別紙様式第２－１

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研究報告の概要

日本での血液透析患者のE型肝炎ウイルス感染率及び輸血による遺伝子型III型のHEV感染例：
E型肝炎ウイルス(HEV)の維持血液透析患者の有病率を調べるため、日本の透析施設で7.6±6.3（平均±標準偏差）（範囲0.3-26.0）年間、血液透析を受けている416人の患者から2003年1月に採取した血清サンプルと、同じ患者から血液透析開始時に採取された血清サンプルを、HEV抗体（IgG）の有無について、ELISA法による院内検査を行った。全体の結果として、2003年1月の時点では39人の患者（9.4%）にHEV抗体（IgG）が認められた。そのうち35人の患者（8.4%）は血液透析の開始時にすでにHEV抗体（IgG）陽性であったが、4人の患者（1%）は血液透析開始後に抗体陽転化した。これら抗体陽転化した４人の、2003年1月以前に定期採取されていた血清サンプルを、HEV抗体とHEV-RNAの存在について検査した。その結果、4人がHEV抗体（IgG）陽転化したのは各々1979年、1980年、1988年、2003年であったことが示され、その後観察期間終了時まで抗体陽性のままであった。この４人にHEV抗体（IgM）は検出されなかったが、3人は日本での地域局在的とされる遺伝子型III型のHEVであった。1979年にHEVに感染した患者については、HEV-RNA検出の21日前に2単位の血液が輸血されており、この二つの血液保存サンプルのうち一つに検出されたHEV-RNAは、患者から採取したものと100%一致した。本試験により、1979年に日本で輸血によるHEV感染が起こっていたことを証明するデータが得られ、また血液透析の治療期間中に新規HEV感染の発生する率は低いことが示された（4/374、1.1%）。

報告企業の意見	今後の対応
輸血後E型肝炎が認められた1例では、輸血された血液の保存サンプルの一方より検出されたHEV-RNAと当該患者のものが同じであったことより、輸血由来の感染が生じていたと考えられる。 本剤の原材料である人血漿の原産国の米国での発生ではないこと、血漿分画製剤によるHEV伝播が疑われる症例は報告されていないこと、並びに、血漿分画製剤では分画精製におけるウイルス不活化／除去工程により、HEVのモデルウイルスとして想定し得るHAVは不活化あるいは除去できることより、血漿分画製剤によるHEV伝播の可能性は極めて低いと考えている。	当該感染症に関し、引き続き、情報の収集を行っていく。 また、同様に同一生物種等から人に感染すると認められる疾病に関する情報情報の収集に努める。

使用上の注意記載状況・その他の参考事項等

２．重要な基本的注意（1）本剤の原材料となる血漿については、FDAで認可された方法でHBs抗原、抗HCV抗体、抗HIV-1及びHIV-2抗体陰性であることを確認し、かつALT(GPT)値でスクリーニングを実施している。さらに、プールした試験血漿については、HBV-DNA、HCV-RNA、HIV-1-RNA、HIV-2-RNA及びHAV-RNAについて核酸増幅検査（NAT）を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。同様に、パルボウイルスB19-DNAについてはプールした試験血漿で核酸増幅検査（NAT）を実施し、10^5IU/mL以下であることを確認した健康人血漿を用いている。本剤の製造工程である、Cohn低温エタノール分画法及び60±0.5℃10時間の液状加熱処理は、HIVをはじめとする各種ウイルスに対し、除去・不活化効果を有することが確認されているが、投与に際しては、次の点に十分注意すること。

血漿分画製剤の現在の製造工程では、ヒトパルボウイルスB19等のウイルスを完全に不活化・除去することが困難であるため、本剤の投与によりその感染の可能性を否定できないので、投与後の経過を十分に観察すること。

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Prevalence of Hepatitis E Virus Infection Among Hemodialysis Patients in Japan: Evidence for Infection With a Genotype 3 HEV by Blood Transfusion

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To investigate the prevalence of hepatitis E virus (HEV) infection among patients on maintenance hemodialysis, serum samples collected in January 2003 from 416 patients who had been undergoing hemodialysis for 7.6 ± 6.3 (mean ± standard deviation) (range, 0.3–26.0) years in a dialysis unit in Japan and serum samples that had been collected from these patients at the start of hemodialysis were tested for IgG antibodies to HEV (anti-HEV IgG) by an "in-house" enzyme-linked immunosorbent assay (ELISA). Overall, 39 patients (9.4%) had anti-HEV IgG in January 2003, and included 35 patients (8.4%) who had already been positive for anti-HEV IgG at the start of hemodialysis and 4 patients (1%) who seroconverted after initiation of hemodialysis. Periodic serum samples that had been collected from the four seroconverted patients were tested for HEV antibodies and HEV RNA. The four patients became positive for anti-HEV IgG in 1979, 1980, 1988, or 2003, and continued to be seropositive until the end of the observation period. Although anti-HEV IgM was not detectable in the four patients, three were infected transiently with apparently Japanese indigenous HEV strains of genotype 3. The patient who contracted HEV infection in 1979 had been transfused with 2 U of blood 21 days before the transient viremia: one of the two stored pilot serum samples had detectable HEV RNA with 100% identity to that recovered from the patient. Our study provides evidence of transfusion-transmitted HEV infection in Japan in 1979, and that the prevalence of de novo HEV infection during hemodialysis was low (1.1% or 4/374).

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KEY WORDS: hepatitis viruses; genotype; PCR; phylogenetic analysis; route of transmission

INTRODUCTION

Hepatitis E, which is caused by hepatitis E virus (HEV), is an important public health concern in many developing countries where sanitation is suboptimal: large epidemics of hepatitis E have been reported in Asia, Africa, and Latin America [Purcell and Emerson, 2001]. Although only sporadic cases of acute hepatitis E have been reported in many industrialized countries including the United States, European countries, and Japan [Harrison, 1999; Purcell and Emerson, 2001; Schlauder and Mushahwar, 2001; Smith, 2001; Okamoto et al., 2003], a significant proportion of healthy individuals in industrialized countries are seropositive for HEV antibodies [Mast et al., 1997; Thomas et al., 1997]. HEV was recently classified as the sole member of the genus *Hepevirus* in the family Hepeviridae. Its

The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB175483–AB175486.

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genome is a single-stranded, positive-sense RNA of approximately 7.2 kb. It consists of a short 5′ untranslated region (UTR) followed by three partially overlapping open reading frames (ORFs: ORF1, ORF2, and ORF3), and then a short 3′ UTR terminated by a poly(A) tract [Reyes et al., 1990; Tam et al., 1991; Huang et al., 1992; Wang et al., 2000].

Extensive genomic diversity has been noted among HEV isolates, and HEV sequences have tentatively been classified into four genotypes (genotypes 1–4). The majority of HEV infections in developing countries are caused by genotype 1; one epidemic of infection with HEV of genotype 2 has been documented in Mexico; and only isolated cases of infection with HEV of genotype 3 or 4 have been described in industrialized nations [Schlauder and Mushahwar, 2001]. Recent studies have indicated that hepatitis E is a zoonosis [Meng et al., 1997, 1998; Erker et al., 1999; Harrison, 1999; Meng, 2000; Halbur et al., 2001; Okamoto et al., 2001; Smith, 2001; Nishizawa et al., 2003; Tei et al., 2003; Yazaki et al., 2003]. Numerous strains of HEV of genotype 3 or 4 have been isolated from pigs in both developing and industrialized countries [Clayson et al., 1995; Chandler et al., 1999; Hsieh et al., 1999; Pina et al., 2000; Garkavenko et al., 2001; van der Poel et al., 2001; Arankalle et al., 2002; Huang et al., 2002; Pei and Yoo, 2002; Wang et al., 2002; Wu et al., 2002; Choi et al., 2003; Takahashi et al., 2003].

In Japan, it has been shown that the circulating HEV strains are polyphyletic [Mizuo et al., 2002] and that the zoonotic food-borne mode of transmission of HEV to humans may play an important role in the occurrence of hepatitis E [Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Tamada et al., 2004]. However, the mode of HEV transmission was not clear in the majority of patients with sporadic acute or fulminant hepatitis E in Japan [Takahashi et al., 2001, 2002a,b; Aikawa et al., 2002; Mizuo et al., 2002; Suzuki et al., 2002]. Recently, a patient who was infected with HEV via transfused blood from a voluntary blood donor was reported, and the authors stated that the potential risk of post-transfusion hepatitis E should be considered even in non- or low-endemic countries including Japan [Matsubayashi et al., 2004]. The majority of patients on maintenance hemodialysis have a history of blood transfusion. Some investigators [Halfon et al., 1994; Ding et al., 2003] observed a high prevalence of anti-HEV antibody among their hemodialysis patients. However, other investigators found only a few anti-HEV-positive patients among their hemodialysis populations [Courtney et al., 1994; Psichogiou et al., 1996; Fabrizi et al., 1997].

Therefore, in the present study, we determined the prevalence of HEV infection among 416 patients undergoing maintenance hemodialysis at a single dialysis unit in Japan using serum samples that had been obtained at the start of hemodialysis and serum samples that had been periodically collected from each patient and stored since the initiation of hemodialysis, to investigate whether hemodialysis and blood transfusion are associated with increased risk of HEV infection. Furthermore, stored pilot serum samples of transfused blood were tested for the presence of HEV RNA to clarify whether a hemodialysis patient who contracted de novo HEV infection 20 days after blood transfusion acquired transfusion-associated HEV infection.

MATERIALS AND METHODS

Serum Samples

Serum samples were collected in January 2003 from a total of 416 hemodialysis patients (age, 60.1 ± 12.6 [mean ± standard deviation, SD] years; 274 men and 142 women) who had been receiving maintenance hemodialysis in the dialysis unit of Masuko Memorial Hospital in Nagoya, Japan, for more than 3 months (7.6 ± 6.3 [range, 0.3–26.0] years). Additionally, stored serum samples that had been obtained from the 416 patients at the start of hemodialysis were used. From the four patients who became seropositive for HEV infection after the initiation of hemodialysis, stored serum samples that had been obtained periodically (semimonthly between 1977 and 2001 and monthly thereafter) were also used in this retrospective analysis. This study conforms to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the ethics committee at the institution. Informed consent was obtained from each patient.

Detection of Antibodies to HEV

The serum samples were tested for the IgG, IgM, and IgA classes of anti-HEV by in-house enzyme-linked immunosorbent assay (ELISA), using purified recombinant ORF2 protein of HEV genotype 4 that had been expressed in the pupae of silkworm, as described previously [Mizuo et al., 2002; Tokita et al., 2003]. The optical density (OD) of each sample was read at 450 nm. The cut-off value used for the anti-HEV IgG assay was 0.152 that for the anti-HEV IgM assay was 0.353, and that for the anti-HEV IgA assay was 0.350. Samples with OD values for anti-HEV IgG, IgM, or IgA equal to or greater than the respective cut-off value were considered to be positive for anti-HEV IgG, IgM, or IgA, respectively. The specificity of the anti-HEV assays was verified by absorption with the same recombinant ORF2 protein that was used as the antigen probe or a mock protein obtained from the pupae of silkworm infected with non-recombinant baculovirus. Briefly, if the OD value of the tested sample was less than 30% of the original value after absorption with the recombinant ORF2 protein and if it was greater than 70% of the original value after absorption with the mock protein, the sample was considered to be positive for anti-HEV. The serum samples were also tested for anti-HEV IgM using a commercially available kit (Genelabs Diagnostics, Pte. Ltd., Singapore Science Park, Singapore).

Detection of HEV RNA

Reverse transcription (RT)-polymerase chain reaction (PCR) was carried out for detection of HEV RNA in

the serum samples. Total RNA was extracted from 100 μl of serum, reverse transcribed, and then subjected to nested PCR with primers targeting the ORF2 region as described previously [Mizuo et al., 2002]. The size of the amplification product of the first-round PCR was 506 base pairs (bp), and that of the second-round PCR was 457 bp. The nested RT-PCR assay was performed in duplicate, and reproducibility was confirmed. To avoid contamination during PCR procedures, the guidelines of Kwok and Higuchi [1989] were strictly observed. The specificity of the RT-PCR assay was verified by sequence analysis as described below. The sensitivity of the RT-PCR assay was assessed as described previously [Mizuo et al., 2002].

Serological Tests for Other Viral Infections

Serum samples were tested for hepatitis B surface antigen (HBsAg) and the corresponding antibody (anti-HBs) by passive hemagglutination using commercial assay kits (Mycell II HBsAg and Mycell II anti-HBs, respectively; Institute of Immunology, Co. Ltd., Tokyo, Japan). The presence of antibody to hepatitis B core antigen (anti-HBc) was determined by hemagglutination inhibition by the method described previously [Iizuka et al., 1992]. The presence of antibody to hepatitis C virus (HCV) (anti-HCV) was determined by a commercially available enzyme immunoassay kit (HCV · EIA II, Abbott Japan, Tokyo, Japan).

Sequence Analysis of PCR Products

The amplification products were sequenced directly on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Genetyx-Mac version 12.2.0 (Genetyx Corp., Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ: National Institute of Genetics, Mishima, Japan) [Ina, 1994]. Sequence alignments were generated by CLUSTAL W (version 1.8) [Thompson et al., 1994]. A phylogenetic tree was constructed by the neighbor-joining method [Saitou and Nei, 1987] based on the partial nucleotide sequence of the ORF2 region (301 nucleotides [nt]). Bootstrap values were determined on 1,000 resamplings of the datasets [Felsenstein, 1985]. The final tree was obtained using the TreeView program (version 1.6.6) [Page, 1996].

Statistical Analysis

Data are presented as the mean ± SD. Statistical analyzes were carried out using the Welch's t-test for comparison of continuous variables between two groups, and the χ^2-test for comparison of proportions between two groups. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Prevalence of Anti-HEV IgG, HBsAg, and Antibodies to HBV and HCV Among Hemodialysis Patients

Serum samples obtained from 416 patients on maintenance hemodialysis at a dialysis unit of a city hospital in Japan in January 2003, were tested for the presence of anti-HEV IgG. Anti-HEV IgG was detected in 39 (9.4%) of the 416 patients, with a higher prevalence among males than among females (11.3% vs. 5.6%) (Table I). The prevalence of anti-HEV IgG tended to be lower among patients aged <40 years than among those aged ≥40 years, but the difference fell short of being statistically significant. There were nine patients with anti-HEV IgG of high OD_{450} value (≥1.000), including two (5.0%) in the age group of 40–49 years, four (3.7%) in the age group of 50–59 years, two (1.4%) in the age group of 60–69 years, and one (1.3%) in the age group of 70–79 years. Of note, two patients had anti-HEV IgG of very high OD_{450} value (≥3.000), but they were negative for both anti-HEV IgM and HEV RNA.

Overall, at the screening conducted in January 2003, 14 patients were positive for HBsAg, 105 patients were negative for HBsAg but positive for anti-HBs and/or anti-HBc, and 53 patients were positive for anti-HCV. There were no significant associations between positivity for anti-HEV IgG and the presence of serological markers of HBV or HCV (Table II).

TABLE I. Age-Specific Prevalence of Anti-Hepatitis E Virus (HEV) IgG Among 416 Patients on Maintenance Hemodialysis at Screening in January 2003, According to Gender and Optical Density (OD) Value of Anti-HEV IgG

	No. of patients with anti-HEV IgG[a]			Anti-HEV IgG with high OD_{450} value of	
Age (years)	Total	Male	Female	≥1.000	≥3.000
23–39	1/33 (3.0%)	0/21	1/12 (8.3%)	0/33	0/33
40–49	3/40 (7.5%)	3/29 (10.3%)	0/11	2/40 (5.0%)	1/40 (2.5%)
50–59	10/109 (9.2%)	9/71 (12.7%)	1/38 (2.6%)	4/109 (3.7%)	1/109 (0.9%)
60–69	17/138 (12.3%)	13/92 (14.1%)	4/46 (8.7%)	2/138 (1.4%)	0/138
70–79	6/77 (7.8%)	5/48 (10.4%)	1/29 (3.4%)	1/77 (1.3%)	0/77
80–91	2/19 (10.5%)	1/13 (7.7%)	1/6 (16.7%)	0/19	0/19
Total	39/416 (9.4%)	31/274 (11.3%)	8/142 (5.6%)	9/416 (2.2%)	2/416 (0.5%)

[a]Cut-off value for anti-HEV IgG was 0.152.

TABLE II. Characteristics of the Anti-HEV IgG-Positive and -Negative Patients at Screening Conducted in January 2003

Features	Anti-HEV IgG-positive (n = 39)	Anti-HEV IgG-negative (n = 377)	*P* value
Age (years)	62.4 ± 10.0	59.9 ± 12.8	0.1540 (NS[a])[b]
Duration of hemodialysis (years)	7.5 ± 7.0	7.6 ± 6.2	0.9671 (NS)[b]
Hepatitis B surface antigen (HBsAg)-positive	0	14 (3.7%)	0.4485 (NS)[c]
Anti-HBs/HBc-positive	8 (20.5%)	97 (25.7%)	0.6028 (NS)[c]
Anti-hepatitis C virus (HCV)-positive	4 (10.3%)	49 (13.0%)	0.8131 (NS)[c]

[a]NS, not significant.
[b]Welch's *t*-test.
[c]χ^2-test.

Prevalence of Anti-HEV IgG Among Hemodialysis Patients at the Start of Hemodialysis

The prevalence of anti-HEV IgG at the start of hemodialysis was surveyed retrospectively by testing stored serum samples that had been obtained from the 416 patients. Anti-HEV IgG was detected in 42 patients (10.1%), including 32 males and 10 females (Table III). Although two patients had anti-HEV IgG of extremely high OD_{450} value (≥3.000), they were negative for both anti-HEV IgM and HEV RNA. Among the 42 patients with anti-HEV IgG at the first examination between January 1977 and October 2002, 35 patients remained seropositive and 7 patients tested negative for anti-HEV IgG in January 2003 (Table IV). On the other hand, among the 374 patients who were negative for anti-HEV IgG at the start of hemodialysis, 370 patients remained negative for anti-HEV IgG but 4 patients were found to be seropositive in the screening test performed in January 2003. In January 2003, 194 patients (46.6%) had a history of blood transfusion; however, the presence of a history of blood transfusion was not significantly associated with seropositivity for anti-HEV IgG.

Detection of HEV RNA in Four Patients who Contracted HEV Infection After the Start of Hemodialysis

The demographic characteristics of the four patients who contracted HEV infection during the observation period of 8.1–24.3 years, presence of a history of blood transfusion, and OD_{450} values of anti-HEV IgG in their serum samples that had been obtained at the start of hemodialysis and at the screening in January 2003 are shown in Table V. In the serum samples obtained in January 2003, the four patients had anti-HEV IgG with OD_{450} value of 0.416–1.348 but were negative for both anti-HEV IgM and HEV RNA. The stored serum samples that had been obtained semimonthly between 1978 and 2001 and monthly between 2002 and 2003 from each of the four patients (Patients 1–4), were tested for anti-HEV IgG and HEV RNA in order to clarify when they contracted HEV infection. Patient 1 was first positive for anti-HEV IgG on November 26, 1979 with an OD_{450} value of 1.381 and remained positive until the end of the observation period (Table VI). The serum sample containing anti-HEV IgG that had been obtained on November 26, 1979 was negative for HEV RNA; however, the serum sample that had been obtained

TABLE III. Age-Specific Prevalence of Anti-HEV IgG Among 416 Patients on Maintenance Hemodialysis at the Start of Hemodialysis, According to Gender and OD Value of Anti-HEV IgG

	No. of patients with anti-HEV IgG[a]			Anti-HEV IgG with high OD_{450} value of	
Age (years)	Total	Male	Female	≥1.000	≥3.000
14–29	2/27 (7.4%)	1/19 (5.3%)	1/8 (12.5%)	1/27 (3.7%)	0/27
30–39	2/52 (3.8%)	2/35 (5.7%)	0/17	1/52 (1.9%)	1/52 (1.9%)
40–49	7/83 (8.4%)	6/47 (12.8%)	1/36 (2.8%)	2/83 (2.4%)	1/83 (1.2%)
50–59	17/127 (13.4%)	15/89 (16.9%)	2/38 (5.3%)	2/127 (1.6%)	0/127
60–69	10/83 (12.0%)	5/57 (8.8%)	5/26 (19.2%)	2/83 (2.4%)	0/83
70–87	4/44 (9.1%)	3/27 (11.1%)	1/17 (5.9%)	0/44	0/44
Total	42/416 (10.1%)	32/274 (11.7%)	10/142 (7.0%)	8/416 (1.9%)	2/416 (0.5%)

[a]Cut-off value for anti-HEV IgG was 0.152.

TABLE IV. Characteristics of 416 Hemodialysis Patients, Stratified by the Presence/Absence of Anti-HEV IgG at the Start of Hemodialysis and the Screening of HEV Infection in January 2003

Presence of anti-HEV IgG at the start of hemodialysis/ at screening (January 2003)	No. of patients	Male (%)	Age (mean ± SD, years)[a]	Duration of hemodialysis[b] (mean ± SD [range], years)	No. of patients with a history of blood transfusion		
					Before the start of hemodialysis	After the start of hemodialysis	Either[c]
Yes/Yes	35	82.9	56.6 ± 11.9	6.3 ± 5.9 [0.3–21.8]	8 (22.9%)	10 (28.6%)	16 (45.7%)
Yes/No	7	42.9	50.6 ± 10.9	13.0 ± 6.9 [3–23.2]	1 (14.3%)	3 (42.9%)	3 (42.9%)
No/Yes	4	50.0	40.0 ± 7.8	18.2 ± 6.4 [8.1–24.3]	2 (50.0%)	4 (100%)	4 (100%)
No/No	370	64.9	52.3 ± 14.3	7.5 ± 6.1 [0.3–26.0]	98 (26.5%)	125 (33.8%)	171 (46.2%)
Total	416	65.9	52.5 ± 14.1	7.6 ± 6.3 [0.3–26.0]	109 (26.2%)	142 (34.1%)	194 (46.6%)

[a]At the start of hemodialysis.
[b]Patients who had been on maintenance hemodialysis for more than 3 months as of January 2003, were enrolled in the present study.
[c]Patients with a history of blood transfusion before the start of hemodialysis and/or after the start of hemodialysis.

14 days earlier (November 12, 1979) had detectable HEV RNA. Similarly, Patient 2 was first positive for anti-HEV IgG on April 21, 1980, with an OD_{450} value of >3.000 and continued to be positive thereafter. Patient 2 had detectable HEV RNA in the serum sample that had been obtained 14 days earlier (April 7, 1980). Patient 3 was first positive for anti-HEV IgG on August 16, 1988, with an OD_{450} value of >3.000 and continued to be positive thereafter. This patient had detectable HEV RNA in two consecutive serum samples, i.e., the serum sample obtained on the day of emergence of anti-HEV IgG (August 16, 1988) and the serum sample that had been obtained 14 days earlier (August 2, 1988). Patient 4 was first positive for anti-HEV IgG on January 6, 2003 with an OD_{450} value of 1.348 and continued to be positive until the end of the observation period. However, HEV RNA was not detectable in any of the stored serum samples of Patient 4, unlike the other three patients, and this was probably due to the lack of a serum sample obtained 14 days before the emergence of anti-HEV IgG: only a serum sample obtained 1 month earlier was available.

Surprisingly, although transient viremia was recognizable in three of the four patients who contracted HEV infection, anti-HEV IgM was not detected in any of the stored serum samples of the four patients, using not only an "in-house" ELISA but also a commercially available ELISA kit supplied by Genelabs. In support of this observation, anti-HEV IgA which can be utilized as an additional confirmatory antibody for recent HEV infection [Chau et al., 1993; Tokita et al., 2003], was not detected throughout the observation period in two patients (Patients 1 and 2) and was only weakly positive within a short period of time or at a single time point in the remaining two patients (Patients 3 and 4, respectively).

Detection of HEV RNA From Pilot Serum Samples of Transfused Blood Units

Patients 2–4 contracted HEV infection 1.5–8.1 years after the start of hemodialysis and had no history of blood transfusion within 1 year before seroconversion to anti-HEV IgG. However, Patient 1 who became positive for HEV RNA in the serum on November 12, 1979, approximately 1 month after initiation of hemodialysis, had received 2 U of blood on October 22, 1979 (3 weeks before detection of HEV RNA in the circulation). Two pilot serum samples of transfused blood units had been stored and were subjected to PCR assay. Of remarkable interest, one of the two pilot samples had detectable HEV RNA, although they were negative for anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA.

Genetic Analysis of HEV Isolates Recovered From Three Viremic Patients and an HEV RNA-Positive Pilot Sample

The three HEV isolates recovered from the transiently viremic patients (Patients 1–3) were named

TABLE V. Past History of Blood Transfusion and Anti-HEV IgG in Four Hemodialysis Patients who Became Seropositive for Anti-HEV IgG After the Start of Hemodialysis

Patient	Sex	Blood transfusion		At the start of hemodialysis				At screening (January 2003)		
		Before the start of hemodialysis	After the start of hemodialysis	Date of sampling	Age (years)	Anti-HEV IgG (OD_{450} value)	Duration (years)	Date of sampling	Age (years)	Anti-HEV IgG (OD_{450} value)
1	Male	No	Yes (1979/10/22)	1979/10/9	31	0.075 (−)	23.2	2003/1/6	54	1.037 (+)
2	Female	Yes (1978/8/14)	Yes (1983/8/11)	1978/10/2	39	0.089 (−)	24.3	2003/1/7	63	0.416 (+)
3	Female	Yes (1985/11/5)	Yes (1986/3/13)	1985/11/25	45	0.068 (−)	17.1	2003/1/14	62	0.496 (+)
4	Male	No	Yes (1999/10/4)	1994/12/12	50	0.012 (−)	8.1	2003/1/6	58	1.348 (+)

TABLE VI. Laboratory Parameters, Anti-HEV Antibody Levels and HEV RNA in Periodic Serum Samples Obtained From Four Hemodialysis Patients With Transient HEV Infection

Patient	Date of sampling	ALT (IU/L)	AST (IU/L)	Anti-HEV (absorbance at 450 nm) IgG-class	IgM-class	IgA-class	HEV RNA
1	1979/10/9[a]	18	14	0.075 (−)	0.029 (−)	0.036 (−)	−
	1979/10/29	12	3	0.107 (−)	0.035 (−)	0.042 (−)	−
	1979/11/12	9	7	0.138 (−)	0.032 (−)	0.039 (−)	+
	1979/11/26	9	8	**1.381** (+)	0.045 (−)	0.085 (−)	−
	1979/12/10	6	8	**2.319** (+)	0.052 (−)	0.081 (−)	−
	1979/12/24	4	2	**2.619** (+)	0.042 (−)	0.090 (−)	−
	1980/1/7	10	6	**2.733** (+)	0.050 (−)	0.099 (−)	−
	1980/1/21	12	3	**2.705** (+)	0.047 (−)	0.110 (−)	−
	1980/2/4	14	5	**2.447** (+)	0.037 (−)	0.073 (−)	−
	1980/2/18	12	7	**2.666** (+)	0.040 (−)	0.086 (−)	−
	1980/4/14	1	1	**2.103** (+)	0.044 (−)	0.095 (−)	−
	1980/10/28	12	8	**1.517** (+)	0.053 (−)	0.079 (−)	−
	1987/1/12	35	22	**0.884** (+)	0.024 (−)	0.052 (−)	−
	1991/1/2	21	26	**0.837** (+)	0.016 (−)	0.014 (−)	−
	1997/1/13	14	15	**0.635** (+)	0.023 (−)	0.035 (−)	−
	2003/1/6[b]	18	14	**1.037** (+)	0.021 (−)	0.027 (−)	−
	2003/11/10	14	9	**1.093** (+)	0.021 (−)	0.031 (−)	−
2	1978/10/2[a]	7	1	0.089 (−)	0.069 (−)	0.031 (−)	−
	1980/3/24	23	20	0.087 (−)	0.056 (−)	0.032 (−)	−
	1980/4/7	4	9	0.087 (−)	0.045 (−)	0.029 (−)	+
	1980/4/21	6	7	**>3.000** (+)	0.078 (−)	0.093 (−)	−
	1980/5/5	6	6	**>3.000** (+)	0.088 (−)	0.096 (−)	−
	1980/5/19	1	1	**>3.000** (+)	0.057 (−)	0.083 (−)	−
	1980/6/2	5	6	**>3.000** (+)	0.076 (−)	0.093 (−)	−
	1980/6/16	5	5	**>3.000** (+)	0.081 (−)	0.091 (−)	−
	1980/6/30	7	6	**>3.000** (+)	0.076 (−)	0.084 (−)	−
	1980/8/11	2	5	**2.819** (+)	0.071 (−)	0.078 (−)	−
	1980/9/22	8	7	**2.804** (+)	0.060 (−)	0.059 (−)	−
	1981/2/2	1	6	**2.210** (+)	0.070 (−)	0.055 (−)	−
	1992/1/7	21	27	**0.483** (+)	0.031 (−)	0.116 (−)	−
	2003/1/7[b]	7	13	**0.416** (+)	0.012 (−)	0.063 (−)	−
	2003/11/11	10	15	**0.454** (+)	0.016 (−)	0.065 (−)	−
3	1985/11/25[a]	49	45	0.068 (−)	0.024 (−)	0.030 (−)	−
	1988/7/5	13	15	0.062 (−)	0.022 (−)	0.032 (−)	−
	1988/7/19	11	11	0.061 (−)	0.023 (−)	0.038 (−)	−
	1988/8/2	11	12	0.058 (−)	0.018 (−)	0.030 (−)	+
	1988/8/16	15	12	**>3.000** (+)	0.034 (−)	**0.664** (+)	+
	1988/8/30	14	12	**>3.000** (+)	0.082 (−)	**0.829** (+)	−
	1988/9/13	11	12	**>3.000** (+)	0.085 (−)	**0.654** (+)	−
	1988/10/11	10	10	**>3.000** (+)	0.079 (−)	**0.450** (+)	−
	1988/10/25	20	17	**>3.000** (+)	0.072 (−)	**0.398** (+)	−
	1988/11/22	10	13	**>3.000** (+)	0.044 (−)	0.318 (−)	−
	1989/3/6	11	14	**>3.000** (+)	0.034 (−)	0.167 (−)	−
	1989/5/29	13	14	**2.776** (+)	0.034 (−)	0.141 (−)	−
	1995/1/10	14	16	**0.807** (+)	0.023 (−)	0.039 (−)	−
	2000/1/2	14	16	**0.517** (+)	0.008 (−)	0.024 (−)	−
	2003/1/14[b]	10	15	**0.496** (+)	0.009 (−)	0.030 (−)	−
	2003/11/4	12	18	**0.349** (+)	0.009 (−)	0.023 (−)	−
4	1994/12/12[a]	15	21	0.012 (−)	0.017 (−)	0.028 (−)	−
	2002/11/11	9	14	0.064 (−)	0.027 (−)	0.054 (−)	−
	2002/12/9	11	11	0.061 (−)	0.028 (−)	0.057 (−)	−
	2003/1/6[b]	10	12	**1.348** (+)	0.107 (−)	**0.355** (+)	−
	2003/2/3	8	12	**1.575** (+)	0.131 (−)	0.343 (−)	−
	2003/3/3	9	9	**1.412** (+)	0.099 (−)	0.284 (−)	−
	2003/4/14	9	13	**1.180** (+)	0.067 (−)	0.262 (−)	−
	2003/11/10	7	12	**0.815** (+)	0.042 (−)	0.208 (−)	−

[a]Date of the start of hemodialysis.
[b]Date of the initial screening in the current study.

HE-JHD1979, HE-JHD1980, and HE-JHD1988, respectively, and the HEV isolate recovered from the HEV RNA-positive pilot serum sample that had been transfused to Patient 1 was named HE-JHD1979d. The 412-nt sequence of ORF2 of these HEV isolates were determined and compared with each other and with that of known human and swine HEV isolates of genotypes 1–4. The HE-JHD1979 and HE-JHD1979d isolates were 100% identical, supporting transfusion-transmitted HEV infection in Patient 1. The three HEV

isolates recovered from transiently viremic patients were 92.0–95.6% similar to each other, and were closely related to the prototype Japanese isolate of genotype 3 (JRA1, see accession no. in Fig. 1) with nucleotide sequence identity of 91.2–94.7%, and were only 79.3–80.5, 74.9–76.5, and 78.8–79.4% similar to the B1 isolate of genotype 1, MEX-14 isolate of genotype 2, and T1 isolate of genotype 4, respectively, in the 412-nt ORF2 sequence. The phylogenetic tree constructed based on the common 301-nt sequence within ORF2 sequence confirmed that the HE-JHD1979, HE-JHD1979d, HE-JHD1980, and HE-JHD1988 isolates obtained in the present study belonged to genotype 3, and that they segregated into the cluster consisting of Japanese HEV strains of the same genotype that had been recovered from humans (HE-JA5, HE-JA6, HE-JA9, HE-JA11, HE-JA21, HE-JA23, HE-JF2, HE-JO-1982, JJT-Kan, and JRA1) and swine (swJ570 and swJ681), supporting the indigenous nature of these HEV isolates.

DISCUSSION

HEV is associated frequently with fecal-contaminated drinking water or poor sanitation conditions in developing countries, and blood transfusion is not considered to be an important cause of HEV transmission as the virus does not produce a chronic carrier state. However, the theoretical possibility of HEV transmission via a parenteral route in developing countries has been suggested due to the high endemicity of HEV; the fact that the majority of HEV infections were subclinical; and documentation of viremia during the incubation period of the disease [Arankalle and Chobe, 1999]. In fact, it has been reported that a substantial proportion of blood donors (3/200 or 1.5%) were positive for HEV RNA and viremic blood donors are able potentially to cause transfusion-associated hepatitis E in areas of high endemicity [Arankalle and Chobe, 1999, 2000]. Such a possibility is also supported in industrialized countries where HEV infection is now considered to be low-endemic, based on the observation that positivity for anti-HEV antibody was more frequent among transfusion recipients than among the same number of non-transfused controls [Mannucci et al., 1994]. However, the epidemiology of HEV infection among hemodialysis patients who have a high rate of a history of blood transfusion and are at increased risk for infection with

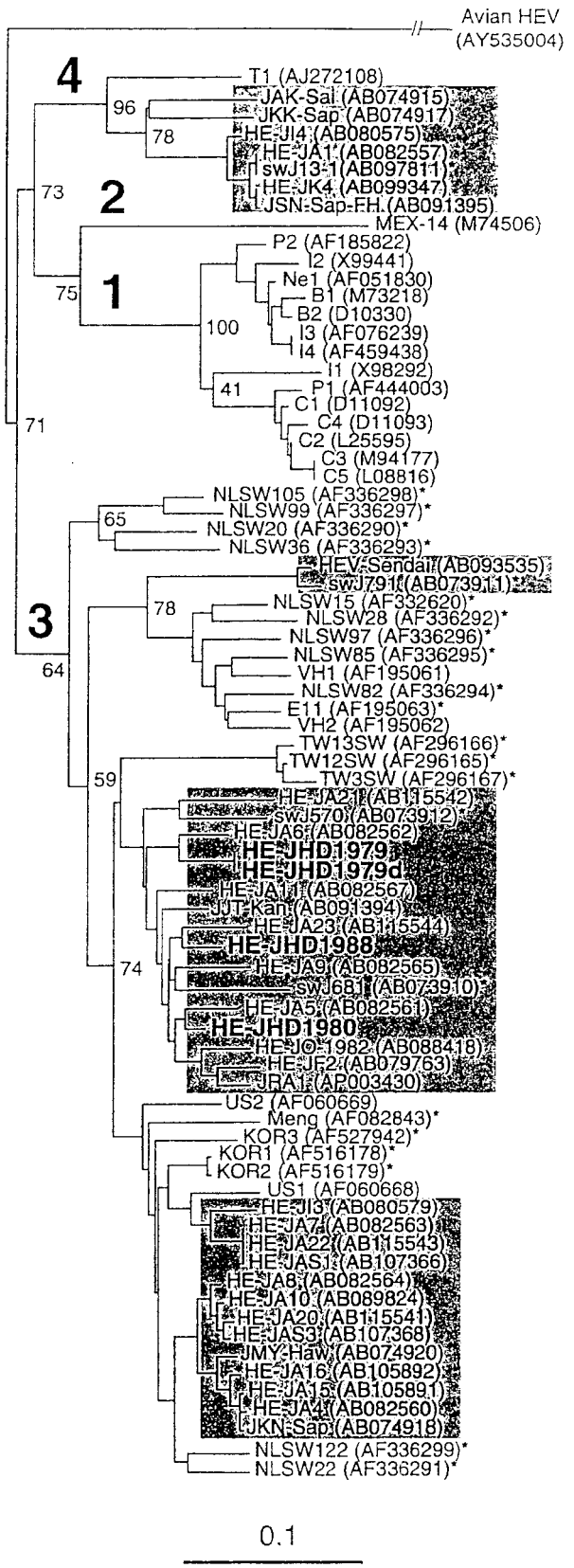

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the open reading frames (ORF)2 region (301 nt) of 77 hepatitis E virus (HEV) isolates, using an avian HEV (AY535004) as an outgroup. In addition to the HE-JHD1979, HE-JHD1979d, HE-JHD1980, and HE-JHD1988 isolates found in the present study which are indicated in bold type, 73 reported HEV isolates of genotypes 1–4 whose common 301-nt sequence is known are included for comparison and their accession nos. are shown in parentheses. The previously reported HEV sequences of genotype 1 are indicated with abbreviations in accordance with the review article by Schlauder and Mushahwar [2001]: B1 and B2 in Burma; C1, C2, C3, C4, and C5 in China; I1, I2, I3, and I4 in India; Ne1 in Nepal; and P1 and P2 in Pakistan. Asterisks denote swine HEV strains. The human and swine HEV isolates of Japan origin are shaded for visual clarity. Bootstrap values are indicated for the major nodes as a percentage obtained from 1,000 resamplings of the data.

blood-borne viruses, has not been fully understood and conflicting results have thus far been reported [Courtney et al., 1994; Halfon et al., 1994; Psichogiou et al., 1996; Fabrizi et al., 1997; Ding et al., 2003].

In the current study, at the first screening for HEV infection of hemodialysis patients at a dialysis unit of a city hospital in Japan conducted in January 2003, a high prevalence (9.4%) of anti-HEV IgG was observed among the 416 patients on maintenance hemodialysis who had been undergoing hemodialysis for 0.3–26 (mean, 7.6) years. However, we did not find significant associations between infection of HEV and infection with other blood-borne viruses such as HBV or HCV, consistent with a previous report [Fabrizi et al., 1997]. When the stored serum samples that had been collected at the start of hemodialysis from the 416 patients were tested for anti-HEV IgG, a high prevalence of 10.1% (42/416) was also observed, suggesting that the majority (89.7% or 35/39) of hemodialysis patients who were positive for anti-HEV IgG in January 2003, had been infected before initiation of hemodialysis. Furthermore, there was no appreciable difference in the prevalence of anti-HEV IgG at the start of hemodialysis between the patients who did or did not have a past history of blood transfusion (8.3% [9/109] vs. 10.7% [33/307], $P = 0.5776$), suggesting that the HEV infection in our hemodialysis patients that was acquired before the initiation of hemodialysis, had not been acquired by blood transfusion in the majority of cases. Of note, the prevalence of anti-HEV IgG at the start of hemodialysis tended to be higher among males than among females (11.7 vs. 7.0%), similar to the reported higher prevalence of anti-HEV IgG among males in the general population and the higher prevalence of HEV-associated hepatitis among male patients who had no history of blood transfusion within one or more years before the onset of disease [Tanaka et al., 2001; Mizuo et al., 2002]. Therefore, we would consider the possibility that our patients who were positive for anti-HEV IgG at the start of hemodialysis had acquired HEV infection in the community. It is likely that differences in the prevalence of HEV in the general population at the regional level, the criteria for inclusion of patients, and the routes of HEV transmission could partly explain the diverse results obtained in previous studies on hemodialysis patients. In Japan, regional differences in the prevalence of clinical and subclinical HEV infection have been reported [Li et al., 2000; Okamoto et al., 2003]. Furthermore, it was found previously that HEV-associated hepatitis was associated significantly with males, higher age (≥40 years) and living in the northern part of Japan [Mizuo et al., 2002]. It has been revealed that the zoonotic food-borne mode of transmission of HEV to humans may play an important role in the occurrence of hepatitis E [Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Tamada et al., 2004]. However, the mode of HEV transmission is unclear in most patients with sporadic acute or fulminant hepatitis E in Japan [Takahashi et al., 2001, 2002a,b; Aikawa et al., 2002; Mizuo et al., 2002; Suzuki et al., 2002]. Two male patients (49 and 52 years of age) were admitted in 2001 and 2002, respectively, to the hospital with our dialysis unit, and were diagnosed retrospectively as sporadic acute hepatitis E, but the mode(s) of HEV transmission could not be specified (unpublished observations). Further studies are needed to elucidate the region-dependent prevalence and mode(s) of clinical and subclinical HEV infection in the general population of Japan including Aichi Prefecture, where our dialysis unit is located.

In the present study, during the mean observation period of 7.7 years after the start of hemodialysis, four hemodialysis patients (1.1% or 4/374) acquired de novo HEV infection. In three of the four patients, the mode(s) of HEV transmission was unclear. However, it was found that the remaining one patient contracted HEV infection by transfusion of HEV-viremic blood in 1979. The HEV isolates, HE-JHD1979 and HE-JHD1979d, recovered from the stored serum sample from the patient and from the stored pilot serum of transfused viremic blood, respectively, were 100% identical in the 412-nt sequence of the ORF2 region, and segregated into genotype 3, and further into a cluster consisting of apparently Japan-indigenous strains. These results support our previous observation that a domestic HEV strain has been present for >2 decades in Japan [Aikawa et al., 2002] and also the recent report of a patient who was infected with HEV by transfused blood from a voluntary blood donor in Hokkaido, Japan [Matsubayashi et al., 2004], where clinical HEV infection is most prevalent. Although HEV infection via blood transfusion does not occur frequently as described above, we found a probable case of transfusion-transmitted HEV infection by molecular approaches. Therefore, the potential risk of post-transfusion hepatitis E should be taken into consideration even in low-endemic countries including Japan.

The four patients who contracted de novo HEV infection after the initiation of hemodialysis, did not have an elevated ALT level even after the appearance of anti-HEV IgG in the circulation, indicating that the HEV infection in the four patients was exclusively subclinical, although we cannot rule out the possibility of mild ALT elevation during the interval of 2 or 4 weeks. Of note, anti-HEV IgM was not detected in any of the four patients, and was also undetectable in all four patients by a commercially available kit: this may argue against the possibility of low sensitivity of the "in-house" ELISA system. It has been reported that IgA anti-HEV test can be utilized as an additional confirmatory test for recent HEV infection [Chau et al., 1993; Tokita et al., 2003]. IgA anti-HEV was also undetectable in two patients but was detectable weakly in the remaining two patients, suggesting that IgA anti-HEV detection is useful for serological diagnosis of acute HEV infection in the absence of IgM anti-HEV. As patients on maintenance hemodialysis have an impaired immune response [Goldblum and Reed, 1980; Girndt et al., 2001; Libetta et al., 2001], they may be unable to raise an adequate antibody response to viral proteins, especially in subclinical infection. Hemodialysis patients also have an

impaired immune response to HCV proteins or to hepatitis B vaccination [Rapicetta, 1992; Devesa et al., 1997]. Although the OD value of anti-HEV IgG in the four patients was lower than that among non-dialysis patients with clinical HEV infection, anti-HEV IgG persisted until the end of the observation period (as of June 2004, when this report was prepared) of 24 years in Patient 1, 24 years in Patient 2, 15 years in Patient 3, and 1 year in Patient 4. Therefore, we would like to consider that underestimation of HEV infection in our studied population by our anti-HEV IgG assay may be small or hopefully negligible.

In conclusion, it was found that approximately 90% of anti-HEV IgG-positive patients who were receiving maintenance hemodialysis for 0.3–26.0 (mean, 7.6) years had already been infected with HEV at the start of hemodialysis, regardless of the presence of a past history of blood transfusion before the initiation of hemodialysis, suggesting that the majority of anti-HEV IgG-positive hemodialysis patients had acquired HEV infection in the community through undefined route(s) but not via blood transfusion. However, a patient with probable transfusion-transmitted HEV infection was identified who was infected with an apparently Japan-indigenous HEV strain of genotype 3 21 days after transfusion of the implicated viremic blood in 1979. Based on the present study and the recent report by Matsubayashi et al. [2004], cases of transfusion-associated HEV infection may have been present for more than two decades in Japan. Therefore, the potential risk of post-transfusion hepatitis E should be taken into consideration even in HEV low-endemic countries including Japan.

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