Acute hepatitis caused by a novel strain of hepatitis E virus most closely related to United States strains

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A unique hepatitis E virus (HEV) strain was identified as the aetiological agent of acute hepatitis in a United States (US) patient who had recently returned from vacation in Thailand, a country in which HEV is endemic. Sequence comparison showed that this HEV strain was most similar, but not identical, to the swine and human HEV strains recovered in the US. Phylogenetic analysis revealed that this new HEV isolate was closer to genotype 3 strains than to the genotype 1 strains common in Asia. The fact that this HEV was closely related to strains recovered in countries where HEV is not endemic and was highly divergent from Asian HEV strains raises the questions of where the patient’s infection was acquired and of whether strains are geographically as localized as once thought.

Introduction

Hepatitis E virus (HEV) is a widespread, enterically transmitted agent that is a serious public health problem in many countries of Asia and Africa. HEV is responsible for epidemic and sporadic hepatitis and is often spread by faecal contamination of drinking water (Purcell, 1996; Balayan, 1997). Furthermore in these countries, antibodies to HEV have been detected in animals such as pigs (Balayan et al., 1990; Clayson et al., 1995), sheep (Usmanov et al., 1994), monkeys (Arankalle et al., 1994) and rats (Karetnyi et al., 1993). Therefore, such animals might be a reservoir of HEV in developing countries.

In Western Europe and the United States (US), clinical cases of hepatitis caused by HEV are rare and most often they have been associated with travel to areas where HEV is endemic. However, novel strains of HEV have been isolated in the US (Schlauder et al., 1998) and in Europe (Schlauder et al., 1999; Zanetti et al., 1999) from patients without a history of travel to regions endemic for HEV. Serological studies in industrialized countries have shown that the prevalence of anti-HEV antibodies is 1–6% among blood donors (Paul et al., 1994; Mast et al., 1997) and is much higher in some populations (Zaaier et al., 1995; Thomas et al., 1997). The cause of this relatively high prevalence of anti-HEV in countries where clinical hepatitis E is rare is unknown. The discovery of a new strain of HEV circulating among swine in the US (Meng et al., 1997) and the detection of anti-HEV antibodies in rodents in the US (Kabrane-Lazizi et al., 1999; Favorov et al., 2000) raise the possibility that an animal reservoir of HEV may exist even in developed countries.

HEV is a small, non-enveloped virus that has a positive-sense, single-stranded RNA genome of approximately 7.5 kb. The genome contains three open reading frames (ORFs): ORF1 encodes the non-structural proteins, ORF2 encodes the capsid protein and ORF3 encodes a protein of unknown function (Reyes et al., 1993). The sequences of ORF2 and ORF3 are well conserved among HEV isolates whereas one region of ORF1 (the hypervariable region) displays significant genetic diversity (Tsarev et al., 1992; Huang et al., 1995). Four genotypes of HEV have been identified, based on comparisons of nearly full-length genomic sequences. In general, different genotypes circulate within different geographical areas. Genotype 1 comprises Southeast and Central Asian isolates from Burma, Pakistan, India and China; genotype 2 comprises a single Mexican isolate; genotype 3 comprises US isolates (two human, one swine); and genotype 4 comprises recently described Chinese isolates (Wang et al., 1999, 2000).

In the present study, we identified the aetiological agent of acute hepatitis in a US patient who had recently returned from a vacation in Thailand. We transmitted the HEV strain from the patient to a monkey and demonstrated that the genome
represented a unique HEV strain related to, but not identical to, US isolates.

Methods

■ Patient history. An HIV-positive patient (W) developed acute hepatitis 6 weeks after returning to the US from a 2 week vacation with three companions in Thailand. One week before leaving the US, he received one dose of killed hepatitis A virus (HAV) vaccine (HAVRIX, SmithKline Beecham). Serum samples were collected from patient W 2 months before he left for Thailand and during the acute phase of the hepatitis.

■ Animal transmission. A rhesus monkey (Macaca mulatta) was inoculated intravenously with 0.2 ml of the first acute phase serum collected from patient W. Serum samples were collected weekly from the monkey for 12 weeks post-inoculation (p.i.) and tested for anti-HEV by ELISA and for levels of serum liver enzymes by standard methods. Faecal samples were collected daily for HEV RNA detection by RT–PCR.

■ Serological tests. Serology for hepatitis A (IgG and IgM anti-HAV), for hepatitis B (HBsAg, IgG and IgM anti-HBc) and for hepatitis C (anti-HCV) were performed using commercial ELISAs (Abbott Laboratories). The detection of antibody to HAV non-structural protein (3C protease) was performed as described previously (Stewart et al., 1992). The detection of anti-HEV antibodies (IgG and IgM) in patient and monkey sera was performed with an in-house ELISA using as antigen a truncated (55 kDa) recombinant HEV capsid protein expressed from baculovirus and purified from SF-9 insect cells (Robinson et al., 1998; Tsarev et al., 1999).

■ RT–PCR. HEV RNA was extracted from 50 µl of patient or monkey serum with TRIzol reagent (Gibco-BRL) or from 100 µl of a 10% suspension of faeces with a QIAamp viral RNA kit (Qiagen). Reverse transcription was performed with random hexamers and a GeneAmp RT–PCR kit according to the manufacturer’s protocol (Perkin Elmer). Detection and titration of viral RNA by RT–PCR were performed with degenerate primers described for amplification of a conserved region in ORF1 and in ORF2 (Wang et al., 2000). These primers were also used to amplify the ORF1 and ORF2 regions for sequencing. Additional primers were designed to amplify three other regions of the genome: these were HVR external antisense 5′CGGGAGCA-GTCTTCCCGGTASGCWGCCTC (nt 2671–2649); HVR internal sense 5′GGTAATAARACCTTCMGSACGWCGKTK (nt 2684–2656); HVR internal antisense 5′AGTCTCCCGGTASGCWGCCTCAAGCCTC (nt 2671–2649); ORF2 internal sense 5′GGCATGATGAAAAC-CCGTGT-CCCAGCGSCWTTCGCTGACCGG (nt 1985–2006); ORF1, 2, 3 internal sense 5′CCCGGTASGCWCGCTCAACGCTC (nt 2671–2649); ORF1, 2, 3 internal antisense 5′CCGGTGTGAAACCGGCGSCWCGKTK (nt 4951–4971); ORF1, 2, 3 external antisense 5′CGAGGTGTGACAAATGTGCCG (nt 4951–4971); ORF1, 2, 3 external antisense 5′CGGGTTGTGAAAACGCCAATGGCGAGC (nt 5381–5362); ORF1, 2, 3 internal sense 5′CCGGCGSCWCGKTK (nt 4951–4971); ORF1, 2, 3 internal antisense 5′CCGGTTGTGAAAACGCCAATGGCGAGC (nt 5381–5362); ORF1, 2, 3 internal antisense 5′CGGAGAATCAACCCTGTCACCCC (nt 5316–5294); ORF2 external sense 5′TGAGAATCAACCCTGTCACCCC (nt 5316–5294); ORF2 external sense oligo(dT)12–18; ORF2 internal sense 5′TTGGTCT-CGGCCAATGGCGGAC (nt 6371–6392); ORF2 internal antisense 5′TTTTTTTTTTTCCAGGGAGCGCGG. Primer positions are numbered according to the sequences published in the GenBank database.

■ Genome characterization. RT–PCR was performed on HEV RNA extracted from patient W serum (conserved region of ORF1 and ORF2) and from a monkey faecal sample (remaining three regions) collected 2 days before the experimentally infected monkey seroconverted to anti-HEV. The cDNAs were synthesized with Superscript II reverse transcriptase (Gibco-BRL).

The PCR conditions used to amplify the different regions of the genome varied. A 100 µl PCR reaction was performed with 10 µl of cDNA or amplification product for first and second round respectively and 50 pmol of each external or internal primer, 2 mM MgCl₂ and 5 U Taq polymerase. For amplification of GC-rich regions, first and second round PCR were carried out in a 50 µl reaction using an Advantage-GC cDNA PCR kit (Clontech). All the thermal procedures were performed in a GeneAmp PCR system (Perkin Elmer).

■ Sequence and phylogenetic analysis. The PCR fragments were purified with a QIAquick gel extraction kit (Qiagen) and cloned with a TA cloning kit (Invitrogen). Both strands were sequenced with an automated DNA sequencer. Nucleotide and amino acid sequences were analysed with the Sequencher 3.0 software (Gene Codes Corp.). Strains representing the four genotypes were compared with GAP (GCG version 9.0, Genetics Computer Group, Madison, WI, USA).

The nucleotide sequences were aligned using PILEUP (GCC) and analysed with PAUPSEARCH and PAUPDISPLAY from the PAUP computer software (4.0) to generate phylogenetic trees. Bootstrap 50% majority-rule consensus trees (midpoint rooting) were obtained by performing heuristic search (optimality criterion, maximum parsimony: all characters equal weight: 1000 replicates). The consensus trees were viewed using the TREEVIEW program.

GenBank numbers: Pakistan (SAR-55), accession no. M80581 (Tsarev et al., 1992); Burma (B1 and B2), accession nos M73218 and D10330 (Tam et al., 1991); Mexico M1, accession no. M74506 (Huang et al., 1992); US1 and US2, accession nos AF066668 and AF066669 respectively (Schlaufer et al., 1998); US swine, accession no. AF082843 (Meng et al., 1998); Italian (It1), accession nos AF10387, AF110309 respectively (Schlauder et al., 1999); Greek, (G1 and G2), accession nos AI10388, AI110391 and AI10389, AI110392 (Schlauder et al., 1999); New Chinese, accession no. AJ272108 (Wang et al., 2000); New Zealand swine (NZ swine), accession nos AF215661, AF200704 (Garbavene et al., 2000).

Results

Patient study

Although the patient did not have symptoms of hepatitis, his serum levels of liver enzymes were elevated [alanine aminotransferase (ALT) 1500, aspartate aminotransferase (AST) 500], indicating that he probably had acute hepatitis. Commercial serological tests for hepatitis B and C were negative. The acute phase serum was positive for antibody to HAV nonstructural protein, 3C proteinase. The test for antibody to HAV virus (anti-HAV), but negative for anti-HAV IgM. Since the patient had received an inactivated HAV vaccine just prior to his trip, it was necessary to determine if the anti-HAV was in response to the vaccination. Current diagnostic commercial assays for HAV detect antibodies only to structural proteins. In natural HAV infection, antibodies against both structural and nonstructural proteins are induced whereas vaccination with inactivated vaccine induces antibodies only to structural proteins (Stapleton et al., 1995). To determine if the anti-HAV antibody was induced by active HAV infection or by vaccination, we performed in-house ELISA for antibody to the HAV nonstructural protein, 3C proteinase. The test for antibody to 3C proteinase was negative, suggesting that the antibodies detected by the commercial HAV test were induced by the recent vaccination.

The serum collected prior to visiting Thailand was negative...
Table 1. Nucleotide and deduced amino acid sequence identity of selected HEV strains and the W isolate

<table>
<thead>
<tr>
<th>HEV strain</th>
<th>Genotype</th>
<th>W strain</th>
<th>ORF1* 221 nt</th>
<th>ORF2* 97 nt</th>
<th>ORF2–3 † end 798 nt</th>
<th>ORF1, 2, 3 § 246 nt</th>
</tr>
</thead>
<tbody>
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<td>US1</td>
<td>3</td>
<td></td>
<td>86 (96)†</td>
<td>85 (94)</td>
<td>87 (84)</td>
<td>86</td>
</tr>
<tr>
<td>Swine (US)</td>
<td>3</td>
<td></td>
<td>85 (93)</td>
<td>90 (94)</td>
<td>87 (85)</td>
<td>89</td>
</tr>
<tr>
<td>Swine (NZ)</td>
<td>?‡</td>
<td></td>
<td>87 (95)</td>
<td>(85) (90)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Italian (It1)</td>
<td>?‡</td>
<td></td>
<td>81 (92)</td>
<td>80 (94)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Greek (Gr1)</td>
<td>?‡</td>
<td></td>
<td>78 (95)</td>
<td>81 (94)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mexico (M1)</td>
<td>2</td>
<td></td>
<td>72 (89)</td>
<td>76 (90)</td>
<td>74 (79)</td>
<td>77</td>
</tr>
<tr>
<td>Burma (B1)</td>
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<td></td>
<td>71 (86)</td>
<td>82 (90)</td>
<td>78 (82)</td>
<td>78</td>
</tr>
<tr>
<td>Pakistan (SAR-55)</td>
<td>1</td>
<td></td>
<td>70 (85)</td>
<td>84 (90)</td>
<td>77 (81)</td>
<td>78</td>
</tr>
<tr>
<td>New Chinese (S15)</td>
<td>4</td>
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<td>73 (90)</td>
<td>72 (87)</td>
<td>78 (81)</td>
<td>77</td>
</tr>
</tbody>
</table>

* Conserved regions.
† Percentage identity of nucleotide or amino acid (parentheses) sequences.
‡ Sequence insufficient to genotype.
§ Amino acids not compared because of reading frame overlap.
NA, Not available.

for anti-HEV, but the two serum samples collected 1 week apart during the acute phase were both strongly positive for IgG and IgM anti-HEV antibodies. HEV RNA was also detected by RT–PCR, indicating that the acute hepatitis in patient W was caused by HEV. The first acute phase serum was positive for HEV sequences at dilutions of $10^{-5}$ and $10^{-7}$ respectively for ORF1 and ORF2. An aliquot of this serum specimen was inoculated into a rhesus monkey. We also tested sera from two of the three travelling companions 10 months after they returned from Thailand. They were negative for anti-HEV.

**Transmission study**

ALT values were elevated in the inoculated monkey on day 7, 14 and 42 p.i with the peak value (188 IU/l) on day 42 p.i. The monkey seroconverted to anti-HEV IgG and anti-HEV IgM 3 weeks after inoculation (day 21). HEV RNA was detected by RT–PCR in serum collected 1 week (day 14) before seroconversion and remained detectable for 3 weeks. The six faecal specimens collected near the time of seroconversion (between days 18 and 30 p.i) were all positive for HEV sequences by RT–PCR: RNA from the faecal specimen collected 2 days before seroconversion was further characterized.

**Sequence analysis of conserved regions in ORF1 and ORF2**

The PCR products corresponding to the most conserved regions in ORF1 and in ORF2, respectively, were sequenced since these regions have been frequently used for the identification of new strains.

The comparison of the ORF1 nucleotide sequence amplified from patient serum indicated that the HEV strain recovered from patient W was most closely related to swine and human strains recovered in the US (85–86% identity) and to the swine strain recovered in New Zealand (87% identity) (Table 1). The sequence identities between the W isolate and the European HEV strains were lower (78–81%). The nucleotide sequence of the W isolate was significantly divergent from those of the Asian, New Chinese and Mexican isolates (70–73% identity). The amino acid sequence comparisons also showed more identity between the W isolate and HEV strains from the US and European groups (92–96%), compared to that observed with the Asian group (85–86%) or with the Mexican isolate (89%). However, the W isolate also had high amino acid identities with NZ swine (95%) and the New Chinese isolate (90%).

In ORF2, the nucleotide identity between the W isolate and US strains (85–90%) was also higher than that observed with the Asian or European HEV strains (80–84%) or with the Mexican strain (78%). The lowest identity (72%) in this case was with the New Chinese strain. The W isolate shared 94% amino acid identity with the US and European groups across this region and approximately 90% identity with all other strains examined except for the New Chinese (87% identity).

We confirmed these data by analysing the sequence of the same regions amplified from stools collected from the infected monkey.

**Sequence analysis of the 3’ portion of the genome**

Approximately 800 bp of the 3’ terminus of ORF2 was amplified from the monkey faecal sample. The HEV strain from
patient W shared approximately 87% nucleotide identity with the US strains (swine and human) within this region and only 74–78% with the Mexican, Asian and New Chinese strains (Table 1). The amino acid sequence comparisons also showed more identity between the patient W strain and the US strains, 84–85% versus 81–82% with the Asian strains and 79% with the Mexican strain.

We also amplified about 250 nt encompassing a portion of ORF1 and the region of ORF2/ORF3 overlap, and compared its sequence with the sequences available for other HEV strains. The nucleotide identities found with swine and human US strains (86–89%) were 9–12% higher than with the genotype 1, 2 or 4 strains (Table 1). As in the US isolates, the W strain had a deletion of 3 nt just downstream of the ORF3 initiation codon (data not shown). The amino acid sequences within this region were not compared because all three reading frames were utilized.

Sequence analysis of the hypervariable region in ORF1

Because the HVR in ORF1 (between nt 2011 and 2325) distinguishes between very closely related strains (Tsarev et al., 1992), we amplified and analysed a fragment of 555 nt in this region. The estimated nucleotide identity between the homologous regions of the W strain and the US strains (US1 and swine) or the Pakistani, Burmese, Mexican and genotype 4 isolates was approximately 82% and 59–61%, respectively (data not shown). We also compared a portion of the HVR sequence of strain W to that of the swine and US1 strains and to a consensus sequence of 17 strains (Fig. 1). The HVR of US human and swine strains has 42–45 nt more than do all the Asian strains and 33–36 nt more than does the Mexican strain (Meng et al., 1998). The W strain also had additional nucleotides in this region but it had only 30 extra nucleotides compared to the Asian strains since it did not have the poly(C) tract characteristic of the US strains (Fig. 1). The New Chinese strain had a similar number of additional nucleotides as did the US strain in this region but lacked the poly(C) tract (data not shown). Because of the high level of diversity and the difference in lengths of the HVR, the exact positions of the 30 extra nucleotides could not be defined relative to the other strains.

Phylogenetic analysis

Phylogenetic analyses of nucleotide sequences were performed to determine more precisely the relationship between the W isolate and other HEV strains. The sequences of the conserved regions of ORF1 and of ORF2 were compared with those of the homologous regions from 18 human and swine isolates. Phylogenetic trees based on alignment of the ORF1 sequence suggested that the W isolate was closer to the NZ swine, US and European HEV strains than to the Asian strains of genotype 1, which were all located at the other end of the tree (Fig. 2A). The New Chinese isolate of genotype 4 was more closely related to the W strain than were the other strains isolated in Asia. A similar tree generated for the ORF2 fragment indicated that all of the US, European and NZ strains now grouped more closely with the W isolate and the Asian strains were closer to the W strain than was either the Chinese genotype 4 isolate or the Mexican genotype 2 strain (Fig. 2B).

The topology of the tree obtained with either the 3'-
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Fig. 2. Phylogenetic trees generated from (A) 221 nt of the conserved region in ORF1, (B) 97 nt of the conserved region in ORF2, (C) 798 nt at the 3' end of ORF2, and (D) 246 nt of the ORF1, 2, 3 overlap region. Branch lengths are proportional to genetic distance. Bootstrap values for the different branches are shown as percentages of trees obtained from 1000 replicates of the data.

Terminal sequence of ORF2 (Fig. 2C) or the overlap region (Fig. 2D) was similar to that obtained with ORF1 sequence in that the W isolate was more closely related to the US strains than to the Asian or Mexican strains. In both cases, the Chinese genotype 4 isolate again formed a distinct branch and was less distant than were the Asian strains (Fig. 2C, D).
Discussion

A novel isolate of HEV was identified in a US patient (W) who had travelled in Thailand. Since HEV is endemic in Thailand, it was thought that the patient probably was infected with HEV in that country. However, the incubation period until onset of disease varies between approximately 4 and 6 weeks so the patient could have been infected either in Thailand or following his return to the US. Nucleotide sequence comparisons from four different regions of the genome all suggested that the HEV strain recovered from patient W was phylogenetically distinct from the genotype 1 or 4 HEV strains commonly isolated in Asia and that it was more closely related to strains of US genotype 3 (Fig. 2 and Table 1). The ORF1 sequence amplified from serum of patient W shared the highest degree of amino acid identity with the US1 isolate, followed closely by the NZ swine HEV and Greek isolates (Table 1). The NZ swine and Greek sequences were not available for the ORF2–3' region but this region showed higher amino acid identity with the US strains than with the Mexican, Burmese, Pakistani or New Chinese strains. The amino acid sequence analysis of the conserved region in ORF2 showed less distinction among the isolates, probably because the amplified sequence compared in ORF2 was only 97 nt and was from a highly conserved region. Thus, in all three genomic regions compared, the amino acid sequence of the W isolate was most closely related to that of strains recovered from areas where HEV is not endemic in the human population.

To define further the relationship between the W isolate and US isolates, we sequenced and analysed the HVR in ORF1. Very low nucleotide or amino acid identities within the HVR have been observed between geographically distant HEV strains. For instance, the US isolates are approximately 85% identical to each other but only 42% identical to the Pakistani strain, 39% identical to the Burmese strain and 32% identical to the Mexican strain in this region (Erker et al., 1999). Furthermore, within this region, US isolates from humans and from swine contain many nucleotide insertions and a unique poly(C) tract, when compared to other strains. The W isolate was similar to the US strains in having many insertions but differed from them by lacking this characteristic poly(C) tract: otherwise the HVR nucleotide sequences were quite similar with an identity of 82% (data not shown). The W strain additionally had a 3 nt deletion in ORF3 as do US strains but not Asian strains (Meng et al., 1998; Erker et al., 1999). Comparison of the HVR of strain W with those of the Pakistani, the Burmese and the New Chinese strains showed lower nucleotide identities (59–61%), consistent with the results of nucleotide comparisons based on conserved regions of ORF1 and ORF2. Therefore, the W strain shared some unique features and considerable homology with the US strains. However, its genotype cannot be assigned until more sequence, preferably that of the entire genome, is obtained.

Originally, all HEV strains from Southeast and Central Asia were classified as genotype 1 but recently another group of viruses from China was found to constitute a new genotype, genotype 4. The only strain isolated from Mexico is of genotype 2 although a new Nigerian strain is more closely related to the Mexican strain than to other HEV strains (Buisson et al., 2000). All these strains were isolated in areas where hepatitis E is endemic or epidemic. In contrast, the viruses isolated in the US, where hepatitis E is rare, form another branch of the phylogenetic tree and constitute genotype 3.

Additional isolates of HEV are being identified at a rapid rate. North African HEV isolates cluster with the Asian strains and constitute what appears to be a separate subtype within genotype 1 (Tsarev et al., 1999). Unique isolates from other countries have been assigned by their discoverers to genotype 4 (Hsieh et al., 1998) or to new genotypes 5–8 (Schlauder et al., 1999, 2000). However, only very limited sequence data (< 400 nucleotides) are available for these latter strains and, as shown by the data in Fig. 2 and Table 1, different degrees of relatedness are predicted when different regions are compared. For instance, the New Chinese strain was closer or more distant to the W strain than were the Asian strains depending on the region. Therefore, until more sequence data are obtained and a consensus is reached on what defines a genotype, these strains, as well as the W strain, are best considered as unclassified.

The fact that the new HEV isolate described in this study was more closely related to the US and European strains than to the Asian strains raises the questions of the origin of this infection in the patient and of the distribution of HEV strains worldwide. Indeed, the report of an isolation of a US/European-like HEV from swine in NZ suggests that the apparent localization of a particular genotype to a defined geographical region may not be as stringent as previously thought. Thus, the true geographical origin of the patient W HEV isolate remains obscure.

References


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