Identification of a novel variant of hepatitis E virus in Austria: sequence, phylogenetic and serological analysis

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We isolated a novel hepatitis E virus (HEV-Au1) variant from a patient in Austria suffering from acute viral hepatitis, who had no known risk factors for acquiring hepatitis E. The clinical presentation and initial serological findings have been reported previously. In this paper we report the results of sequence and phylogenetic analysis of HEV products from viral RNA isolated from acute phase serum. The results show that HEV-Au1 is significantly divergent from other HEV isolates. The nucleotide identity of analysed fragments from the novel isolate ranges from 76.6 to 78.4% when compared to isolates from endemic regions and 84.6 to 87.9% when compared to isolates from non-endemic regions. Divergent results were obtained when serum samples taken from the convalescent phase of disease were tested with three different immunoassays (EIAs). An EIA based on United States isolate-specific peptides showed enhanced reactivity whereas EIAs based on recombinant proteins derived from prototype HEV strains from Burma and Mexico were unable to detect antibodies to HEV (anti-HEV) in late phase serum. The findings verify the presence of an additional HEV variant in an industrialized country and provide information about possible problems in detecting anti-HEV.

Introduction

Hepatitis E, once thought to be a disease confined to endemic foci in the Indian subcontinent, is now recognized as the major cause of enterically transmitted non-A, non-B hepatitis in many developing countries worldwide, where it occurs both in sporadic and in epidemic forms. In these areas epidemics and spread of hepatitis E virus (HEV), the aetiological agent of hepatitis E, are predominantly caused by contaminated water due to inadequate sanitation practices.

HEV is a non-enveloped virus, approximately 27–30 nm in diameter, with a positive-sense, single-stranded RNA genome of about 7–2 kb (Reyes et al., 1990; Tam et al., 1991). The viral genome consists of three discontinuous, partially overlapping open reading frames (ORFs) encoding three independent polyproteins. For diagnostic purposes two immunodominant epitopes have been identified by immuno-screening HEV clones from expression libraries: one of these epitopes, known as ‘3-2’, mapped to the carboxyl end of ORF 2 and the other, ‘4-2’, mapped to the carboxyl end of ORF 3 (Yarbough et al., 1991). Enzyme linked immunosorbent assays (ELISA) are the preferred screening format. ELISAs are commonly based on recombinant proteins derived from two prototype strains of HEV which were cloned from isolates from outbreaks in Burma and Mexico.

In industrialized countries that do not border on HEV endemic areas, HEV-associated outbreaks have not been reported. Occasionally, sporadic cases of hepatitis E can be observed in travellers returning from HEV endemic regions, but hepatitis E is commonly assumed to play a minor role in cases of acute non-A, non-B, non-C hepatitis in patients without history of travel. Therefore, industrialized countries are to be considered as HEV non-endemic areas.

Recently, some cases of acute hepatitis infection associated with HEV seroreactivity in patients without history of travel or other risk factors from the United States (US) and Europe have been shown to be due to infection with a number of novel variants of HEV that were significantly divergent from previously described isolates of HEV (Schlauder et al., 1998,
1999a; Zanetti et al., 1999). We recently reported on the clinical findings for a patient from Graz, Austria, with an acute hepatitis E infection (Worm et al., 1998). This patient had reported no recent travel to regions where HEV is considered endemic. Here we present molecular evidence that the infection was associated with an HEV isolate distinct from all other published sequences of HEV.

**Methods**

**Diagnosis.** Serum samples obtained during the acute and convalescent phases from a patient who exhibited hepatitis E-associated hepatitis in Austria had been collected and stored at −20 °C for subsequent testing. The clinical presentation of the patient was reported previously (Worm et al., 1998). In a near-2 year follow-up the patient remained healthy after recovery from acute hepatitis.

The diagnosis of acute hepatitis E was primarily based on positive test results for IgG and IgM class antibodies to HEV using a commercially available immunoassay (ELISA). In follow-up controls, anti-HEV IgM and IgG class antibodies declined rapidly (Worm et al., 1998).

We analysed acute phase serum by RT–PCR to amplify HEV RNA. Nucleotide sequences of amplified HEV PCR products were compiled and compared to published nucleotide sequences of HEV isolates. Follow-up serum samples obtained after 3 and 7 weeks as well as 6 and 9 months after onset of disease were tested for anti-HEV with three different ELISAs.

**RT–PCR.** Sequences were identified using RNA extracted from 50 µl of serum with the Ultraspec RNA Isolation System (Biotexc) as described by the manufacturer.

RT–PCR was performed using the GeneAmp RNA PCR kit essentially according to the manufacturer’s instructions (Perkin Elmer) as previously described (Schlauder et al., 1999a) with ORF 1 and ORF 2 degenerate PCR primers designed to match regions of identity between Burmese, Mexican and US isolates.

**Sequence analysis.** PCR products of the appropriate size were sequenced as previously described (Schlauder et al., 1999a). Phylogenetic analyses were done with the PHYLIP package, version 3.5c (Felsenstein, 1993). Distance matrices from nucleotide sequence alignments were determined with the DNADIST program utilizing the Kimura 2-parameter model. Phylogenetic trees were generated with FITCH. The robustness of the trees was determined by bootstrap resampling of the multiple-sequence alignments (1000 sets) with the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE. Bootstrap values of greater than 70% are regarded as providing evidence for a phylogenetic grouping. The final trees were produced using RETREE (PHYLIP) with the midpoint rooting option and the graphical output was created with TREVIEW (Page, 1996).

Abbreviations used for the various HEV isolates and the GenBank accession numbers or referenced sequences utilized in the analyses are given below. Full-length HEV sequences are: Burmese isolates, B1 (M73218), B2 (D10330); Chinese isolates, C1 (D11092), C2 (L25547), C3 (M94177), C4 (D11093), CT1 (A272108); Indian isolates, I1 (X98292), I2 (X99441); Pakistani isolate, P1 (M60581); Mexican isolate, M1 (M74506); United States isolates, U1 (AF006968), U2 (AF006969); and swine isolate, S1 (AF011921). Partial HEV sequences (ORF 1/2) are: Italian isolate, I1 (AF103387/A110390); Greek isolates, G1 (AF110388/AF110391), G2 (AF10389/A110392); Austrian isolate, Au1 (AF279122/AF279123).

The largest region from ORF 1 available for comparison for these sequences is the 371 base region spanning nucleotides 80 to 450, based on the original Burmese isolate 1 numbering (Tam et al., 1991). The largest region from ORF 2 available for the analysis spans a region of 148 bases between nucleotide positions 6322 and 6469.

**ELISAs.** Two commercial anti-HEV assays (HEV EIAs) are utilized for routine diagnostic work-up in most laboratories as well as in published studies on hepatitis E (e.g., seroepidemiological studies). The first assay (test system 1) employs four recombinant proteins from a Burmese (B) and a Mexican (M) strain (B3-2, M3-2 comprising the 42 C-terminal amino acids encoded by ORF2; B4-2, M4-2 comprising the 33 C-terminal amino acids encoded by ORF3). The second assay (test system 2) employs two recombinant proteins from a Burmese strain (SG-3 comprising the C-terminal 327 amino acids encoded by ORF2; 8-5 comprising the full-length 123 amino acid protein encoded by ORF3).

In addition a third, strain-specific, ELISA test system utilizing synthetic peptides based on the US strains of HEV (Schlauder et al., 1998) was used to detect anti-HEV in the convalescent phase of the disease. These peptides, which represent epitopes that are important for detecting antibodies to HEV, are also located at the C termini of the ORF3 and ORF2 proteins and are designated 4-2 and 3-2e, respectively.

**Results**

Evidence for a unique HEV isolate (HEV-Graz, Au1) in Europe comes from the identification and analysis of nucleotide sequences of ORF 1 and ORF 2 amplified from an Austrian patient. Comparisons of these sequences with other HEV isolates for which sequence data over this range are available are shown in Table 1. Au1 exhibits 81–1−87–9% nucleotide identity for ORF1 and 83–1−89–2% identity for ORF2 compared to the variants isolated from non-endemic regions such as the US and Europe. In contrast, the identities of the Austrian isolate to the group 1, group 2 or group 4 isolates represented by the Burmese-type isolates, the Mexican isolate and the Chinese isolate, CT1 (Wang et al., 2000), range between 76–6% and 78–4%. The Austrian isolate appears to be more related to the isolates from non-endemic regions. However, the diversity in ORF1 between the Austrian isolate and the most related isolate, U1, is 12–1%. This is greater than the diversity between the most diverse group 1 isolates, comparing sequence data for this region. The uniqueness of this Austrian isolate is also apparent in the phylogenetic analysis of the region from ORF1 presented as a phylogenetic tree in Fig. 1.

Au1 represents a branch that is distinct from the US group of isolates as well as the other European isolates (I1, G1, G2). Therefore, Au1 potentially represents an additional member in a recently proposed numerical nomenclature for HEV genotypes (Schlauder et al., 1999a; Wang et al., 1999).

Different serological test results were obtained when comparing three anti-HEV assays (EIAs). With the two currently available EIAs based on recombinant proteins derived from Burmese/Mexican isolates, anti-HEV declined rapidly and was no longer detectable in late-phase convalescent serum. At 3 weeks after presentation the patient was negative with
Table 1. Percentage nucleotide identity between isolates of HEV over a 371 base ORF1 fragment and a 148 base ORF2 fragment

Isolates represented are Burmese (B1, B2), Chinese (C1, C2, C3, C4, CT1), Indian (I1, I2), Pakistan (P1), Mexican (M1), Swine (S1), United States (U1, U2), Greek (G1, G2), Italian (It1) and Austrian (Au1).

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| % Nucleotide Identity ORF2 |
one assay (test system 2) and weakly positive with the other (test system 1). At 7 weeks and 24 weeks after presentation, no reactivity was indicated with these ELISAs.

With a third ELISA based on US isolate-specific peptides (Schlauder et al., 1998) enhanced reactivity was observed relative to Burmese/Mexican-based ELISAs. In contrast to the results observed with the latter, serum remained positive up to 9 months after the acute phase using the 4-2 peptide based on the US sequence. The enhanced reactivity of the US-based peptide was previously observed in sera from patients infected with the US strain of HEV. Patient sera from endemic regions were reactive with ELISAs based on the Burmese, Mexican and US strains. In contrast, in some cases, patients infected with the US strain exhibited little or no reactivity with the Burmese and Mexican ELISAs but were strongly reactive to the US-based ELISAs (Schlauder et al., 1998).

Discussion

Until recently, there has been little data to support the possibility that hepatitis E should be considered in non-ABC hepatitis in patients from industrialized countries without history of travel. Even though cases of sporadic HEV infection have been reported from industrialized countries all over the world (Zaaier et al., 1992; Chapman et al., 1993; Heath et al., 1995; Skidmore & Sherrat, 1996; Kwo et al., 1997) studies on the aetiology of non-ABC hepatitis showed no evidence that HEV might be the causative agent (Buti et al., 1994; Pham et al., 1994).

However, the low incidence of HEV-associated hepatitis is in contrast to anti-HEV seroprevalence data. Seropidemiological studies revealed a constant anti-HEV prevalence in countries not endemic for HEV disease. In all industrialized countries where seropidemiological studies have been carried out in a healthy population, e.g. blood donors, an anti-HEV prevalence of at least 1–2% has been found independent of the type of tests that were used (Dawson et al., 1992). Moreover, in some studies a considerably higher anti-HEV prevalence was found in subpopulation groups, suggesting the possibility of parenteral HEV transmission, e.g. via blood, blood products or in persons with high-risk sexual practices (Balayan, 1997; Arankalle & Chobe, 1999). In an Austrian study 2·3% of patients without hepatitis and 8·3% of patients with hepatitis tested positive for antibodies to HEV (Hoffmann & Holzmann, 1995). In most seropidemiological studies the majority of persons with anti-HEV-positive test results had no known history of hepatitis, suggesting a high rate of subclinical HEV infections.

To explain the low incidence of acute HEV infection in industrialized countries it was supposed that anti-HEV seroprevalence data might not be reliable, either due to cross-reaction with an unknown agent and/or due to lack of appropriate reagents (Balayan, 1997). The specificity of the HEV IgG ELISA was tested in epidemics and is as high as 99%. However, results from studies comparing the performance of various EIAs by determining anti-HEV in healthy subjects from industrialized countries did not confirm the near-100%
specificity for any of the tested assays (Mast et al., 1998). A previous study comparing test system 1 and 2 for serum samples from patients with acute hepatitis showed highly discrepant results (Quiroga et al., 1996). Interestingly test system 1, which showed an enhanced reactivity in the Austrian patient, was judged to be relatively insensitive in comparison to test system 2.

Serological testing of the convalescent sera from the Austrian patient showed a negative anti-HEV status after proven infection with HEV as demonstrated utilizing commercial assays. However, a lack of IgG reactivity with Burmese/Mexican-based ELISAs has been reported for endemic areas as well (Wang et al., 1999; Ke et al., 1996). Therefore, further studies are needed to assess whether the lack of reactivity might at least partially be due to genetic variability of new HEV isolates.

In spite of the inconsistency of serological test results, the detection of a novel ‘non-endemic’ HEV isolate demonstrates that hepatitis E infection may occur in industrialized countries even in patients without history of travel and should therefore be considered as a potential cause of acute non-A–D hepatitis.

Sequence and phylogenetic analyses increase our insight into the geographical distribution of HEV isolates and make it possible to characterize the epidemiology of HEV-associated disease. In endemic regions comparison of nucleotide sequences in isolates derived from patients of a single outbreak showed a high degree of identity (Aggarwal et al., 1999). Substantial genetic diversity in one isolate in comparison to others from the same outbreak may be interpreted as a sporadic case of hepatitis E (Aggarwal et al., 1999). Genetic drift does not seem to be important for HEV (Gouvea et al., 1997). Therefore, novel HEV isolates from non-endemic areas possibly represent genetic variants from a separate reservoir with a distinct evolution. The genetic difference and the lack of travel history in patients infected with these novel isolates makes the existence of a reservoir of HEV isolates in so-called ‘non-endemic’ regions plausible. Inoculation via contaminated water, which is common for endemic areas, is unlikely. Spread and accumulation of cases of HEV infection have not been reported. Therefore, a zoonotic reservoir has been suggested. This is supported by the detection of HEV RNA by PCR in stool and serum samples from wild and domestic animals in HEV endemic regions (Clayson et al., 1996). There is also increasing evidence that HEV plays a potential role as a heptatis-causing pathogen in some animal species in industrialized countries: hepatitis E infection is highly prevalent in herds of swine in industrialized countries. A high anti-HEV serum prevalence has been reported for the US (Schlauder et al., 1999b), Australia (Chandler et al., 1999) and a German region (Hartmann et al., 1998) near to Austria.

Recent findings show a close relationship between gene sequences of ‘endemic’ and ‘non-endemic’ HEV variants isolated in the same region from swine and humans with HEV-associated hepatitis (Meng et al., 1997; Hsieh et al., 1999).

Furthermore, successful cross-species infection has been demonstrated in HEV-antibody-free pigs with human isolates and in primates with swine hepatitis E virus (Meng et al., 1998), as well as in primates with a human isolate of HEV (Erker et al., 1999).

Evidence for HEV infection of wild rats has been reported from the US (Kabrane-Lazizi et al., 1999). Therefore, genetic diversity within the ‘non-endemic’ group of human HEV isolates (U1, U2, G1 and 2, H1 and Au1) might be due to divergent geographical evolution as well as to separate reservoirs.

In conclusion, the isolation of a novel HEV variant in Austria in a patient without history of travel implies the possibility of HEV strains native to Western countries. The genotype variation of this novel isolate in comparison to published novel isolates from the US and Europe might be in accordance with a geographical distribution reported from endemic regions. However, distinct reservoirs and the potential for multiple serotypes have to be considered.

References


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