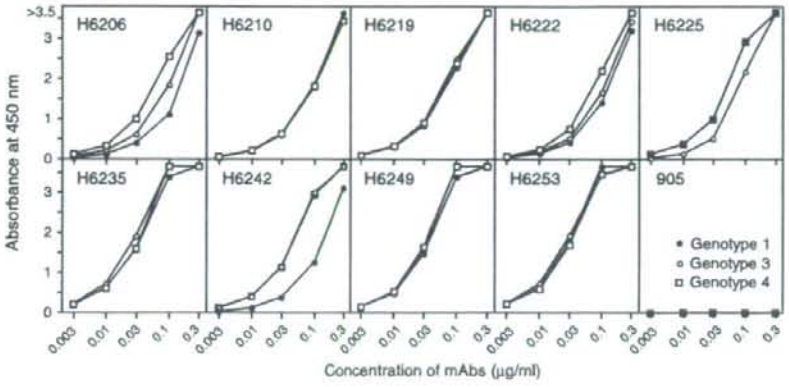


**Fig. 1** Binding of nine mAbs in increasing concentrations with the immobilized recombinant HEV ORF2 protein of genotype 1, 3, or 4. As a negative control, an mAb (No. 905) raised against a secretory protein (hepatitis B e antigen) of hepatitis B virus [39] was used

dependent reactivity. The reactivity with the three distinct recombinant proteins was comparable for H6222 and H6225, although H6225 showed lower binding affinity with the genotype 3 protein and H6222 showed lower binding affinity with the genotype 1 and 3 proteins. In addition, H6242 showed lower reactivity with the genotype 1 protein, and H6206 showed the highest reactivity with the genotype 4 protein and the lowest reactivity with the genotype 1 protein.

To investigate the spatial relationships of the epitopes recognized by each of the nine mAbs, pairwise competitive ELISA was performed at various mAb concentrations of 0.3–300 μg/ml (Fig. 2). As for six mAbs of H6206, H6219, H6222, H6235, H6242, and H6253, when 300 μg/ml of mAb was added as a competitor, the binding of biotinylated mAb to rHEV ORF2-G4 protein was almost completely inhibited by a homologous mAb and five heterologous mAbs at 71.2–100%, but was only partially inhibited by H6210, H6225, and H6249 at ≤30.8, ≤54.8, and ≤56.8%, respectively, suggesting that H6206, H6219, H6222, H6235, H6242, and H6253 share the same or overlapping epitopes. In contrast, H6210 at a concentration of 300 μg/ml could only partially inhibit the binding of biotinylated-H6225 and biotinylated-H6249 to rHEV ORF-G4 protein at 38.0 and 27.4%, respectively. Similar relationships showing partial inhibition were seen between H6225 and biotinylated-H6210 or -H6249 (54.2 and 52.5%, respectively) and between H6249 and biotinylated-H6210 or -H6225 (55.4 and 48.3%, respectively). An mAb (No. 905) that recognizes a secretory protein of hepatitis B virus (hepatitis B e antigen) served as the negative control and showed no significant inhibition of biotinylated-H6210, -H6225, and -H6249 (0–4.2% inhibition). These results suggested that each of these three mAbs (H6210, H6225, and H6249) probably recognizes a different but spatially related epitope.

Western blotting was carried out against the purified rHEV ORF2-G4 protein that had or had not been denatured at 100°C for 5 min in the presence of 2-mercaptoethanol. The nine mAbs bound with the undenatured rHEV ORF2-G4 protein to various degrees in Western blotting (Fig. 3). Figure 3 showed that the untreated protein mainly migrated

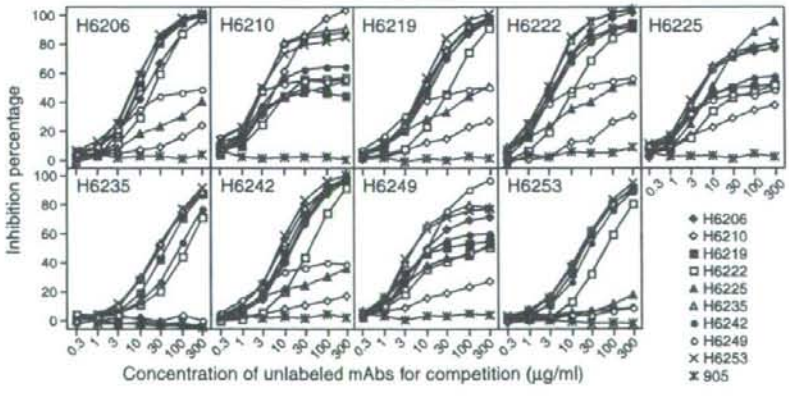


**Fig. 2** Competition of non-labeled mAb in increasing concentrations (0.3–300 μg/ml) against biotinylated mAb (10 μg/ml) for binding with a recombinant HEV ORF2 protein of genotype 4 immobilized on a solid support. An mAb (No. 905) was used as a negative control

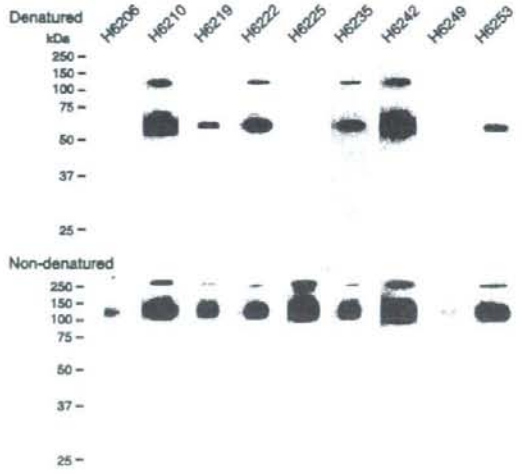


**Fig. 3** Western blotting of a recombinant HEV ORF2 protein (aa 111–660) of genotype 4 by mAbs. A recombinant ORF2 protein (rHEV ORF2-G4) that had (*top*) or had not (*bottom*) been denatured in the presence of 2-mercaptoethanol at 100°C for 5 min, was run on SDS-PAGE and tested for binding with nine mAbs raised in the present study

**Table 1** Reactivity of mAbs with HEV particles evaluated by immuno-capture PCR

| mAb no. | Copies of captured HEV (% of total HEV) per well | | |
|---|---|---|---|
| | Genotype 1[a] (22,929 copies)[d] | Genotype 3[b] (33,883 copies)[d] | Genotype 4[c] (295 copies)[d] |
| H6206 | 135 (0.6) | 248 (0.7) | 3 (1.0) |
| H6210 | 28 (0.1) | 66 (0.2) | 4 (1.4) |
| H6219 | 753 (3.3) | 181 (0.5) | 2 (0.7) |
| H6222 | 63 (0.3) | 127 (0.4) | 5 (1.7) |
| H6225 | **22,685 (98.9)** | **32,528 (96.0)** | **292 (99.0)** |
| H6235 | 60 (0.3) | 187 (0.6) | 8 (2.7) |
| H6242 | 152 (0.7) | 210 (0.6) | 8 (2.7) |
| H6249 | 29 (0.1) | 136 (0.6) | 7 (2.4) |
| H6253 | 178 (0.8) | 206 (0.6) | 3 (1.0) |
| 905[e] | 54 (0.2) | 135 (0.4) | 8 (2.7) |

[a] Fecal supernatant from a patient who contracted infection of genotype 1 HEV

[b] Fecal supernatant from a patient who contracted infection of genotype 3 HEV

[c] Fecal supernatant from a patient who contracted infection of genotype 4 HEV

[d] Total copy number of HEV applied to each well

[e] mAb against hepatitis B e antigen as a negative control [39]

as a dimer with an approximate molecular weight of 125 kDa, along with minute amounts of higher oligomers, and that the treated protein migrated as a monomeric form of 61 kDa as reported previously [28], along with minute amounts migrating as 125 kDa dimers. Among the three mAbs (H6210, H6225, and H6242) with the strongest binding with the untreated protein, H6225 showed little or no reactivity with the monomeric form, while H6210 and H6242 were reactive with both the monomeric and dimeric forms.

## Reactivity with HEV particles

To determine whether the mAbs could capture native HEV particles in feces, real-time RT-PCR was used to detect HEV RNA captured by the mAbs quantitatively. As indicated in Table 1, only one mAb, H6225, was able to capture HEV efficiently regardless of genotype. Neither the remaining eight mAbs raised in the present study nor the control mAb (No. 905) could capture the virus.

## Neutralization of HEV by mAb H6225

An mAb, H6225, was tested for its ability to neutralize an HEV strain of genotype 3 (JE03-1760F) in a recently developed cell culture system [46]. H6225 at a concentration of 2, 20, or 200 μg/ml was mixed with an equal volume of the diluted virus stock containing $8.0 \times 10^5$ copies/ml of HEV, kept at room temperature for 60 min, and then inoculated on monolayers of PLC/PRF/5 cells in a 6-well microplate. When cells were inoculated with HEV that had been incubated with 100 μg/ml of control mAb (No. 905), HEV RNA was first detected in the culture medium 14 days post-inoculation (dpi) and continued to be detectable up to the end of the observation period of 60 days, reaching a maximum load of $9.4 \times 10^4$ copies/ml (Fig. 4a). In contrast, in wells inoculated with HEV that had been incubated with 100 μg/ml of mAb H6225, HEV RNA was not detectable throughout the observation period of up to 60 days. In wells with a lower concentration of H6225 (10 or 1 μg/ml), HEV RNA was not detectable until 46 and 18 dpi, respectively, and the HEV viral load continued to be low ($<10^4$ copies/ml) up to the end of the observation period, indicating that the infection caused by non-neutralized virus or escape virus was significantly delayed. Even when cells were inoculated with a tenfold higher load of HEV ($4.0 \times 10^6$ copies/ml) that had been incubated with 100 μg/ml of H6225, the harvested culture supernatants were negative for HEV RNA throughout the observation period of 60 days (Fig. 4b). Reflecting the higher viral load of HEV inoculated, HEV RNA was first detected 30 dpi when HEV was incubated with 10 μg/ml of H6225.

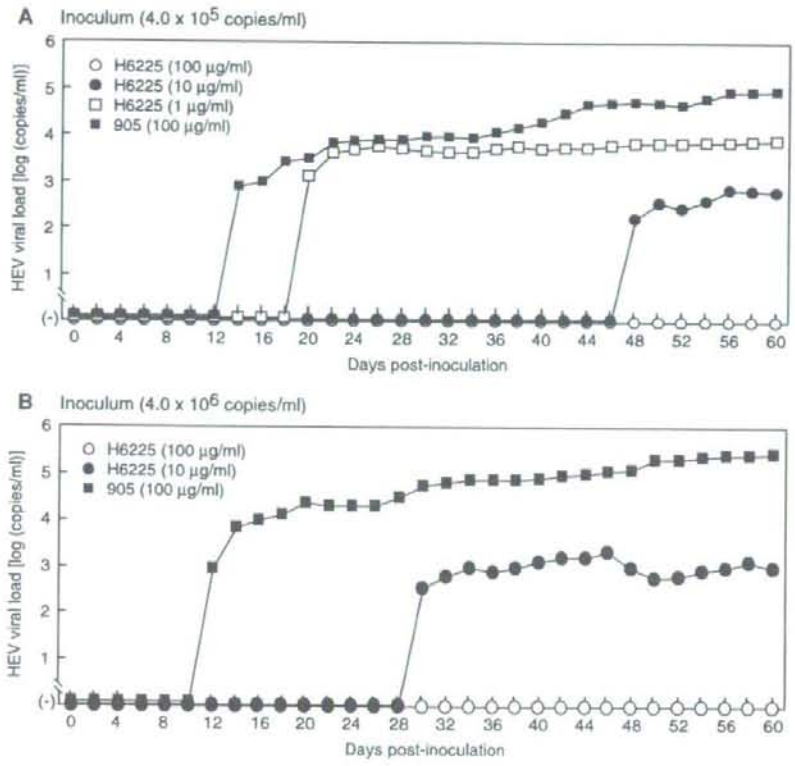


**Fig. 4** Quantitation of HEV RNA in culture supernatants of PLC/PRF/5 cells inoculated with a fecal supernatant containing an HEV (JE03-1760F) strain with a viral load of $4.0 \times 10^5$ copies/ml (**a**) or $4.0 \times 10^6$ copies/ml (**b**) that had been mixed with mAb H6225 (1, 10, or 100 µg/ml) or a negative control mAb [No. 905 (100 µg/ml)] and cultured for the indicated number of days

## Discussion

In the present study, nine mAbs against a recombinant HEV ORF2 protein of genotype 4 (rHEV ORF2-G4) were produced and characterized, and one of these mAbs could efficiently neutralize the infectivity of HEV in a cell culture system. Production of mAbs against HEV ORF2 proteins or peptides has already been reported by several research groups. Briefly, 14 mAbs against the recombinant ORF2 protein of the genotype 1 Sar-55 Pakistan strain of HEV were first isolated by phage display from a cDNA library of chimpanzee antibody genes [36, 37]. Seven mouse mAbs were generated following immunization with a fusion ORF2 protein with a hexahistidine tag, representing the C-terminal 267 aa of the 660-aa capsid protein of the Chinese genotype 1 HEV strain, that was expressed in *Escherichia coli* (*E. coli*) [34]. In addition, eight murine mAbs were raised against the recombinant peptide E2, comprising aa 394–606 of the capsid protein of a Chinese genotype 1 HEV strain, which was expressed as a nonfusion protein in *E. coli* [52], and 27 murine mAbs were produced using a recombinant ORF2 protein of Pakistan HEV Sar-55 that was expressed in an insect cell line as an immunogen [6]. Although a number of antigenic determinants have been identified within ORF2 [14, 15, 19, 34], it is unlikely that all of them represent neutralization epitopes. In fact, among the 56 mAbs thus far reported in the literature, neutralizing activity was demonstrated in five mAbs including two chimpanzee mAbs (HEV#4 and HEV#31) that were isolated by phage display [36], and three murine mAbs (8C11 and 8H3 [52]; Mab 7 [6]). In the present study, only one mAb (H6225) exhibited neutralizing activity. The remaining eight mAbs obtained in the present study may recognize epitopes that are not accessible on the surface of virions.

Propagation and production of HEV in vitro had been attempted in various continuous cell lines including the cell line (PLC/PRF/5) used in the present study [7–9, 13, 18, 22, 23, 50] and in primary hepatocytes from nonhuman primates [2, 12, 43–45, 48]. However, an in vitro cell culture system supporting efficient multiplication of HEV had not been established until recently. The neutralizing activity of mAbs 8C11 and 8H3 was tested in rhesus monkeys [52], and that of Mab 7 by an RT-PCR-based seroneutralization assay [6]. The RT-PCR-based seroneutralization assay involved the detection of the genomic

RNA of HEV virions adsorbed on the surface of PLC/PRF/5 cells in culture. The mAb Mab 7 blocked adsorption of HEV to the surface of PLC/PRF/5 cells in culture, suggesting neutralization capability of Mab 7. However, only those antibodies that block binding of virus to cells would be regarded as neutralizing, and antibodies that neutralize at a step after binding would not be seen as neutralizing in their test. In addition to neutralization tests in rhesus monkeys [36], a quantitative cell-culture assay was conducted to determine the neutralizing ability of two chimpanzee mAbs (HEV#4 and HEV#31) by detecting infected HepG2/C3A cells by immunofluorescence microscopy [4]. However, progenies of inoculated HEV (Akluj and Sar-55, both genotype 1) were not released in the supernatant of culture cells, and attempts to determine neutralization ability in vivo and in vitro gave inconsistent results [4]. Summarizing the difference between the present study and previously reported studies with regard to the immunogen and evaluation of neutralizing activity of anti-HEV mAbs, our mAbs were generated following immunization with a genotype 4 HEV ORF2 protein, and the neutralizing activity of mAbs was evaluated in an efficient cell culture system for HEV that can release high-titer progenies in the culture medium.

Recently, using a fecal suspension with high HEV load ($2.0 \times 10^7$ copies/ml) as an inoculum, obtained from a Japanese patient who contracted domestic infection of genotype 3 HEV (the JE03-1760F strain), an efficient cell culture system for HEV in PLC/PRF/5 cells was developed which yielded the highest HEV load of up to $10^8$ copies/ml in the culture supernatant, and five generations of serial passages of culture supernatant was successfully propagated [46]. The JE03-1760F genome had 29 unique nucleotides that were not seen in any of the 25 reported HEV isolates of the same genotype over the entire genome, with 6 amino acid substitutions in the ORF1 protein [41]. It seems likely that these substitutions could be candidates for mutations associated with the capability of efficient replication in a cell culture system. In a previous study [46], serum samples with IgG, IgM, and IgA classes of anti-HEV antibodies that were obtained from patients infected with HEV of genotype 1, 3 or 4 during the convalescent phase neutralized the genotype 3 JE03-1760F strain in PLC/PRF/5 cells, and serum samples obtained from patients with IgG anti-HEV antibodies 8.7 or 24.0 years after the onset of HEV infection that are detectable by in-house ELISA [28, 40] also prevented propagation of HEV in PLC/PRF/5 cells. Taken together, the established efficient cell culture system for HEV is useful for evaluating the neutralizing activity of various specimens containing polyclonal or monoclonal anti-HEV antibodies in vitro.

Although a single serotype has been proposed, the HEV genomes are extremely heterogeneous and are classified into four major genotypes, and further into several subtypes (subgenotypes) within each genotype [3, 20, 30, 35]. The effect of sequence heterogeneity on the antigenic properties of HEV, especially on the neutralization epitope(s), has not been fully investigated. In the present study, five of the nine mAbs obtained reacted equally with three recombinant HEV ORF2 proteins of genotypes 1, 3, and 4 at all mAb concentrations tested, while the remaining four mAbs showed genotype-dependent reactivity at the lower mAb concentrations of 0.01–0.1 μg/ml. Of note, mAb H6225 exhibited relatively lower binding affinity with a recombinant genotype 3 ORF2 protein (rHEV ORF2-G3) immobilized on a solid support but was able to cross-neutralize a genotype 3 HEV strain (JE03-1760F) in PLC/PRF/5 cells efficiently when 100 μg/ml of H6225 was mixed with HEV prior to inoculation. Even when cells were inoculated with the genotype 3 HEV strain with a viral load of $4.0 \times 10^6$ or $4.0 \times 10^5$ copies/ml that had been mixed with a lower concentration of H6225 (10 μg/ml), HEV RNA in culture supernatant continued to be undetectable until 28 or 46 dpi, respectively, suggesting that, due to incubation with H6225 prior to inoculation, the infection of the virus was inhibited at least in part, and the release of HEV progenies in the culture supernatant was markedly delayed. Meng et al. [24] reported that antibodies against the pB166 protein (aa 452–617 of the ORF2 protein of the HEV Burma strain) neutralized the Burma (genotype 1), Pakistan (genotype 1), MEX-14 (genotype 2), and US1 (genotype 3) strains, but neutralized the Morocco (genotype 1) strain with lower efficiency. In addition, in the rhesus neutralization assay, chimpanzee mAb HEV#31 neutralized both the Sar-55 and MEX-14 strains completely, but was unable to neutralize the infection of Meng (genotype 3) [4]. Therefore, although H6225 can efficiently capture HEVs of genotypes 1, 3, and 4 and, at present, only a single strain of HEV (JE03-1760F) can be propagated in our culture system, it should be investigated in future studies whether H6225 is able to neutralize HEV strains with significant sequence divergence.

In conclusion, one mAb (H6225) against a recombinant genotype 4 HEV ORF2 protein that can efficiently neutralize the infection of a heterologous HEV strain (JE03-1760F, genotype 3) in our recently established cell culture system was obtained in the present study, which furthered the usefulness of the cell culture system capable of supporting efficient propagation of infectious HEV. Since discrepant results have been reported on the neutralization capability of mAbs against HEV ORF2 protein in vivo (in rhesus monkeys) and in vitro (by a quantitative cell culture assay using immunofluorescence microscopy) [4], it would be important to investigate whether our culture system is as convincing as animal models for evaluation of the neutralization activity of HEV antibodies.

**Acknowledgments** This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labor and Welfare of Japan.

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Arch Virol (2008) 153:1703–1713
DOI 10.1007/s00705-008-0179-6

ORIGINAL ARTICLE

# Monoclonal antibodies raised against the ORF3 protein of hepatitis E virus (HEV) can capture HEV particles in culture supernatant and serum but not those in feces

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Received: 9 June 2008 / Accepted: 23 July 2008 / Published online: 5 August 2008


**Abstract** Ten murine monoclonal antibodies (MAbs) against a synthetic peptide corresponding to the well-conserved, C-terminal 24-amino acid portion of ORF3 protein of hepatitis E virus (HEV) were produced and characterized. Immunofluorescent assays using the anti-ORF3 MAbs revealed accumulation of ORF3 protein in the cytoplasm of PLC/PRF/5 cells transfected with ORF3-expressing plasmid or inoculated with cell-culture-generated HEV. The anti-ORF3 MAbs could capture HEV particles in culture medium and serum at variable efficiency of up to 61 and 49%, respectively, but not those in feces. By sandwiching between immobilized and enzyme-labeled anti-ORF3 MAbs in ELISA, ORF3 antigen was detected in the culture media with an HEV RNA titer of $>10^6$ copies/ml and increased in parallel with the increase in HEV load. HEV progenies in the culture supernatant, with ORF3 protein on the surface, banded at a low buoyant density of 1.15 g/cm$^3$ in sucrose. A representative anti-ORF3 MAb (TA0536) could partially neutralize the infection of cell-culture-generated HEV in a cell culture system. These results indicate that ORF3 protein, at least its C-terminal portion, is present on the surface of HEV virions released from infected cells and support a previously proposed assumption that ORF3 protein is associated with virus release from infected cells.

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## Introduction

Hepatitis E virus (HEV) has long been known as a major cause of acute viral hepatitis in many developing countries where the sanitation conditions are suboptimal, with occasional travel-related cases of hepatitis E in industrialized countries [8]. However, sporadic cases of hepatitis E that are not related to travel to endemic areas have been increasingly recognized in numerous industrialized countries including the USA, European countries and Japan, where autochthonous hepatitis E appears to be an emerging disease [1, 12, 14, 19, 22, 29, 35]. Accumulating lines of evidence suggest that hepatitis E is a zoonosis [24–26, 32–34, 38, 41, 42]. Recently, convincing evidence that HEV might be transmitted by eating raw or undercooked meat was obtained [47, 52]. HEV is transmitted primarily by the fecal-oral route [3, 31], but persons with HEV infection go through a viremic phase, resulting in the potential of blood-borne spread of the infection [2, 23, 28]. Hepatitis E is typically a self-limiting disease with variable severity, presenting as acute icteric hepatitis with clinical symptoms similar to those of hepatitis A [8]. HEV infection runs an acute course, normally resulting in resolution within a few weeks after onset, with mortality ranging from 0.5 to 1% among the general population to as high as 20% among infected pregnant women [8, 17]. Recently, chronic or persistent HEV infection in organ-transplant recipients has been described [11, 16].

HEV is a non-enveloped virus with a single-stranded, positive-sense RNA genome of 7.2 kb [8] and is classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [4]. The genome is capped and polyadenylated, and contains a short 5′ untranslated region (UTR) followed by three open reading frames (ORFs: ORF1,

ORF2 and ORF3), and then a short 3′UTR [45]. The 5′ two-thirds of the genome contains ORF1, which encodes non-structural proteins involved in viral replication and viral protein processing. ORF2 occupies the 3′-terminal part of the genome and encodes a 660-amino acid (aa) capsid protein. ORF3 overlaps with ORF2 and encodes a small protein. Recently, it was demonstrated that ORF2 and ORF3 proteins are encoded by a bicistronic subgenomic RNA [10], and that initiation of translation at the third in-frame AUG codon of ORF3 is essential for viral infectivity in vivo [9, 13].

There are four recognized genotypes of HEV that infect humans: genotypes 1 and 2 are thought to infect humans and non-human primates exclusively, whereas genotypes 3 and 4 have been isolated from humans as well as pigs, wild boars, a deer and a mongoose [24, 30, 39, 47]. Genotype 1 is responsible for the majority of HEV infections in developing countries in Asia and Africa; genotype 2 consists of strains that are found not only in Mexico but also in African countries; genotype 3 is widely distributed throughout the world; and genotype 4 is distributed exclusively in Asian countries [4, 21, 33, 37].

Recently, using a fecal suspension with high HEV load ($2.0 \times 10^7$ copies/ml) as an inoculum, obtained from a Japanese patient who contracted domestic infection of genotype 3 HEV (the JE03-1760F strain) [43], we developed an efficient cell culture system for HEV in PLC/PRF/5 and A549 cells, which yielded the highest HEV load of $10^8$ copies/ml in the culture supernatant, and the HEV strain was successfully propagated for 24 generations of serial passages of culture supernatant [46; unpublished observations].

It has been suggested that the ORF3 protein may be involved in HEV virion morphogenesis and viral pathogenesis [48, 49] and may be associated with virion release from infected cells [7], but it remains unknown whether or not ORF3 protein is a component of virions. In the present study, monoclonal antibodies (MAbs) against a synthetic peptide corresponding to the C-terminal 24-aa portion of ORF3 protein were generated and characterized, and their ability to bind HEV virions in cell culture, serum and fecal specimens was evaluated. Using a newly developed enzyme-linked immunosorbent assay (ELISA) method with two pairs of anti-ORF3 MAbs, HEV ORF3 antigen was detected in the culture media, and the amount of ORF3 antigen increased in parallel with the HEV viral load. Furthermore, one representative anti-ORF3 MAb that was capable of more efficiently binding cell-culture-produced HEV virions was assessed for its ability to neutralize HEV in a recently established cell culture system [46].


**Fig. 1** Amino acid sequences of three synthetic peptides representing the well-conserved, C-terminal portion of ORF3 protein. The ORF3 amino acid sequence of aa 90–113 of the JE03-1760F strain (genotype 3) is depicted at the top. Three synthetic peptides with an additional C at their N termini (spORF3-C24, spORF3-C10L and spORF3-C10P) are illustrated in the middle, and the sequence corresponding to spORF3-C24 is shown for 27 genotype 3 HEV isolates, 31 genotype 4 HEV isolates, 17 genotype 1 HEV isolates, and one genotype 2 HEV isolate at the bottom, whose entire genomic sequence is retrievable from GenBank/DDBJ/EMBL databases as of May 20, 2008. The number of HEV isolates with the indicated amino acid sequence is shown on the *right*

## Materials and methods

### Preparation of HEV ORF3 synthetic peptides

An oligopeptide (spORF3-C24) with a sequence of CLG ATS PSA PPL PPV VDL PQL GLR R, covering aa 90–113 at the C-terminus of the ORF3 protein of the JE03-1760F strain (accession no. AB301710), was synthesized by the F-moc peptide technique and purified by high-pressure liquid chromatography; C at the N-terminus was added to facilitate its conjugation to keyhole limpet hemocyanin or bovine serum albumin (BSA; Oriental Yeast Co. Ltd, Tokyo, Japan). Additionally, two shorter oligopeptides with C at the N-terminus spanning aa 104–113 of the ORF3 protein of various HEV strains were synthesized, with a sequence of CVD LPQ LGL RR (spORF3-C10L) and with a sequence of CVD LPQ PGL RR (spORF3-C10P): the C-terminal 24-aa region of the ORF3 protein is well-conserved among HEV isolates (Fig. 1).

### Production of MAbs

MAbs were raised against spORF3-C24 conjugated with keyhole limpet hemocyanin by a method described

elsewhere [51] with slight modifications. Briefly, BALB/c mice were injected twice intraperitoneally with 73 μg of the spORF3-C24 conjugated with keyhole limpet hemocyanin in complete Freund's adjuvant (DIFCO Laboratories, Detroit, MI, USA) on day 0 and in incomplete adjuvant (DIFCO Laboratories) on day 14, followed by intravenous injection of 36 μg of the same antigen (without adjuvant) on day 42, 3 days before fusion. NS-1 myeloma cells were fused with immunized spleen cells at a ratio of 1:10. The screening of MAbs was done by ELISA using the spORF3-C24 conjugated with BSA immobilized on wells of an immunoplate (part no. 762071; Greiner Bio-One GmbH, Frickenhausen, Germany). Bound antibodies were detected by peroxidase-conjugated sheep affinity-purified antibody to mouse IgG or IgM (Cappel/MP Biochemicals, Solon, OH, USA). Hybridomas secreting antibodies against spORF3-C24 of desired specificity were propagated in the peritoneal cavity of mice that had been made ascitic by the injection of 2,6,10,14-tetramethylpentadecane. Ascites fluid was harvested approximately 10 days after implantation, and γ-globulin fractions were precipitated with 2 M $(NH_4)_2SO_4$ and then purified by gel filtration in Sephadex G-200 (GE Healthcare UK Ltd, Buckinghamshire, England). MAbs were tested for their immunoglobulin class/subclass using a mouse monoclonal antibody isotyping kit (Bio-Rad Laboratories, Hercules, CA, USA).

### ELISA for determination of binding with synthetic HEV ORF3 peptides

Wells of microplates (Greiner Bio-One GmbH) were coated with 100 μl of synthetic peptides [spORF3-C24, spORF3-C10L, or spORF3-C10P: 5 μg/ml in 10 mM phosphate-buffered saline, pH 7.5 (PBS)] and incubated at 25°C for 18 h. Two hundred microliters of PBS containing 0.1% (wt/vol) BSA (PBS-BSA) was added. The microplates were incubated at room temperature for 2 h with shaking. The blocking buffer was discarded, and each well was washed with saline containing 0.05% (vol/vol) Tween 20 (Bio-Rad Laboratories). One hundred microliters of MAbs (0.001–3 μg/ml) diluted with PBS-BSA was added to each well. The microplates were incubated at room temperature for 1 h with gentle agitation and were then washed with washing solution (0.05% Tween 20 in saline). One hundred microliters of PBS containing 25% (vol/vol) fetal bovine serum (FBS; Sigma–Aldrich, St Louis, MO, USA) and peroxidase-conjugated goat IgG fraction to mouse IgG (whole molecule) (Cappel/MP Biochemicals) or peroxidase-conjugated affinity-purified anti-mouse IgMμ [mu-chain-specific (goat)] (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) was added to each well. The microplates were incubated at room temperature for 1 h with gentle agitation and then washed. One hundred microliters of tetramethylbenzidine (TMB) soluble reagent (BioFX Laboratories Inc., Owings Mills, MD, USA) as a substrate was added to each well. The plate was incubated at room temperature for 30 min in the dark, and then 100 μl of TMB stop buffer (BioFX Laboratories Inc.) was added to each well. The optical density (OD) of each sample was read at 450 nm.

### Plasmid construction

An expression plasmid for the full-length ORF3 protein (aa 1–113) of the HEV JE03-1760F strain, pCI-HEVORF3, was constructed as follows. The coding sequence of full-length ORF3 was amplified by reverse transcription (RT)-PCR using the primer set, sense primer [5′-GCT AGC CAC CAT GGG ATC ACC ATG CGC CCT-3′ (an *Nhe*I site is underlined)] and antisense primer [5′-ACT CTA GAT TCA ACG GCG CAG CCC CAG CT-3′ (a *Xba*I site is underlined)] and KOD Plus ver. 2 (TOYOBO, Osaka, Japan). The amplified blunt-ended fragment was added with an A-overhang using the A-Addition Kit (Qiagen KK, Tokyo, Japan) and then cloned into pT7 Blue T vector (Novagen, Madison, WI, USA). After confirmation by restriction enzyme digestions and nucleotide sequencing, the insert was excised and ligated into the *Nhe*I-*Xba*I site of pCI vector (Promega, Madison, WI, USA), generating a recombinant plasmid, pCI-HEVORF3.

### Plasmid transfection

A hepatocarcinoma cell line (PLC/PRF/5) obtained from American Type Culture Collection (ATCC no. CRL-8024: Manassas, VA, USA) was grown as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) that had been heat-inactivated at 56°C for 30 min, 100 U/ml of penicillin G, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin B at 37°C in a humidified 5% $CO_2$ atmosphere, as described previously [46]. The PLC/PRF/5 cells in a 24-well plate (BD Sciences, San Jose, CA, USA) were transfected with 0.5 μg of pCI-HEVORF3 or pCI vector using TransIT-LT1 reagent (Mirus Bio, Madison, WI, USA) according to the manufacturer's instructions and incubated at 37°C for 48 h. The transfected cells were subjected to immunofluorescent assay as described below.

### Indirect immunofluorescence

PLC/PRF/5 cells transfected with pCI-HEVORF3 or pCI vector, and PLC/PRF/5 cells on the 12th day after inoculation with cell-culture-produced HEV of the JE03-1760F strain (third passage: $3.0 \times 10^7$ copies/well) [46], were fixed in 4% (vol/vol) paraformaldehyde at room temperature for

20 min and permeabilized in 0.2% (vol/vol) Triton X-100 at room temperature for 10 min. After washing with PBS, the fixed cells were incubated with an anti-HEV ORF3 MAb (10 μg/ml in PBS) at 37°C for 1 h. After washing with PBS, the cells were stained with Alexa Fluor 488-conjugated anti-mouse IgG or IgM (Molecular Probes, Invitrogen) (2 μg/ml in PBS) at 37°C for 1 h. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Roche Diagnostics K·K, Tokyo, Japan). Images of stained cells were obtained using a BZ-8000 digital microscope (Keyence, Osaka, Japan), and the obtained images were processed with BZ-Analyzer software (Keyence), or, alternatively, images were obtained using an IN Cell Analyzer 1000 (GE Healthcare).

Biotinylation of MAbs and immuno-capture PCR

One milliliter of PBS containing MAb (2 mg/ml) was mixed with 27 μl of 10 mM Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL), with a molecular ratio of IgG to biotin of 1:20, and incubated on ice for 2.5 h. The biotinylated MAbs were purified by gel filtration using a PD-10 column (GE Healthcare) and aliquoted and stored at −20°C until assay.

To evaluate the virus-binding ability of MAbs, immuno-capture PCR was performed. Briefly, the wells of a Reacti-Bind Streptavidin High Binding Capacity Coated Plate (Pierce Biotechnology) were washed with saline three times, and 100 μl of 1 μg/ml biotinylated MAb in PBS-BSA containing 0.2% Tween 20 was added to each well. The wells were incubated with gentle shaking at room temperature for 1 h and were then washed five times with saline. One hundred microliters of sample containing HEV of genotype 3 (fecal supernatant, serum or culture medium) was added to each well and incubated with shaking at room temperature for 2 h and then incubated without shaking at 4°C overnight. The solution in each well was removed, and the wells were washed three times with saline. One hundred fifty microliters of Trizol-LS reagent (Invitrogen) and 50 μl of distilled water were directly added twice to each well. The RNA was then extracted and subjected to quantitative detection of HEV RNA as described below.

Quantitation of HEV RNA

Quantitation of HEV RNA was performed by real-time detection RT-PCR according to a previously described method [15] with a slight modification. In brief, extracted RNA was subjected to real-time RT-PCR with a Quanti-Tect Probe RT-PCR Kit (Qiagen KK), sense primer (5′-GGT GGT TTC TGG GGT GAC-3′), antisense primer (5′-AGG GGT TGG TTG GAT GAA-3′), and a probe consisting of an oligonucleotide with a 5'-reporter dye (FAM) and a 3′-quencher dye (TAMRA) (5′-FAM-TGA TTC TCA GCC CTT CGC-TAMRA-3′) in a LightCycler Apparatus (Roche Diagnostics K·K.). Thermal cycler conditions were 50°C for 20 min at stage 1; 95°C for 15 min at stage 2; and 45 cycles of 95°C for 0 s and 60°C for 60 s at stage 3. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate, and the mean value was adopted.

ELISA for detection of HEV ORF3 antigen

Wells of microplates were coated with 50 μl of MAbs (TA0512 or TA0536: 20 μg/ml in saline) and incubated at 25°C for 18 h. The antibody solution was discarded, and each well was washed with washing solution. Fifty microliters of culture supernatant containing cell-culture-produced HEV and 0.2% Tween 20 was added to each well. The microplates were incubated at room temperature for 1 h with gentle agitation and were then washed with washing solution. Fifty microliters of PBS containing 25% FBS and biotinylated MAb TA0546 was added to each well. The microplates were incubated at room temperature for 1 h with gentle agitation and then washed. Fifty microliters of PBS containing 25% FBS and peroxidase-conjugated streptavidin (Pierce Biotechnology) was added to each well. The microplates were incubated at room temperature for 1 h with gentle agitation and then washed. Fifty microliters of TMB soluble reagent was added to each well. The plate was incubated at room temperature in the dark for 30 min, and then 50 μl of TMB stop buffer was added to each well. The OD of each sample was read at 450 nm.

Equilibrium centrifugation in a sucrose density gradient

A sucrose density gradient was prepared in an SW28 tube [5.6 ml of 60%, 4.2 ml each of 50, 40, 30 and 20%, and 2.1 ml of 10% (wt/wt) sucrose in TE buffer supplemented with 150 mM NaCl (TEN)]. Five milliliters of culture supernatant ($2.0 \times 10^8$ copies/ml) of HEV-infected PLC/PRF/5 cells was layered onto the surface of the gradient and overlaid with 5 ml of TEN. The tube was centrifuged at $82{,}705 \times g$ at 10°C for 48 h, and then 1-ml fractions were recovered from the surface. The density of each fraction was measured by refractometry.

Neutralization assay in a cell culture system

PLC/PRF/5 cells were grown as described above. One hundred microliters of culture supernatant containing cell-culture-generated HEV of the JE03-1760F strain ($8.0 \times 10^5$ copies/ml) as an inoculum that had been diluted with PBS without $Ca^{2+}$ and $Mg^{2+}$ [PBS(−)]

containing 0.2% (wt/vol) BSA (Sigma–Aldrich) and filtered, was mixed with an equal volume of each of the MAb solutions (TA0536 or 905: 0.2 or 2 mg/ml) and kept at room temperature for 1 h. Monolayers of PLC/PRF/5 cells in wells (diameter of 3.5 cm) of a six-well microplate (IWAKI, Tsukuba, Japan) were washed three times with 1 ml of PBS(−), and 0.2 ml of the virus stock mixed with MAb was inoculated onto the cells. One hour after inoculation at room temperature, the solution was removed and 2 ml of maintenance medium was added. The maintenance medium used for virus culturing consisted of 50% DMEM and 50% medium 199 (Invitrogen) containing 2% heat-inactivated FBS and 30 mM $MgCl_2$ at final concentration. Other supplements were the same as those in the growth medium. The culture was done at 35.5°C in a humidified 5% $CO_2$ atmosphere. On the day following inoculation, the inoculated cells were washed five times with 1 ml of PBS(−), and then 2 ml of maintenance medium was added. Then, every other day, one half (1 ml) of the culture medium was replaced with fresh maintenance medium, and the collected medium was stored at −80°C until virus titrations were performed. The HEV load was determined for all or selected series of culture supernatants from the inoculated wells, and representative data were used.

## Results

### Production and characterization of MAbs

Ten hybridoma clones secreting MAbs against synthetic HEV ORF3 peptide [spORF3-C24 (Fig. 1)], designated TA0503, TA0512, TA0523, TA0529, TA0531, TA0536, TA0537, TA0541, TA0546, and TA0559, were obtained in the present study. Three of these (TA0512, TA0531, TA0537) were of the IgG1 subclass, two (TA0536 and TA0541) were of the IgG2a subclass, and the remaining five were of the IgM class. All ten MAbs reacted specifically with the spORF3-C24 peptide, which was used as an immunogen in a manner dependent on the concentration of MAb added as the primary antibody (Fig. 2). At the concentrations of 0.3, 1, and 3 μg/ml, the OD values of antibody reacting with the immobilized spORF3-C24 peptide were exclusively >3.0 for all ten MAbs (data not shown for the concentrations of 1 and 3 μg/ml in Fig. 2). Of note, TA0559 reacted equally well with two additional peptides [spORF3-C10L and spORF3-C10P (Fig. 1)] covering aa 104–113 of the HEV ORF3 protein at concentrations of >0.03 μg/ml (Fig. 2). Two other MAbs (TA0529 and TA0546) showed lower binding affinity to spORF3-C10L, having the same amino acid at position 109 as the spORF3-C24 used as the immunogen, and even lower binding affinity to spORF3-C10P. The remaining seven MAbs reacted with neither of the 11-mer synthetic peptides.

To further explore the specificity of the ten MAbs, they were used in indirect immunofluorescence against full-length ORF3 protein expressed in PLC/PRF/5 cells using plasmid pCI-HEVORF3 (Fig. 3a). All ten MAbs showed strong reactivity with the ORF3 protein in the cytoplasm of the transfected cells. Although three MAbs (TA0529, TA0546, and TA0559) gave non-specific signals in the pCI-transfected cells, which served as a negative control, reactivity of the remaining seven MAbs was not detectable in the negative control cells, ensuring specific reactivity with the ORF3 protein. MAbs TA0536 and TA0541 showed a strong signal in the cytoplasm and cell membrane of the PLC/PRF/5 cells inoculated with cell-culture-generated HEV (Fig. 3b; data not shown for

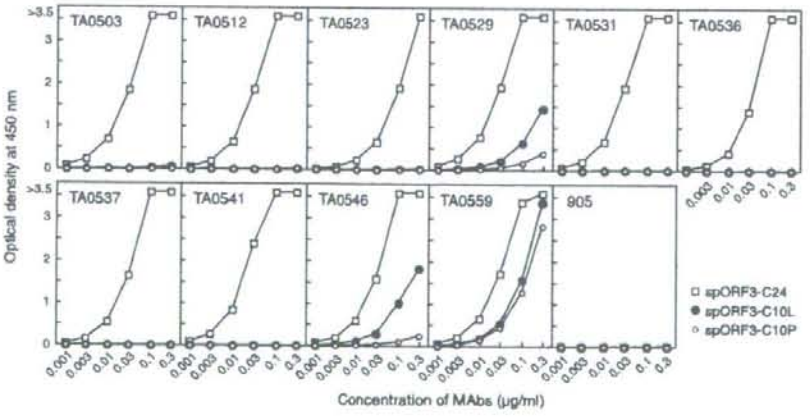


**Fig. 2** Binding of ten MAbs in increasing concentrations with the immobilized synthetic peptide (spORF3-C24, spORF3-C10L, or spORF3-C10P) corresponding to the C-terminal portion of the HEV ORF3 protein. As a negative control, a MAb (No. 905) raised against a secretory protein (hepatitis B e antigen) of hepatitis B virus [40] was used

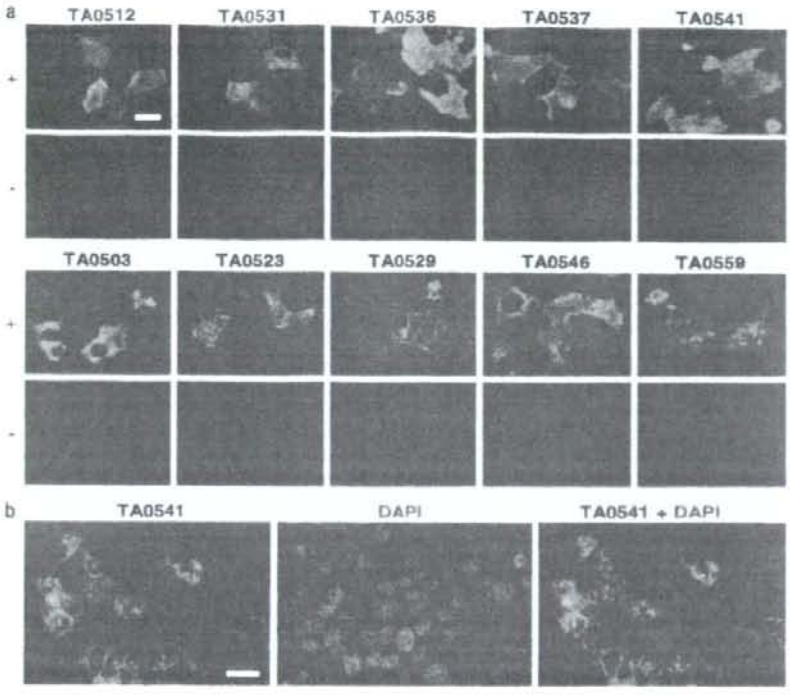


**Fig. 3** Indirect immunofluorescence staining of ORF3 protein in PLC/PRF/5 cells with anti-ORF3 MAbs. **a** PLC/PRF/5 cells transfected with pCI-HEVORF3 (+) or pCI (−), were stained with each of the ten anti-ORF3 MAbs obtained in the present study, or with irrelevant MAb No. 905 [40] followed by AlexaFluor 488-conjugated anti-mouse IgG or IgM (data not shown for No. 905). Images of stained cells were obtained using an IN Cell Analyzer 1000 (GE Healthcare). **b** PLC/PRF/5 cells inoculated with cell-culture-produced HEV of the JE03-1760F strain [46] were incubated with MAb TA0541 and then stained with AlexaFluor 488-conjugated anti-mouse IgG. Nuclei were stained with DAPI. Images were obtained using a BZ-8000 digital microscope (Keyence). *Bar* 20 μm

TA0536), suggesting that the anti-ORF3 MAbs obtained in the present study, represented by MAb TA0541, are also reactive with the ORF3 protein in HEV-infected cells.

Reactivity with HEV particles

In an attempt to examine whether the anti-ORF3 MAbs could capture HEV particles in culture supernatant and those in serum and feces obtained from infected patients, real-time RT-PCR was performed to detect HEV RNA captured by the MAbs quantitatively (Table 1). As expected, anti-ORF2 MAb (H6225) [44], used as a positive control, could efficiently capture fecal HEV. None of the ten anti-ORF3 MAbs raised in the present study nor the negative control MAb (No. 905) [40] could capture HEV in feces. Notably, however, at least seven of the ten anti-ORF3 MAbs were able to capture HEV in culture supernatant at an efficiency of 41.7–60.5%, and two MAbs (TA0529 and TA0536) could bind serum HEV at an efficiency of 42.0–48.8%. Even after three successive immuno-capture experiments of the uncaptured fraction with MAb TA0529 or TA0536, the percentage of bound HEV did not reach 100%, and unbound HEV accounted for 10.2–16.6% of cell-culture-produced HEV and 40.9–41.5% of serum HEV (Table 2).

Detection of ORF3 antigen by ELISA in culture supernatant of HEV-infected cells

When culture supernatants containing HEV progenies of the JE03-1760F strain released in the culture medium [46] were inoculated on monolayers of PRF/PLC/5 cells in a 6-well microplate, HEV RNA was first detected in the culture supernatant at 2 days post-inoculation (dpi) at $2.0 \times 10^2$ copies/ml, and its load increased gradually, reaching $2.2 \times 10^8$ copies/ml at 60 dpi (Fig. 4). By sandwiching between MAb TA0512 immobilized on a solid support and biotinylated MAb TA0546 in ELISA, HEV ORF3 antigen was detected in the culture media after 22 dpi, when the HEV RNA titer was $>3.0 \times 10^5$ copies/ml, and the amount of HEV ORF3 antigen increased in parallel with the increase in HEV viral load (Fig. 4). When ELISA with TA0536 and TA0546 was applied to the culture supernatants, HEV ORF3 protein was also detected at 22 dpi and thereafter, with increasing OD value.

Distribution of ORF3 antigen-positive particles in sucrose density fractions of culture supernatant of HEV-infected cells

A culture supernatant of HEV-infected cells was subjected to ultracentrifugation in a sucrose density gradient.

**Table 1** Reactivity of MAbs with HEV particles evaluated by immuno-capture PCR

| MAb no. | Copies of captured HEV (% of total HEV) per well | | |
|---|---|---|---|
| | Culture supernatant[a] (52,283 copies)[d] | Serum[b] (6,569 copies)[d] | Feces[c] (13,602 copies)[d] |
| TA0503 | 10,660 (20.4) | 372 (5.7) | 30 (0.2) |
| TA0512 | **26,020 (49.8)**[e] | 2,438 (37.1) | 6 (0.0) |
| TA0523 | 2,262 (4.3) | 14 (0.2) | 7 (0.1) |
| TA0529 | **31,630 (60.5)** | **3,206 (48.8)** | 21 (0.2) |
| TA0531 | **21,780 (41.7)** | 696 (10.6) | 49 (0.4) |
| TA0536 | **30,220 (57.8)** | **2,759 (42.0)** | 35 (0.3) |
| TA0537 | 15,650 (29.9) | 104 (1.6) | 16 (0.1) |
| TA0541 | 6,574 (12.6) | 115 (1.8) | 3 (0.0) |
| TA0546 | **26,410 (50.5)** | 1,838 (28.0) | 16 (0.1) |
| TA0559 | **26,450 (50.7)** | 2,524 (38.4) | 21 (0.2) |
| H6225[f] | **27,450 (52.5)** | 881 (13.4) | **12,930 (95.1)** |
| 905[g] | 111 (0.2) | 8 (0.1) | 24 (0.2) |

[a] Culture supernatant of PLC/PRF/5 cells that had been inoculated with a culture-generated variant of the JE03-1760F HEV strain (genotype 3)

[b] Serum sample from a patient who contracted infection of genotype 3 HEV

[c] Fecal supernatant containing the JE03-1760F HEV strain

[d] Total copy number of HEV applied to each well

[e] Values of >40% are highlighted with boldtype

[f] MAb against capsid protein of HEV [44] as a positive control

[g] MAb against hepatitis B e antigen [40] as a negative control

Fractions were tested for HEV RNA titer and for the amount of ORF3 antigen-positive particles detectable by immuno-capture PCR with MAb TA0536. The amount of ORF3 antigen-positive particles peaked at the fraction of 1.15 g/cm$^3$, which contained the highest HEV RNA titer (Fig. 5).

## Neutralization of HEV by MAb TA0536

MAb TA0536 was tested for its ability to neutralize a cell-culture-produced HEV of the JE03-1760F strain in a recently developed cell culture system [46]. TA0536 at a concentration of 200 μg/ml or 2 mg/ml was mixed with an equal volume of the diluted culture supernatant containing $8.0 \times 10^5$ copies/ml of HEV, kept at room temperature for 60 min, and then inoculated onto monolayers of PLC/PRF/5 cells in a six-well microplate. When cells were inoculated with HEV that had been incubated with 1 mg/ml of control MAb (No. 905), HEV RNA was first detected in the culture medium at 8 dpi and continued to be detectable up to the end of the observation period of 60 days, reaching a maximum load of $6.8 \times 10^7$ copies/ml (Fig. 6a). In contrast, in wells inoculated with HEV that had been incubated with 1 mg/ml of MAb TA0536, HEV RNA was not detectable until 32 dpi, indicating that infection caused by non-neutralized virus or escape virus was significantly delayed. In wells with a lower concentration of TA0536

**Table 2** Bound HEV after repeated immuno-capture by MAbs

| MAb no. | Copies of captured HEV (% of total HEV) per well | |
|---|---|---|
| | Culture supernatant[a] | Serum[b] |
| TA0529 | 46,784[c] | 7,558[c] |
| Captured (first) | 27,820 (59.5) | 3,581 (47.4) |
| Captured (second)[d] | 12,390 (26.5) | 494 (6.5) |
| Captured (third)[e] | 1,784 (3.8) | 389 (5.2) |
| Uncaptured[f] | 4,790 (10.2) | 3,094 (40.9) |
| TA0536 | 46,831[c] | 7,642[c] |
| Captured (first) | 26,450 (56.5) | 3,405 (44.6) |
| Captured (second)[d] | 9,388 (20.0) | 708 (9.3) |
| Captured (third)[e] | 3,219 (6.9) | 360 (4.7) |
| Uncaptured[f] | 7,774 (16.6) | 3,169 (41.5) |

[a] Culture supernatant of PLC/PRF/5 cells that had been inoculated with a culture-generated variant of the JE03-1760F HEV strain (genotype 3)

[b] Serum sample from a patient who contracted infection of genotype 3 HEV

[c] Total copy number of HEV applied to each well

[d] Bound HEV after immuno-capture of the uncaptured fraction in the first reaction

[e] Bound HEV after immuno-capture of the uncaptured fraction in the second reaction

[f] HEV in the uncaptured fraction in the third reaction. This value was obtained by subtracting the number of copies of HEV captured during three successive immuno-captures from the total number of copies of HEV that had been applied to each well

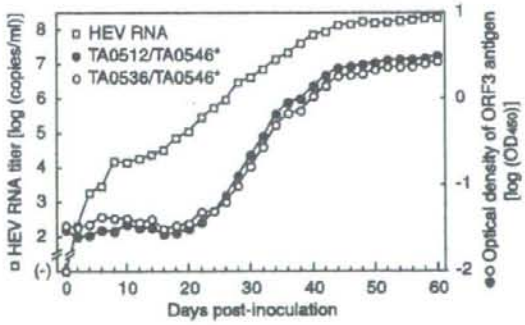


**Fig. 4** Quantitation of HEV RNA and detection of ORF3 antigen by ELISA in culture supernatants of PLC/PRF/5 cells inoculated with cell-culture-generated HEV of the JE03-1760F strain [46]. By sandwiching between MAb TA0512 or TA0536 immobilized on a solid support and biotinylated MAb TA0546 (with an asterisk) in ELISA, ORF3 antigen was detectable at 22 dpi and thereafter, with increasing OD values, in parallel with the HEV viral load

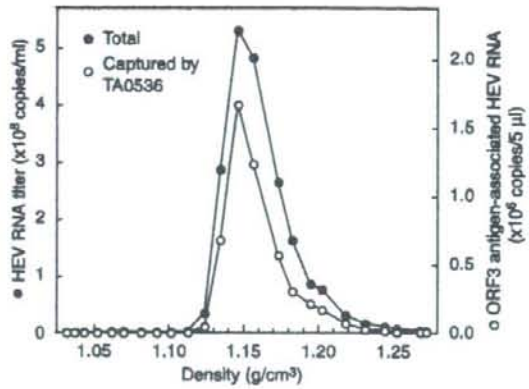


Fig. 5 Density-gradient fractionation of HEV in culture medium. Total HEV RNA and ORF3 antigen-associated HEV RNA in each fraction in a density gradient with sucrose was quantitated by real-time RT-PCR and immuno-capture RT-PCR using MAb TA0536 as a probe, respectively

(100 µg/ml), HEV RNA was detectable after 8 dpi, similar to those in the wells with control MAb (No. 905) (Fig. 6b).

## Discussion

Although the ORF3 protein contains only 113 (genotype 3) or 114 (genotype 1, 2 and 4) amino acids rather than the 122 or 123 reported previously, it has been postulated to carry out numerous functions [18, 49, 50]. Previous studies with expressed recombinant ORF3 protein have demonstrated that the ORF3 protein is phosphorylated and interacts with the cell cytoskeleton as well as with non-glycosylated recombinant ORF2 protein [48, 53]. In addition, it has been suggested that the ORF3 protein has regulatory functions through binding to multiple cellular proteins containing the src homology three domains and through activation of mitogen-activated protein kinase [18]. However, it was unknown whether ORF3 protein is a component of virions. Of interest, using the MAbs raised against the synthetic peptide corresponding to the C-terminal 24-aa portion of the ORF3 protein and the recently established efficient cell culture system for HEV infection and propagation, the present study revealed for the first time that ORF3 protein is present on the surface of cell-culture-generated HEV virions and on the surface of HEV in the circulation of infected patients, despite the lack of ORF3 protein on the surface of fecal HEV, suggesting that ORF3 protein is a component of virions, at least when it is released from infected cells.

Several research groups have used MAbs or polyclonal antibodies raised against HEV ORF3 proteins or peptides for Western blot analysis and/or immunofluorescent

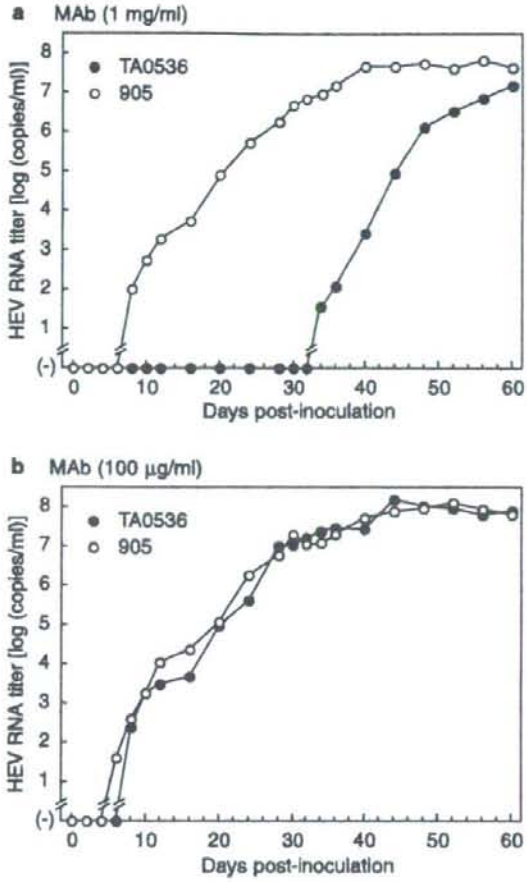


Fig. 6 Quantitation of HEV RNA in culture supernatants of PLC/PRF/5 cells inoculated with cell-culture-generated HEV of the JE03-1760F strain [46] with a viral load of $4.0 \times 10^5$ copies/ml that had been mixed with MAb TA0536 or a negative control MAb (No. 905) at 1 mg/ml (**a**) or 100 µg/ml (**b**) and cultured for the indicated number of days

analysis of the expressed ORF3 proteins [5–7, 9, 18, 48–50, 54]. However, the characteristics of these antibodies have not been described in detail. In the present study, ten MAbs against a synthetic peptide (spORF3-C24) corresponding to the well-conserved C-terminal, 24-aa sequence of the HEV ORF3 protein were produced and characterized. These ten anti-HEV ORF3 MAbs could be classified into at least three groups based on reactivity with three synthetic peptides (spORF3-C24, spORF3-C10L, and spORF3-C10P). The IgM-class MAb TA0559 exhibited strong reactivity with all three peptides, suggesting that TA0559 recognizes the extreme C-terminal portion of the ORF3 protein (aa 104–113 in HEV genotype 3) and can bind the ORF3 protein of essentially all genotype 3 and 4 HEVs,

irrespective of the amino acid of position 109 (Leu or Pro) of the ORF3 protein (Fig. 1). Two other MAbs TA0529 and TA0546 were of the IgM class and were reactive with two peptides (spORF3-C24 and spORF3-C10L) but not with the remaining one peptide (spORF3-C10P) with Pro at position 109 of the ORF3 protein, suggesting that these two MAbs also recognize the epitope within the extreme C-terminal 10-aa sequence of the ORF3 protein, in which the presence of Leu at aa 109 may be pivotal. When used in immunofluorescent analysis, these three MAbs in common gave non-specific signals in the pCI-transfected cells, which served as a negative control. Although further efforts to determine the appropriate reaction conditions for these three MAbs in immunofluorescent assay are required, a search in TFASTX (version 3.4t26 [DDBJ]) [36], as of May 20, 2008, revealed the presence of cellular proteins including N-acetyltranferase 1 (BC020205), DNA methyltransferase (AF202565), and hyaluronan synthetase 2 (AY941178) with amino acid sequences that are 70% identical to the C-terminal 10-aa sequence of the ORF3 protein, suggesting the possibility of non-specific reactions by means of molecular mimicry to the host's proteins. The remaining 7 MAbs were of the IgM, IgG1, or IgG2a class and showed high reactivity with only spORF3-C24 peptide, suggesting that these seven MAbs recognize an epitope present on the N-terminal portion of the peptide, most likely aa 90–103 of the ORF3 protein.

The anti-ORF3 MAbs obtained in the current study could capture HEV particles in culture medium and serum at variable efficiency of up to 61 and 49%, respectively, but not those in feces, indicating that ORF3 protein is present on the surface of HEV virions released from infected cells. MAb TA0529, which is reactive with an epitope in the C-terminal portion of the spORF3-C24 peptide, and MAb TA0536, which was assumed to recognize the N-terminal part of the peptide, were found to bind cell-culture-generated HEV particles and HEV particles in the serum from a hepatitis E patient in the acute phase, indicating that at least the C-terminal 24-aa region of the ORF3 protein is accessible on the surface of HEV virions in culture medium released from infected cells and in the circulation egressed from the infected liver of the hosts. The binding efficiency of the anti-HEV ORF3 MAbs did not reach near 100% even after three successive immuno-capture assays in the present study. However, HEV virions in culture supernatant were captured by MAbs TA0529 and TA0536 at an increased efficiency of approximately 90% with prior treatment of cell-culture-produced HEV particles with 3% Tween 20 (data not shown). HEV progenies in the culture supernatant, with ORF3 protein on the surface, banded at a density of 1.15 g/cm$^3$ in sucrose (Fig. 5), contrasting with HEV in a fecal suspension with a markedly higher buoyant density of 1.27–1.28 g/cm$^3$ (data not shown). Of note, Lemon and Binn [20] reported that a substantial proportion of hepatitis A virus (HAV), another non-enveloped hepatitis virus, released from infected cells banded at a lower density in CsCl (1.14–1.18 g/ml) than normal, neutralizable virus (1.32 g/ml) and that chloroform extraction of virus resulted in a substantial reduction of the non-neutralizable fraction (to less than 1%), suggesting that non-neutralizable virions might be associated with lipids and implying an important role for cell membranes in the assembly and release of HAV in vitro. It was postulated that the ORF3 protein binds to the ORF2 capsid protein [48]. Therefore, it is tempting to speculate that HEV virion might be released from infected cells as a lipid-associated particle and combine with ORF3 protein, and then the cell membrane and ORF3 protein may be dissociated from the virion after excretion into the biliary and intestinal tracts. Supporting our assumption, it has been reported that bluetongue virus, a non-enveloped virus belonging to the family *Reoviridae*, can leave the host cells as enveloped particles by budding from the plasma membrane, mediated by the viral non-structural protein NS3, although the envelope is unstable and is rapidly lost [27]. The ORF2-specific MAb (H6225) captured a minor fraction of viral particles from serum (Table 1). Therefore, it is possible that the epitope of H6225 on serum HEV is masked by ORF3 and lipid membrane.

Reflecting the presence of ORF3 protein on the surface of HEV virions in culture medium, one representative MAb (TA0536) could partially neutralize the infectivity of cell-culture-produced HEV in a cell culture system. In our previous study [44], one MAb (H6225) raised against a recombinant genotype 4 HEV ORF2 protein that was able to capture HEV virions in fecal suspensions at a high efficiency of >96% regardless of genotype could neutralize completely (at the MAb concentration of 100 μg/ml) or partially (at 1 or 10 μg/ml) infection of the HEV JE03-1760F strain in a fecal specimen in a cell culture system. In contrast, in the present study, one representative anti-HEV ORF3 MAb (TA0536) that exhibited higher binding affinity with HEV virion in culture medium and serum (58 and 42%, respectively) could partially neutralize the passage of cell-culture-generated HEV to PLC/PRF/5 cells when 1 mg/ml of MAb was administered. Although the neutralization by TA0536 was only partial, most likely due to the co-existence of HEV virions in culture medium whose C-terminal portion of the ORF3 protein is not accessible on the surface, the obtained results support our notion that ORF3 antigen detectable by anti-ORF3 MAbs raised in the present study is present on the surface of a proportion of HEV particles in culture supernatant. It is reasonable that MAb TA0536 did not neutralize the infection of the JE03-1760F strain in a fecal specimen in a cell culture system (data not shown), since fecal HEV

particles do not seem to possess ORF3 protein on the surface.

In conclusion, ten MAbs against a synthetic peptide corresponding to the C-terminal 24-aa portion of the HEV ORF3 protein were produced and characterized in the present study. Using the anti-ORF3 MAbs and the recently established efficient cell culture system for HEV infection and propagation, we demonstrated for the first time that ORF3 protein is present on the surface of cell-culture-generated HEV and serum HEV, despite the lack of ORF3 protein on the surface of fecal HEV, and that a representative anti-ORF3 MAb (TA0536) could partially neutralize the infection of HEV in culture supernatant. At present, however, we cannot rule out the possibility that the ORF3 protein becomes inaccessible in fecal HEV virions because of a capsid maturation event (which may occlude ORF3 and prevent MAb neutralization). Further studies are needed to elucidate the exact localization of ORF3 on viral particles. The present study strongly supports the assumption by Emerson et al. [7], who proposed that ORF3 protein is associated with virion release from infected cells. ORF3 protein may be a component of HEV virions, at least when the virions are released from infected cells, similar to the NS3 protein of bluetongue virus [27]. To demonstrate the assumption, construction of an infectious cDNA clone of the JE03-1760F strain that is able to replicate efficiently in PLC/PRF/5 and A549 cells and its variants, including an ORF3-deficient mutant, would be extremely useful.

**Acknowledgments** This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labor and Welfare of Japan.

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Virus Research 137 (2008) 86–96

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# Mutational events during the primary propagation and consecutive passages of hepatitis E virus strain JE03-1760F in cell culture☆

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ARTICLE INFO

*Article history:*
Received 2 March 2008
Received in revised form 8 June 2008
Accepted 13 June 2008
Available online 26 July 2008

*Keywords:*
RNA viruses
Hepatitis E virus
Mutation
Cell culture
Entire genome

ABSTRACT

We recently developed a cell culture system for hepatitis E virus (HEV) in PLC/PRF/5 cells, using a genotype 3 HEV (JE03-1760F strain). Thirteen generations of consecutive passages of culture supernatant were successfully carried out in PLC/PRF/5 cells, with the highest HEV load reaching $10^8$ copies/ml in the culture medium. Based on continuous release of progenies into culture medium, 50% tissue culture infectivity doses were estimated to be $2.0 \times 10^3$ copies for wild-type JE03-1760F and $1.4 \times 10^2$ copies for p13 (progeny in the thirteenth passage). Earlier appearance and greater increase in the yield of progenies in the culture supernatant were evident in p13 compared with wild-type. The cell culture-produced variants in primary propagation (p0) and consecutive passages (p5 [fifth passage], p10 [tenth], and p13) differed from the wild-type virus by 1, 9, 18, and 19 nucleotides (nt), respectively, over the entire genome of 7226 nt, excluding the poly(A) tail. Three of five non-synonymous mutations in p13 were shared by a variant (fifth passage) in another series of passages of JE03-1760F. These results suggest that adaptation of HEV variants to growth in vitro is associated with a limited number of mutations similar to hepatitis A virus.



## 1. Introduction

Hepatitis E virus (HEV) is an important human pathogen responsible for enterically transmitted acute hepatitis with a worldwide distribution. In developing countries in Asia, Africa and Latin America where sanitation conditions are suboptimal, HEV infection is transmitted via the fecal-oral route through virus-contaminated water or food (**Emerson and Purcell, 2007**). In industrialized countries including the United States, European countries and Japan, antibodies against HEV have been detected in a significant proportion of healthy individuals (**Thomas et al., 1997; Boutrouille et al., 2007; Gotanda et al., 2007**), suggesting frequent subclinical HEV infection, and domestically acquired sporadic cases of acute hepatitis E have been reported (**Kwo et al., 1997; Mizuo et al., 2002; Widdowson et al., 2003; Mansuy et al., 2004; Amon et al., 2006; Dalton et al., 2007; Peron et al., 2007**). Increasing lines of evidence indicate that hepatitis E is a zoonosis, and animal reservoirs of HEV exist (**Meng et al., 1997b, 1998; Harrison, 1999; Smith, 2001; Takahashi et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Meng, 2005**).

HEV is currently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (**Emerson et al., 2004**). It is a single-stranded, positive sense, polyadenylated RNA molecule of approximately 7.2 kb in size with short 5′ and 3′ untranslated regions (UTRs) (**Tam et al., 1991; Wang et al., 2000**). The viral genome contains three open reading frames (ORFs). ORF1 at the 5′ end encodes nonstructural proteins involved in virus replication and virus protein processing. ORF2 at the 3′ end encodes a 660-amino acid (aa) capsid protein. ORF3 encodes a small multifunctional protein (113-114 aa) that is essential for viral infectivity *in vivo* (**Tyagi et al., 2004; Huang et al., 2007**): in ORF3, the third in-frame AUG codon is regarded as the authentic initiation site (**Graff et al., 2005b; Huang et al., 2007**). Due to the extensive genomic diversity noted among HEV isolates, HEV sequences have been classified into four genotypes, represented by the Burmese strain (genotype 1), Mexican strain (genotype 2), U.S. strain (genotype 3) and the new Chinese strain (genotype 4) (**Schlauder and Mushahwar, 2001; Lu et al., 2006; Okamoto, 2007**).

Propagation of HEV in vitro had been attempted in various continuous cell lines (**Kazachkov et al., 1992; Huang et al., 1995, 1999; Li**

☆ The entire nucleotide sequences of seven HEV isolates reported herein have been assigned GenBank/EMBL/DDBJ accession numbers AB362839–AB362843 and AB425830–AB425831.

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doi:10.1016/j.virusres.2008.06.005

et al., 1996; Meng et al., 1996, 1997a; Wei et al., 2000) and in primary hepatocytes from nonhuman primates (Kane et al., 1984; Arankalle et al., 1988; Tsarev et al., 1994; Tam et al., 1996, 1997). However, an in vitro cell culture system supporting efficient multiplication of HEV had not been established. Recently, using a fecal suspension with high HEV load ($2.0 \times 10^7$ copies/ml) as an inoculum, obtained from a Japanese patient who contracted domestic infection of genotype 3 HEV (the JE03-1760F strain), we developed an efficient cell culture system for HEV in PLC/PRF/5 and A549 cells, which yielded the highest HEV load of $10^8$ copies/ml in the culture supernatant, and successfully propagated five generations of serial passages of culture supernatant (Tanaka et al., 2007). The JE03-1760F genome had 29 unique nucleotides that were not seen in any of the 25 reported HEV isolates of the same genotype over the entire genome, resulting in 6 amino acid substitutions in the ORF1 protein (Takahashi et al., 2007).

In the present study, we quantified HEV RNA in the culture supernatants of PLC/PRF/5 and A549 cells that had been inoculated with the JE03-1760F strain. Next, we characterized the changes that occurred in the HEV RNA sequence following long-term primary propagation and during consecutive passages in PLC/PRF/5 cells. It is hoped that the obtained data can shed light on the adaptation of HEV to growth in cell culture on the molecular basis.

## 2. Materials and methods

### 2.1. Virus

The HEV strain (JE03-1760F) was recovered from a fecal specimen of a 67-year-old Japanese patient in the acute phase of domestic infection with a genotype 3 HEV (Tanaka et al., 2007). The fecal suspension in Tris–HCl buffer (10 mM, pH 7.5) was clarified by centrifugation at $6200 \times g$ at 4 °C for 10 min, and the resulting clear supernatant was aliquoted as virus stocks, and then stored at −80 °C. The virus stock contained $2.0 \times 10^7$ HEV RNA copies/ml (Takahashi et al., 2007; Tanaka et al., 2007).

### 2.2. Cell culture

A hepatocarcinoma cell line (PLC/PRF/5) or a lung cancer cell line (A549) purchased from American Type Culture Collection (ATCC nos. CRL-8024 and CCL-185, respectively: Manassas, VA) was grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; PAA Lab GmbH, Pasching, Austria), 100 U/ml of penicillin G, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin B at 37 °C in a humidified 5% $CO_2$ atmosphere, as described previously (Tanaka et al., 2007).

### 2.3. Virus inoculation and passage

For virus infection, confluent cells were trypsinized and diluted 1:4 in medium, and 2 ml was added to wells (diameter of 3.5 cm) of a 6-well microplate (IWAKI, Tsukuba, Japan) 1 or 2 days before virus infection. Monolayers of cultured cells in a 6-well microplate were washed three times with 1 ml of phosphate-buffered saline without $Ca^{2+}$ and $Mg^{2+}$ [PBS(−)], and inoculated with 0.2 ml of the virus stock that had been diluted with PBS(−) containing 0.2% (w/v) bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO) and filtrated through two microfilters with a pore size of 0.45 and 0.22 μm (Millex-GV; Millipore Corp., Bedford, MA). In serial passages, 0.2 ml of culture supernatant that had been filtrated through a 0.22-μm microfilter was inoculated on a monolayer of PLC/PRF/5 cells. After having been inoculated at room temperature for 1 h, the solution was removed and 2 ml of maintenance medium was added. The maintenance medium used for virus culturing was a 1:1 mixture of DMEM and medium 199 (Invitrogen) containing 2% (v/v) heat-inactivated FCS and 30 mM $MgCl_2$ at final concentration: other supplements were the same as in the growth medium. Culture was performed at 35.5 °C in a humidified 5% $CO_2$ atmosphere. On the day following inoculation, the inoculated cells were washed five times with 1 ml of PBS(−), and thereafter 2 ml of maintenance medium was added. Then, every other day since day 2 after inoculation, one-half (1 ml) of the culture medium was replaced with fresh maintenance medium, and harvested media were stored at −80 °C until virus titrations. Duplicate, triplicate or pentaplicate sets of inoculum were inoculated in parallel on cultivated cells in a 6-well plate. The HEV load was determined for all or selected series of culture supernatants from the inoculated wells, and representative data were adopted.

### 2.4. Quantification of HEV RNA

The HEV RNA level was determined by real-time detection reverse transcription (RT)-PCR with a slight modification of the original method (Jothikumar et al., 2006). In brief, total RNA was extracted from 2 to 100 μl of culture medium with the TRIzol-LS reagent (Invitrogen), and subjected to RT-PCR with the QuantiTect Probe RT-PCR Kit (Qiagen, Tokyo, Japan) with sense primer (5′-GGT GGT TTC TGG GGT GAC-3′), antisense primer (5′-AGG GGT TGG TTG GAT GAA-3′), and a probe consisting of an oligonucleotide with a 5′-reporter dye (FAM) and a 3′-quencher dye (TAMRA) (5′-FAM-TGA TTC TCA GCC CTT CGC-TAMRA-3′) on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Thermal cycler conditions were 50 °C for 30 min at stage 1; 95 °C for 15 min at stage 2; and 50 cycles of 94 °C for 15 s, 56 °C for 30 s and 76 °C for 30 s at stage 3. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate or triplicate and the mean value was adopted.

### 2.5. Amplification of full-length HEV genome

Total RNA was extracted from 200 μl of culture medium and subjected to cDNA synthesis followed by nested PCR of eight overlapping regions including the extreme 5′- and 3′-terminal regions: the amplified regions excluding the primer sequences were nucleotides (nt) 1–53 (53 nt), nt 25–1252 (1228 nt), nt 1081–2074 (994 nt), nt 2020–3136 (1117 nt), nt 3088–4682 (1595 nt), nt 4633–6362 (1730 nt), nt 6324–7181 (858 nt) and nt 7083–7226 (144 nt) for all isolates. The extreme 5′-end sequence (nt 1–53) was determined by a modified rapid amplification of cDNA ends (RACE) technique called RNA ligase-mediated RACE (RLM-RACE) with the First Choice RLM-RACE kit (Ambion, Austin, TX), as described previously (Okamoto et al., 2001). Amplification of the 3′-end sequence [nt 7083–7226 (144 nt): poly(A) tail excluded] was performed by the RACE method as described previously (Okamoto et al., 2001).

### 2.6. Sequence analysis of PCR products

The amplification product was sequenced on both strands directly or after cloning into pT7Blue T-Vector (Novagen Inc., Madison, WI), using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequence analysis was performed using Genetyx-Mac 12.2.7 (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). Sequences were aligned 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 entire

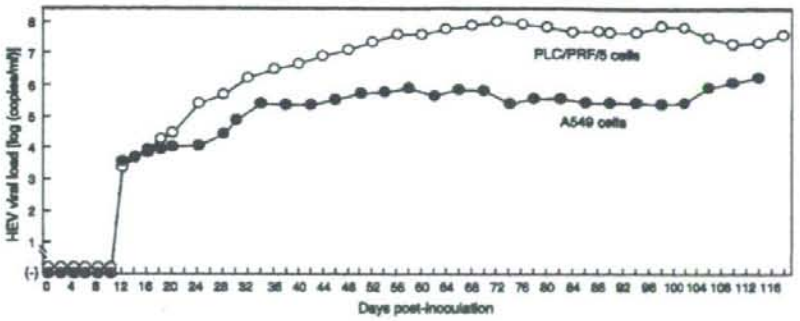


Fig. 1. Quantification of HEV RNA in culture supernatants of PLC/PRF/5 or A549 cells that were inoculated with a fecal supernatant containing the JE03-1760F HEV strain and cultured for the indicated number of days.

genome. Bootstrap values were determined on 1000 resamplings of the data sets (**Felsenstein, 1985**).

## 3. Results

### 3.1. Growth and mutations of HEV in PLC/PRF/5 and A549 cells

Fecal supernatant containing the JE03-1760F strain was inoculated on fresh monolayers of PLC/PRF/5 and A549 cells at the viral load of $2.5 \times 10^5$ copies per well. HEV RNA appeared in the culture media of both PLC/PRF/5 and A549 cells on the 12th day post-inoculation (dpi) and persisted in the culture media of PLC/PRF/5 and A549 cells up through the end of the 118- or 114-day observation period, respectively (**Fig. 1**). The HEV viral load in the culture supernatant of PLC/PRF/5 cells became $1.2 \times 10^7$ copies/ml on 48 dpi and continued to be $>10^7$ copies/ml thereafter, with a maximum load of $9.7 \times 10^7$ copies/ml. Whereas, the HEV RNA titer in the culture supernatant of A549 cells reached $2.4 \times 10^5$ copies/ml on 34 dpi and continued to be in the range of $10^5$ or $10^6$ copies/ml, with a maximum load of $1.8 \times 10^6$ copies/ml. During cell culture, a cytopathic effect (CPE) was not observed in the PLC/PRF/5 nor A549 cells.

To examine mutations in the HEV genome that appeared during primary propagation of the wild-type JE03-1760F strain in cell culture, cell culture-generated variants including the 118d/Alex isolate obtained from the 118-dpi culture supernatant of PLC/PRF/5 cells and the 114d/A549 isolate obtained from the 114-dpi culture supernatant of A549 cells, were sequenced over the entire genome. The 118d/Alex and 114d/A549 isolates each had a genomic length of 7226 nt, excluding the poly(A) tract at the 3′-terminus, and possessed three major ORFs, similar to the wild-type JE03-1760F isolate (accession no. AB301710). In both cell culture-produced variants, ORF1, ORF2 and ORF3 encoded 1703 aa (nt 26–5134), 660 aa (nt 5172–7151), and 113 aa (nt 5161–5499), respectively. The 5′UTRs of the two isolates comprised a common sequence of 25 nt, while their 3′UTRs consisted of 75 nt. The 118d/Alex and 114d/A549 isolates differed by only 5 nt over the entire genome, and shared identical amino acids in all three ORFs (**Table 1**). Upon comparison with the wild-type HEV JE03-1760F isolate, 118d/Alex differed by merely 4 nt, including 2 nt (nt positions 2086 and 6743) that each consisted of two distinct nucleotides of U and C, while 114d/A549 was different by only 1 nt (mixture of U and C in contrast to U alone in wild-type at nt position 1402).

### 3.2. Serial passages of HEV in PLC/PRF/5 cells

HEV progenies released in the culture medium were passaged 13 times in PRF/PLC/5 cells (Experiment A in **Table 2**). The results of Passages 0–5 have been reported (**Tanaka et al., 2007**). In the primary propagation (Passage 0), HEV RNA became detectable in the culture supernatant on 10 dpi at $1.1 \times 10^4$ copies/ml and its load increased to $6.4 \times 10^5$ copies/ml on 28 dpi (**Fig. 2A**). Probably due to the low viral load of the inoculum, HEV RNA in the first passage (Passage 1) was not detectable during 5 weeks in the harvested culture medium, but it appeared on 36 dpi and reached a maximum load of $8.6 \times 10^6$ copies/ml on 56 dpi. In Passage 2 with inoculation of the

**Table 1**
Comparison of the sequences of two HEV variants (118d/Alex and 114d/A549) in cell culture during primary propagation over the entire genome with wild-type HEV (JE03-1760F)

| Nucleotide position | Region | Nucleotide | | | Amino acid | |
|---|---|---|---|---|---|---|
| | | Wild-type | 118d/Alex[b] | 114d/A549[c] | Position | Substitution |
| 1402 | ORF1 | U | U | Y[d] | 459 | - |
| 2086 | ORF1 | C | Y | C | 687 | - |
| 3133 | ORF1 | U | C | U | 1036 | - |
| 3715 | ORF1 | U | C | U | 1230 | - |
| 6743 | ORF2 | U | Y | U | 524 | - |

[a] Nucleotide mutations are shaded for visual clarity.
[b] 118d/Alex: HEV isolate obtained from the 118-dpi culture supernatant of PLC/PRF/5 cells that had been inoculated with JE03-1760F.
[c] 114d/A549: HEV isolate obtained from the 114-dpi culture supernatant of A549 cells that had been inoculated with JE03-1760F.
[d] Y, mixture of U and C.