis E in a Japanese patient infected with a genotype III hepatitis E virus in 1993

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Serum samples collected periodically from a 40-year-old Japanese woman who had not travelled abroad and who had contracted sporadic acute hepatitis E in 1993 were semi-quantitatively tested by enzyme immunoassay for IgM, IgA and IgG antibodies to hepatitis E virus (HEV). Anti-HEV IgM and IgA antibody levels were the highest (1 : 2400 dilution and 1 : 3400 dilution, respectively) on day 9 after the onset of hepatitis and then decreased rapidly in a parallel manner. Anti-HEV IgG antibody levels were the highest (1 : 17000 dilution) on day 145 and then decreased gradually but remained at high titres (1 : 2200 dilution) even 8-7 years after the onset of hepatitis. An HEV isolate, HE-JA10, recovered from the patient’s serum at admission was closely related to a genotype III strain isolated in the United States (US1), with 92-2% identity over the full-length genome, and was most closely related to the JMY-Haw isolate of Japanese origin (95-4% identity).

Hepatitis E virus (HEV) is a major cause of epidemic and sporadic, enterically transmitted non-A, non-B hepatitis in many developing countries in Asia, Africa and Latin America (Purcell & Emerson, 2001a). Recently, there is growing consensus that (i) HEV-associated hepatitis also occurs among individuals in industrialized countries with no history of travel to areas endemic for HEV (Harrison, 1999; Purcell & Emerson, 2001a; Schlauder & Mushahwar, 2001) and (ii) HEV is a zoonotic virus, as suggested by the close genetic relationship between human and swine viruses (Erker et al., 1999; Hsieh et al., 1999; Meng et al., 1997, 1998). The genome of HEV is a single-stranded, positive-sense RNA of approximately 7-2 kb and contains a short 5’ untranslated region (UTR), three open reading frames (ORFs 1–3) and a short 3’ UTR terminated by a poly(A) tract (Reyes et al., 1990; Tam et al., 1991). HEV sequences have been classified tentatively into four major genetic groups (genotypes I–IV) (Schlauder & Mushahwar, 2001). Worldwide, the majority of HEV infections are caused by genotype I, while only isolated cases of infection with HEV of genotype III or IV have been described in the United States, Europe, Argentina, Taiwan and China (Hsieh et al., 1999; Kwo et al., 1997; Pina et al., 2000; Schlauder et al., 1998, 1999, 2000; Wang et al., 1999, 2000, 2001; Worm et al., 2000; Zanetti et al., 1999). In Japan, multiple HEV strains of genotype III or IV have been isolated from patients with acute hepatitis of non-ABC aetiology who had never been abroad (Takahashi et al., 2001, 2002a, b; Mizuo et al., 2002), and swine HEV strains of genotype III have been isolated from farm pigs in Japan (Okamoto et al., 2001). These results indicate that heterogeneous HEV strains are circulating in Japan and that domestically infected hepatitis E occurs in Japan, where HEV infection had been considered to be non-endemic. However, the molecular and serological characteristics of sporadic acute hepatitis E in industrialized countries, including Japan, are not fully understood. Therefore, in the present study we tested serum samples that had been obtained periodically from a 40-year-old Japanese female who contracted sporadic acute hepatitis E in 1993 [patient 10 in our previous report (Mizuo et al., 2002)] for the relative titres of anti-HEV IgM, IgA and IgG antibodies and for HEV RNA; the entire genomic sequence of the HEV strain isolated from the infected patient was determined also to define its genomic characteristics.

To detect anti-HEV IgG and IgM antibodies, ELISAs were performed using purified recombinant ORF2 protein of HEV genotype IV that had been expressed in the pupae.

The entire nucleotide sequence of the HE-JA10 isolate reported herein has been assigned DDBJ/EMBL/GenBank accession no. AB089824.
of silkworm, as described previously (Mizuo et al., 2002). For the anti-HEV IgA assay, peroxidase-labelled rabbit IgG (Fab') against human IgA (Dako) was used in place of the enzyme-labelled anti-human IgG or IgM antibodies. The cut-off absorbance value (read at a wavelength of 450 nm) used for the anti-HEV IgG assay was 0·152 and that for the anti-HEV IgM assay was 0·353. The tentative cut-off absorbance value for the anti-HEV IgA assay was determined to be 0·350, according to the method described previously (Mizuo et al., 2002). The relative titres of anti-HEV IgG, IgM or IgA antibodies were determined by endpoint ELISA; i.e. the serum dilution that would give the absorbance value (measured at a wavelength of 450 nm) of each cut-off point was estimated by testing multiple dilutions of the serum.

To quantify HEV RNA, total RNA was extracted from 100 μl of serum or its dilutions, reverse-transcribed and then subjected to nested PCR with ORF2-specific primers, as described previously (Mizuo et al., 2002). The highest dilution of serum (10^5) found positive was estimated and converted to the titre ml^{-1} of serum.

To sequence the full length of the genome, a central 7 kb sequence of the HEV genome (HE-JA10) was divided into six overlapping sections and amplified by PCR. These six overlapping fragments were nt 43–1270 (1228 nt), 1238–2641 (1404 nt), 2623–3905 (1283 nt), 3899–5327 (1429 nt), 5273–6398 (1126 nt) and 6362–7145 (784 nt) (primer sequences excluded). The extreme 5' end sequence (nt 1–70) was determined by a modified RACE technique, RNA ligase-mediated RACE (RLM-RACE), using the First Choice RLM-RACE kit (Ambion). Amplification of the extreme 3' end sequence [nt 7119–7244, excluding the poly(A) tract at the 3' terminus] was attempted by RACE according to the method described previously (Okamoto et al., 2001). Amplification products were sequenced on both strands either directly or after cloning into the pT7BlueT vector (Novagen) and sequence analysis was performed as described previously (Takahashi et al., 2002b). Phylogenetic trees were constructed using the neighbour-joining method (Saitou & Nei, 1987) and final trees were obtained using the TREEVIEW program, version 1.6.6 (Page, 1996).

In September 1993, a 40-year-old woman visited an internal medicine clinic and presented with general malaise and nausea. Because she had elevated liver function tests [1019 IU alanine aminotransferase (ALT) l^{-1} serum and 590 IU aspartate aminotransferase (AST) l^{-1} serum], she was admitted to our hospital on day 6 from disease onset and followed for 281 days. The results of the initial tests were as follows: ALT, 541 IU l^{-1}; AST, 174 IU l^{-1}; total bilirubin, 2·4 mg dl^{-1}; and thymol turbidity test, 19·0 KU (normal, 0–4 KU). Liver pathology on biopsy specimens obtained on day 9 from disease onset showed typical findings of acute viral hepatitis, unaccompanied by morphological cholestasis. On admission, HEV RNA was detected at an endpoint dilution of 1:1000 and IgM, IgA and IgG classes of anti-HEV antibodies were detected, each with a relative titre of >1:2000 dilution. The titre of HEV RNA decreased rapidly and HEV viraemia continued up through day 23. The anti-HEV IgM antibody level was the highest (1:2400 dilution) on day 9 and then decreased rapidly, in parallel with anti-HEV IgA antibody levels. The relative titre of anti-HEV IgG antibodies was highest on day 145 (1:17 000 dilution) and then gradually decreased but remained at high titres (1:2200 dilution) even 8–7 years after the onset of hepatitis (Table 1).

The HE-JA10 isolate had a genomic length of 7244 nt, excluding the poly(A) tract at the 3' terminus, and possessed three major ORFs, similar to those reported for human and swine HEV isolates. ORFs 1–3 have a coding capacity of 1709 (nt 26–5152), 660 (nt 5190–7169) and 122 aa (nt 5152–5517), respectively. Comparison of the HE-JA10 genome against reported HEV genomes of genotypes I–IV, whose entire or nearly entire nucleotide sequences

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**Table 1.** Laboratory parameters and relative titres of anti-HEV antibodies and HEV RNA in serum samples obtained periodically from the patient with HEV-associated hepatitis

<table>
<thead>
<tr>
<th>Days (years) after onset</th>
<th>Total bilirubin (mg dl^{-1})</th>
<th>ALT (IU l^{-1})</th>
<th>AST (IU l^{-1})</th>
<th>Relative titre of anti-HEV assay</th>
<th>HEV RNA (PCR titre ml^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT</td>
<td>1019</td>
<td>590</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>NT</td>
<td>2·4</td>
<td>541</td>
<td>1:6 700</td>
<td>10^4</td>
</tr>
<tr>
<td>6</td>
<td>2·1</td>
<td>281</td>
<td>79</td>
<td>1:14 000</td>
<td>10^2</td>
</tr>
<tr>
<td>9</td>
<td>0·8</td>
<td>39</td>
<td>25</td>
<td>1:13 000</td>
<td>10^2</td>
</tr>
<tr>
<td>23</td>
<td>0·5</td>
<td>18</td>
<td>19</td>
<td>1:10 000</td>
<td>–</td>
</tr>
<tr>
<td>47</td>
<td>0·6</td>
<td>16</td>
<td>17</td>
<td>1:12 000</td>
<td>–</td>
</tr>
<tr>
<td>61</td>
<td>0·4</td>
<td>17</td>
<td>18</td>
<td>1:17 000</td>
<td>–</td>
</tr>
<tr>
<td>145</td>
<td>0·4</td>
<td>18</td>
<td>17</td>
<td>1:15 000</td>
<td>–</td>
</tr>
<tr>
<td>173</td>
<td>0·5</td>
<td>19</td>
<td>18</td>
<td>1:8 400</td>
<td>–</td>
</tr>
<tr>
<td>287</td>
<td>0·5</td>
<td>12</td>
<td>16</td>
<td>1:2 200</td>
<td>&lt;1:100</td>
</tr>
<tr>
<td>3186 (8·7)</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The normal ranges of total bilirubin, ALT and AST are 0·2–1·0 mg dl^{-1}, 4–34 IU l^{-1} and 9–31 IU l^{-1}, respectively. NT, Not tested.
are known (see legend to Fig. 1), revealed that HE-JA10 was most closely related to JMY-Haw, with identities of 95.4, 95.2, 96.0 and 98.4 % in the nucleotide sequence of the full genome, ORF1, ORF2 and ORF3, respectively. The HE-JA10 isolate was closely related to a genotype III isolate in the United States (US1) with 92.2% identity over the full-length genome and only 73.5–75.6 % similar to the human and swine HEV isolates of genotypes I, II and IV. The phylogenetic tree constructed based on the full genomic sequence confirmed that HE-JA10 belongs to genotype III and is most closely related to JMY-Haw (data not shown). The 5’ UTR of HE-JA10 comprised 25 nt, with a sequence beginning GCGAGCACC, similar to the 5’ UTRs of the P1 strain of genotype I, the MEX-14 strain of genotype II, the swine strain of genotype III (swJ570) and the HE-JI4 strain of genotype IV, which had been determined by RLM-RACE (Fig. 1a), the presence of a cap structure being taken into consideration (Kabrane-Lazizi et al., 1999). Therefore,
it is very likely that functional HEV genomes of all four genotypes have the extreme 5’ end sequence starting with GCAGACCCAC and that the extra nucleotides of G, AG or TCGACAGGGG at the very extreme 5’ end are not essential. The 3’ UTR of the HE-JA10 genome consisted of 72 nt and this region showed appreciable diversity, differing by 4.2–19.4 % compared with the seven HEV isolates of genotype III and by 26.4–36.8 % compared with the 18 HEV isolates of the other three genotypes whose extreme 3’ end sequences are known (Fig. 1b). In the amino acid sequences of ORFs 1–3, the HE-JA10 isolate also had highest identities with JMY-Haw at 99-1, 99-2 and 98-4 %, respectively. As illustrated in Fig. 1(c), HE-JA10 was most closely related to JMY-Haw and JKN-Sap in the amino acid sequence of the hypervariable region of the ORF1 protein, differing by only 7 aa in each case, but differing from the remaining five isolates of the same genotype in this particular region by 18–33 aa. The hypervariable region of HE-JA10 ORF1 displayed only up to 1 aa difference among the five clones obtained, in contrast with the N terminus of the E2 protein of hepatitis C virus (Hijikata et al., 1991; Weiner et al., 1991) and the V3 loop of the gp120 protein of human immunodeficiency virus type 1 (Meyerhans et al., 1989).

When the common 299 nt sequence of ORF2 of HE-JA10 was compared with those of the 35 human and 35 swine strains of genotypes I–IV (see Fig. 2 for names of strains), the HE-JA10 isolate shared a nucleotide identity of between 72-3 and 98-3 %. Of interest, HE-JA10 shared nucleotide identities of between 91-6 and 95-7 % with 27 genotype III HEV isolates obtained from pigs in different geographical regions of the United States (Huang et al., 2002). The phylogenetic tree constructed based on the partial ORF2 sequence of 299 nt confirmed that HE-JA10 belonged to genotype III and that it was segregated into a cluster consisting of four human HEV isolates of Japanese origin (JKN-Sap, JMY-Haw, HE-JA4 and HE-JA8), with a nucleotide identity of between 96-7 and 98-3 % (Fig. 2). Another cluster consisting of five human (JRA1, HE-JA5, HE-JA6, HE-JA9 and HE-JA11) and two swine (swJ570 and swJ681) HEV isolates of Japanese origin was recognized in the other branch of genotype III.

In the present study, it was found that a genotype III HEV strain was present in Japan in the early 1990s before the emergence of a novel HEV variant of genotype III (US1) in a 62-year-old white male who contracted acute hepatitis in 1995 in the United States (Kwo et al., 1997; Schlauder et al., 1998); this was the first case to be identified in an industrialized country from a patient who had no history of travel to endemic areas and who was infected with a non-Asian/African HEV strain of genotype III or IV. The infected patient in the current study also had no history of travel to areas endemic for HEV and did not report contact with persons who had travelled to endemic areas or contact with farm pigs or rodents, although there are accumulating lines of evidence that animals may act as natural hosts of HEV (Erker et al., 1999; Hsieh et al., 1999; Huang et al., 2002; Meng et al., 1997, 1998, 2002; Pina et al., 2000; Wang et al., 2002; Wu et al., 2002). The increasing globalization of food markets and increased overseas travel for business and pleasure have increased the potential of introducing HEV from not only developing countries but also industrialized countries into Japan. Japanese people have a habit of eating raw fish and other uncooked seafood, both those caught in Japan and those imported from many countries in the world, including the United States, Europe, Argentina, New Zealand and Taiwan, where HEV of genotype III is known to circulate (Garkavenko et al., 2001; Pina et al., 2000; Schlauder et al., 1999, 2000; Wang et al., 2001; Worm et al., 2000). The HE-JA10 isolate obtained from our patient was nearer to the human and swine strains isolated in the United States (US1, US2, swUS and swUS01–swUS27), with the highest identity of 95-7 % in the 299 nt sequence of ORF2, than to human or swine isolates from Taiwan and Europe, indicating that HE-JA10 may be an American-like strain. However, HE-JA10 shared a nucleotide identity of 98-3 % with HE-JA8, which was recovered in 2001 from a Japanese patient who lived in Iwate and who had never been abroad: Iwate is located in the northern part of Honshu Island and is

**Fig. 1.** (a) Comparison of the 5’-terminal sequences of HEV isolates. The 5’-terminal sequences of 21 HEV isolates whose extreme 5’ end sequences are available are aligned for comparison. The initiation codon of ORF1 is boxed. In addition to the HE-JA10 isolate obtained in the present study, four isolates whose extreme 5’ end sequence had also been determined by the RLM-RACE technique are indicated by asterisks. (b) Comparison of the 3’-terminal sequences of HEV isolates. The 3’-terminal sequences of 26 HEV isolates whose sequences preceding the poly(A) tract at the 3’ terminus are available are aligned for comparison. The termination codon of ORF2 is boxed. (c) Comparison of the amino acid sequences of the hypervariable region in the ORF1 protein of HEV isolates. The sequences of the hypervariable region of the ORF1 protein of 28 HEV isolates whose entire or nearly entire sequences are available are aligned for comparison. Genotype numbers I–IV are in accordance with the recent report by Schlauder & Mushahwar (2001) and are indicated before the slash of each isolate name. Dashes indicate nucleotides/amino acids that are identical to the top sequence, while slashes denote deletions of nucleotides/amino acids. The accession nos of the 26 isolates are as follows: AB089824 (HE-JA10 isolate); AB074920 (JMY-Haw); AB074918 (JKN-Sap); AF060668 (US1); AF060669 (US2); AF082843 (swUS); AP003430 (JRA1); AB073912 (swJ570); AF051830 (Ne1); AF076239 (I3); AF185822 (P2); AF459438 (I4); D10330 (B2); D11092 (C1); D11093 (C4); L08816 (C5); L25547 (C2); L25595 (C6); M73218 (B1); M80581 (P1); M94177 (C3); X98292 (I1); X99441 (I2); M74506 (MEX-14); AJ272108 (T1); AB074915 (JAK-Sai); AB074917 (JNN-Sap); and AB080575 (HE-JI4).
approximately 450 km from Tokyo where the ‘HE-JA10’ patient lived. In addition, HE-JA10 shared between 96·1 and 98·3 % identity in the 412 nt sequence of ORF2 with the four recently reported Japanese HEV isolates of genotype III (HE-JA4, HE-JA8, JMY-Haw and JKN-Sap) (Takahashi et al., 2002a; Mizuo et al., 2002), suggesting that it is likely that genotype III HEV isolates had already been domestic in the early 1990s and were widespread in the past in Japan;

Fig. 2. Phylogenetic tree constructed using the neighbour-joining method based on the partial nucleotide sequence (299 nt; nt 6039–6337 of the HE-JA10 genome) of the ORF2 region of 71 human and swine HEV isolates. In addition to the 27 reported human and swine HEV isolates of genotypes I–IV whose entire or nearly entire sequences are known, 43 reported isolates of genotype III (accession nos are indicated in parentheses) whose partial sequences of 300, 301, 304, 412, 421 or 436 nt have been determined, as well as the HE-JA10 isolate obtained in the present study (boxed), were included for comparison. For simplicity, the abbreviated names (swUS01–swUS27) are used for the 27 swine strains (isolated in the United States) reported by Huang et al. (2002). All human and swine HEV strains isolated in Japan are indicated by bold typeface for visual clarity. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings (Felsenstein, 1985).
however, we cannot rule out the possibility of an outside source for genotype III strains, since the HE-JA7 and HE-JI3 strains were interspersed among American swine and human strains in the phylogenetic tree based on the partial ORF2 sequences (Fig. 2).

Anti-HEV IgM and IgA antibodies were detectable in our patient for more than 6 months after the onset of disease. Furthermore, anti-HEV IgG antibodies continued to be detected and remained at high levels for more than 8 years after the onset of disease. Khuroo et al. (1993) reported that anti-HEV IgG antibodies were detected in 47 % of patients with HEV infection after 14 years. However, as Krawczynski & Aggarwal (1999) pointed out, it might be difficult to determine the exact duration of persistence of anti-HEV IgG antibodies in endemic areas because of repeated exposure to HEV. On the other hand, in Japan, a country with low endemicity and where clinical HEV infection rarely occurs, there seems to be little or no repeated exposure. The chance of repeated exposure may be negligible in our patient, as supported further by the fact that the patient’s husband who lives with the patient was negative for anti-HEV IgG antibodies both in 1993 and 2002. Therefore, we speculate that anti-HEV IgG antibodies persist for more than 10 years after the onset of sporadic acute hepatitis E in industrialized countries with low endemicity as well. Regarding the duration of seropositivity for anti-HEV IgM, it has been reported that, of sera collected from patients during various hepatitis E outbreaks 3–4 and 6–12 months after the onset of jaundice, 50 and 40 %, respectively, were positive for anti-HEV IgM antibodies (Favorov et al., 1996). Our patient continued to be positive for anti-HEV IgM antibodies for more than 9 months during the convalescent phase. Taken together, seropositivity for anti-HEV IgM antibodies can be regarded as the marker of choice as a diagnostic indicator of recent HEV infection in both developing and industrialized countries. As for anti-HEV IgA antibodies, it is unclear whether our assay is detecting both dimeric secretory IgA and monomeric IgA. However, it seems that anti-HEV IgA antibodies can be utilized as an additional confirmatory antibody for recent HEV infection, as suggested by Chau et al. (1993) who detected anti-HEV IgA antibodies in serum samples obtained from patients who had acute waterborne hepatitis in southern Somalia and Pakistan.

In conclusion, the finding that genotype III HEV was present in Japan in the early 1990s raises the questions of when variant HEV strains emerged and how widespread they were in industrialized countries. The increasing globalization of the world marketplace and increased overseas travel may facilitate the spread of HEV variants into industrialized countries that were believed to be non-endemic. The reported high prevalence of anti-HEV antibodies in a number of animal species, such as pigs, rats and mice, may suggest that multiple sources of exposure to HEV may exist in the general population in industrialized countries that are not at apparent risk for exposure to HEV (Purcell & Emerson, 2001b). Whether the domestic spread of HEV infection in industrialized countries is via zoonosis and/or food deserves further analysis.

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