transfer genes *in vitro*. The fluorescence of GFP-expressing cells was observed under a fluorescence microscope. Although the percentages of fluorescence-positive cells were not so high (11.2% of NIH3T3 cells, 19.6% of RK-13 cells, 21.0% of COS-7 cells and 20.1% of HepG2 cells), all of the cell lines used in this study showed positive reactions (Figure 3). In contrast, no fluorescence-positive cells were observed when the cells were incubated with plasmid DNA alone or intact VLPs in the presence of plasmid DNA (data not shown). We next tried gene transduction *in vivo*. Mice that had orally received a vaccine of DNA expressing HIV env gp120 of the NL432 strain (pJWNL432) that was encapsulated in VLPs were killed 2 days after immunization, and the expression of HIV env protein in the digestive tract was examined. HIV env protein was found in epithelial cells of the small intestine by immunohistochemistry (Figure 4), indicating that the HEV structure necessary for the entry of HEV into target cells had been preserved in refolded VLPs and that the DNA encapsulated in HEV-VLPs had been delivered to intestinal tissues.

Systemic and mucosal HIV-specific humoral immune responses in mice that had orally received a vaccine of HIV DNA encapsulated in VLPs

Mice were orally or subcutaneously immunized four times at 1-week intervals with pJWNL432 either naked or encapsulated in HEV-VLPs. The serum levels of HIV env-specific IgG antibodies in mice that had received loaded VLPs were significantly higher than those in mice that had received naked DNA ($P<0.05$ at 12 wpi, Figure 5a and e). Moreover, specific IgA was detected at high levels in sera of mice that had received loaded VLPs but not in sera of mice that had been immunized subcutaneously ($P<0.05$ at 12 wpi, Figure 5b and f). HIV env-specific IgA was only detected in fecal extracts of mice that had orally received pJWNL432-encapsulated HEV-VLPs (Figure 5d and h). No specific IgG was detected in any of the fecal samples (Figure 5c and g). The levels of HIV env-specific IgG antibodies detected in sera from subcutaneously and orally immunized mice were the same (Figure 5a and e). HEV-specific IgA was detected in both sera and fecal extracts of mice that had been orally administered VLP but not in sera or fecal extracts of mice that had been immunized subcutaneously (Figure 5j and l). Both orally and subcutaneously immunized mice showed HEV-specific IgG in sera (Figure 5i) and fecal extracts (Figure 5k).

Elicitation of HIV-specific cytotoxic T lymphocytes at systemic and mucosal sites by oral administration of a vaccination of HIV DNA encapsulated in VLPs

Cytotoxic T lymphocyte (CTL) responses in the spleen, mesenteric lymph nodes (MLN) and Payer's patches (PP) were investigated at 5 weeks after the first immunization. Mice that had orally received pJWNL432 encapsulated in HEV-VLPs showed HIV env epitope-specific CTL responses in the spleen, MLN and PP, whereas cells from the same tissues in mice that had received naked DNA vaccine did not show any CTL activity (Figure 6a). The P18 peptide is a dominant HIV env CTL and Th cell epitope in BALB/c mice and is restricted to the H-2D^d allele. These effector cell functions derived from our experiments were inhibited by either anti-CD8 or -H-2D^d monoclonal antibody (mAb) (Figure 6b,c), indicating that oral immunization of mice with a vaccine of HIV env DNA-encapsulated HEV-VLPs elicited CD8$^+$ and MHC class I-restricted CTLs both locally and systemically.

Discussion

A large number of pathogens gain access to the human body via mucosa such as oral, nasal or genital mucosa. The best defense against these predominantly mucosal

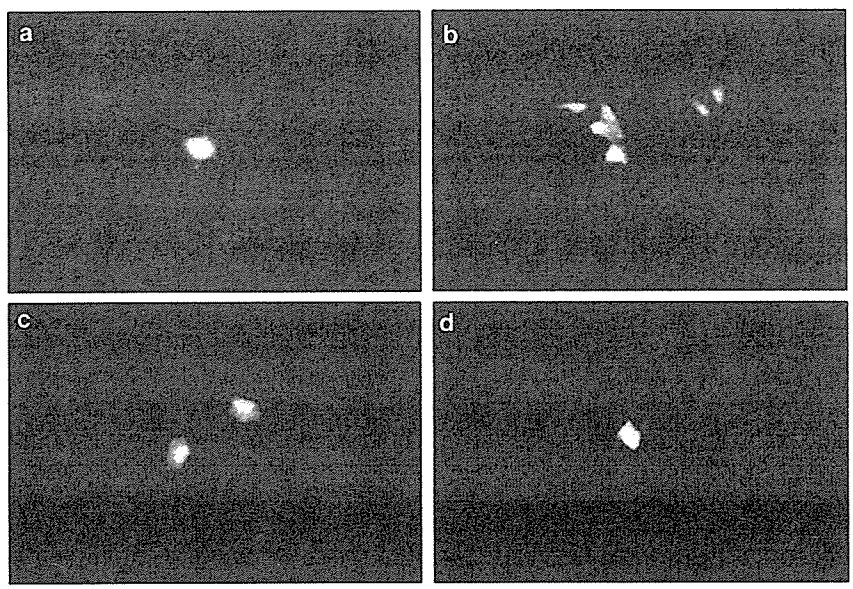

Figure 3 *Expression of GFP in cells transfected with plasmid DNA encapsulated in HEV-VLPs: (a) NIH/3T3 cells (mouse); (b) RK-13 cells (rabbit); (c) COS-7 cells (monkey); and (d) HepG2 cells (human).*

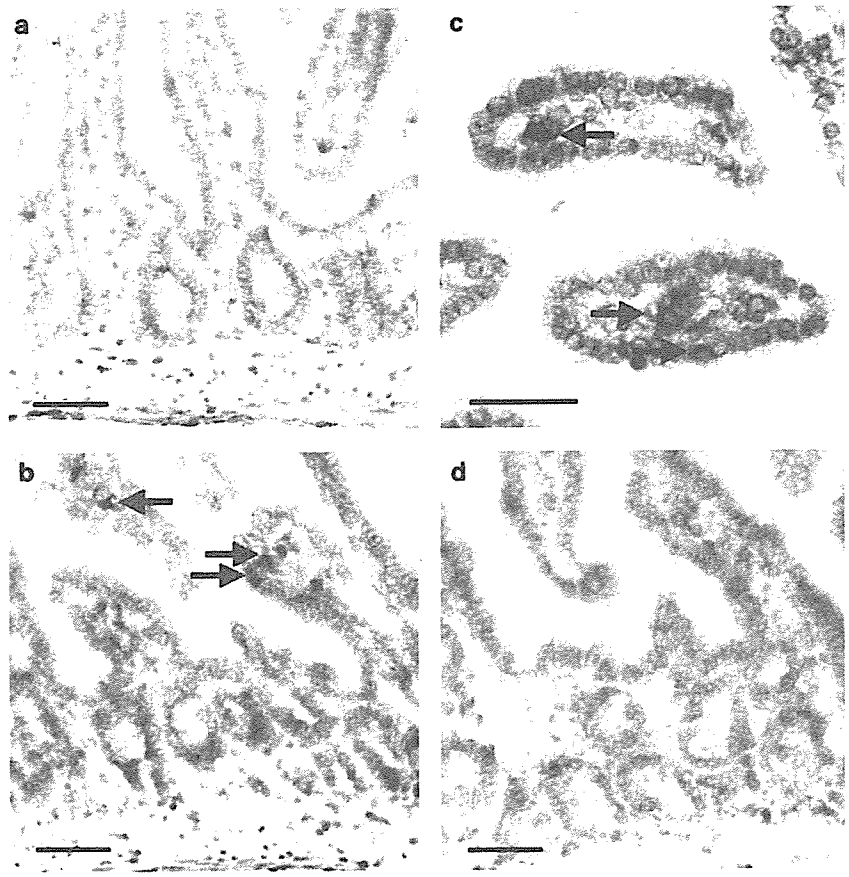

Figure 4 *Immunostaining of serial sections of small intestine tissue from mice 2 days after oral administration of pJWNL432-encapsulated VLPs. HIV env proteins were observed in epithelial cells (arrow) (b, c), and control mAb did not show any positive reactions (d). Control mice were also administered pJWNL432 without VLP encapsulation (a). Bar marker represents 50 μm.*

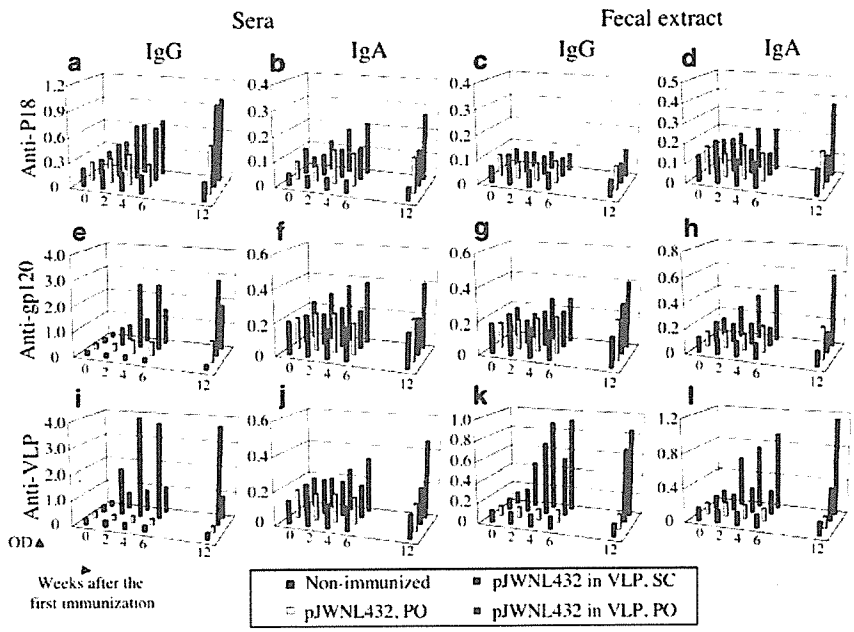

Figure 5 *IgG (a, c, e ,g, i and k) and IgA (b, d, f, h, j and l) levels in sera (a, b, e, f, i and j) and fecal extracts (c, d, g, h, k and l) of immunized mice. Mice were orally (■) or subcutaneously (□) administered pJWNL432 encapsulated in VLP or naked (▩). Symbols indicate HIV env-specific antibody levels. Background levels to HIV env in nonimmunized mice (▨) are also shown. The IgG and IgA antibody levels are expressed as OD at dilutions of 1:100 and 1:2 for serum and fecal extracts, respectively. The mean OD values ±s.e.s were obtained from five mice/group.*

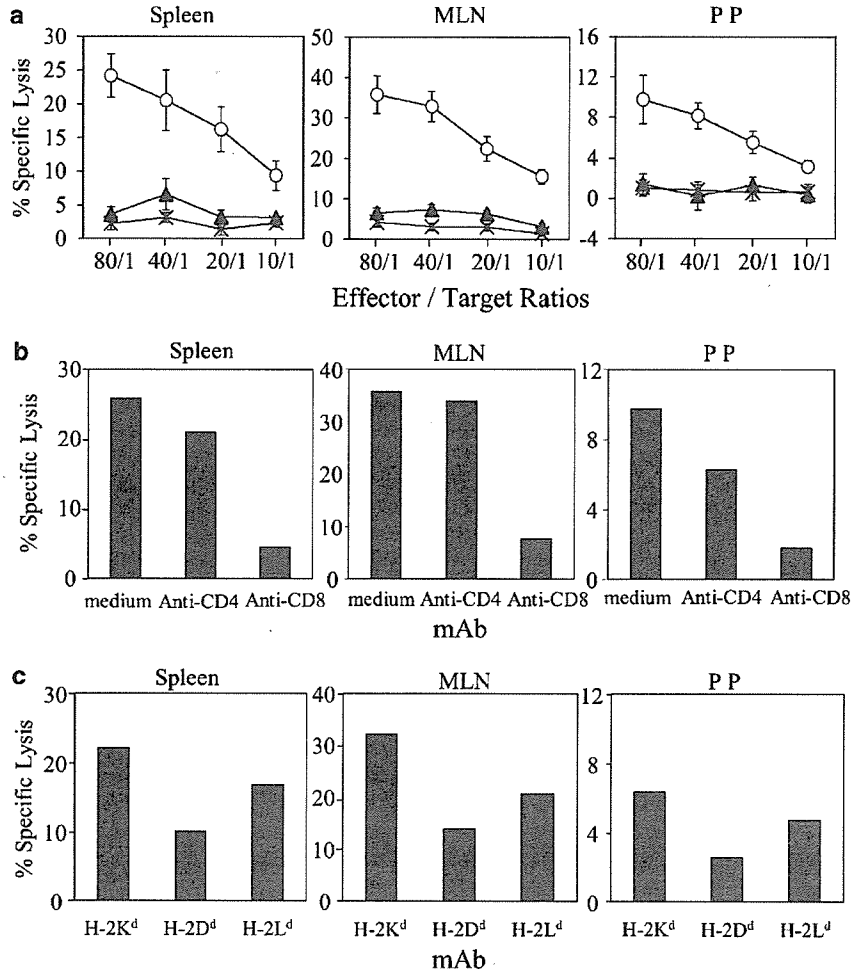

Figure 6 Spleen, MLN and PP cells from mice orally administered pJWNL432-encapsulated VLPs elicited CTL. (a) Mice were orally administered pJWNL432 encapsulated in VLPs (circles) or naked (triangles). Results for nonimmunized controls are also shown (×). (b) Effector cells obtained from the spleen, MLN and PP cells of mice orally administered pJWNL432-encapsulated VLPs are mediated CD8$^+$ cells. Lytic activities of effector cells were assessed in the presence of anti CD4 mAb, anti-CD8 mAb or medium. Effector:target ratio was 80:1. (c) HIV env-specific lysis was restricted by MHC class I. Effector cells were examined for P18-specific lytic activities in the presence of anti-H-2K^d, anti-H-2D^d or H-2L^d mAb. The percentage of P18-specific lysis was calculated as (% lysis of target cells labeled with P18)−(% lysis of target cells labeled with control peptide). Each value is the mean percentage of the specific lysis values obtained from five mice.

pathogens is mucosal vaccines that are capable of inducing both systemic and mucosal immunity. Recent evidence has shown that DNA vaccination can confer protection against a number of infectious agents, including viruses and bacteria, although peripheral immunization with naked DNA is less than optimal for stimulating mucosal immunity.[12,13] In fact, it is quite difficult to induce both mucosal and systemic immune responses by oral administration of naked DNA. This study demonstrated that an orally administered DNA vaccine encapsulated in an orally transmissible virus-derived VLP induced both mucosal and systemic immunity.

The delivery of a DNA vaccine for induction of mucosal immune responses is usually achieved by gene transfer to the upper nasopharynx-associated lymphoid tissue (NALT), upper airway, salivary glands and tonsils.[5,14] Despite its obvious convenience, oral administration is rarely successful, since it is quite difficult to protect plasmid DNA from the environment in the digestive tract. The efficacy of orally delivered DNA vaccine to NALT is improved by encapsulating plasmid DNA in poly (lactide-coglycolide) (PLG) microparticles for protection against the gastric environment.[15,16] The immune responses to particle-borne DNA immunizations by means such as utilization of a gene gun or PLG differ from those to DNA immunizations without particles.[13] It is thought that the microparticles are actively taken up by cells such as macrophages or M cells of PP of the small intestine and thus facilitate the presentation of antigens to local immune systems.[15,17] This mechanism is the same as that of gene gun immunization of a DNA vaccine, that is, phagocytic cells such as macrophages or dendritic cells take up plasmid DNA delivered by a gene gun. The delivered gene is expressed only in these cells.[18] Similarly, only mucosal immunity was induced in mice by oral administration of DNA-encapsulated PLG microparticles.[15,16] It is likely that the mechanism underlying immune recognition of

HEV-VLP infection is similar to that of direct intramuscular or subcutaneous DNA immunization without the use of particles. Protein expressed by HEV-VLP-infected cells is recognized by the immune surveillance system, resulting in the elicitation of Ag-specific immune responses. We showed in this study that genes could be expressed in epithelial cells in the small intestine after delivery by HEV-VLPs (Figure 4). It is plausible that HEV-VLPs, which are derived from an orally transmissible virus, were incorporated into HEV-permissive epithelial cells in the small intestine, because they retained structures and properties similar to those of HEV particles, producing an infection similar to that induced naturally.[19] The Ag-expressing cells might be recognized by intraepithelial lymphocytes or submucous antigen-presenting cells by the same mechanism as that in the case of general virus infection.

An HEV-VLP has several advantages as a vector of DNA. Firstly, in our experience, large amounts can be easily obtained from standard cultivation protocols compared with the amounts of other VLPs obtained. The yield of purified HEV-VLPs collected from a culture supernatant of 50–100 μg/ml is more than 100 times greater than that of other VLPs. Secondly, the outcome of gene delivery in humans can be predicted using conventional laboratory animals, since HEV naturally infects various animals as well as humans through the same infectious route and target cells.[10,20] Thirdly, HEV-VLPs are stable at room temperature. Fourthly, anti-HEV immune responses had no effect on DNA administration in the present study, and this might be related to the neutralizing antibody for preventing infection with HEV. Neutralizing antibodies to HEV for inhibiting infection have not yet been found. This is also the case for HCV. The mechanism by which HEV is eliminated by antibodies is thought to be antibody-dependent cell-mediated cytotoxicity (ADCC). The effect of induction of immune responses to DNA vaccine in our system is not clear. Thus, HEV-VLPs are an attractive vaccine vector in developing countries because these VLP can be preserved without the requirement of any particular equipment. Finally, we have reported that an HEV-VLP can carry foreign amino-acid sequences as a part of the ORF2 protein exposed on the particle surface without any morphological or biological alteration.[10] Liposomal vectors resembling retroviral envelopes endowed with targeting molecules for gene delivery have been reported. The vicronectin receptor, $\alpha_v\beta_3$-integrin, is commonly upregulated on malignant melanoma cells, and liposome carrying an Arg-Gly-Asp (RGD) integrin-binding motif has been used for a system to deliver DNA to these tumor cells.[21] It has also been reported that targeting DNA to M cells by intranasal administration for the induction of mucosal and systemic responses can be achieved by formulating DNA with polylysine linked to viral adhesion.[22] It may be possible to design chimeric ORF2 proteins carrying these targeting molecules to retarget HEV-VLP to particular cell types.

Oral vaccination has obvious advantages for a field trial in a large-scale public health vaccination program.[23] From a practical standpoint, oral administration is less stressful for vaccine recipients and does not require professional skill for the vaccine administration. Moreover, delivery of vaccines via the intestinal tract is considered to be inherently safer than systemic injection. Encouraging results of phase I trials using Norwalk virus VLPs have recently been reported.[24] Trials using DNA vaccines for infectious and malignancy diseases have also been conducted.[25] The results of the present study suggest that oral administration of DNA vaccine encapsulated in oral transmissible virus VLPs, HEV-VLPs, is effective for inducing both humoral and cellular immunity locally as well as systematically. HEV-VLPs might be useful not only for vaccination but also as a vector in human gene therapy.

Materials and methods

Mice

BALB/c female mice were purchased from Clea Japan (Tokyo, Japan) and were housed in the Laboratory Animal Center of Mie University School of Medicine during the experimental period.

Peptide synthesis

The peptides used in this study were the HIV env CTL epitope (HIV 308-322, RIQRGPGRAFVTIGK; P18)[26] and a control peptide (HCV nonstructural protein 5 CTL epitope MSYSWTGALVTPCAAE; P17).[27]

Plasmid DNA

A highly efficient mammalian expression vector, pJW4303,[28] was used for efficient expression of HIV env gp120 of the NL432 strain.[29] Various sizes of plasmid DNA were also used for the *in vitro* packaging experiment (3.162 kb: pUC118; 5.93 kb: pJW322; 8.63 kb: pJWSIVenv; 11.2 kb: pABWN).

Production and purification of HEV-VLPs

HEV-VLPs were produced and purified by previously described methods.[10,11] Briefly, Tn5 cells maintained in Excel 405 serum-free medium (JRH, KS) were infected with the recombinant baculovirus expressing HEV-ORF2 at an m.o.i. of >5 and cultured for 6 days. The supernatant was harvested and the recombinant baculovirus in the supernatant was pelleted by ultracentrifugation at 10 000 *g* for 30 min at 4°C. The VLPs in the supernatant were collected by further ultracentrifugation at 100 000 *g* for 2 h at 4°C. Pelleted VLPs were then resuspended in 10 mM potassium-[2-(*N*-morpholino) ethanesulfonic acid] (MES) buffer (pH 6.2) and purified on a CsCl equilibrium density gradient. The purified HEV-VLPs were spun down and resuspended in potassium-MES buffer and kept at 4°C.

DNA packaging

Plasmid DNA was encapsulated into HEV-VLPs according to a previously described procedure.[30] Purified VLPs (50 μg) were disrupted by incubation in 180 μl of a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA and 20 mM dithiothreitol. Following 30 min of incubation at room temperature, 200 μg (20 μl) of each plasmid in 50 mM Tris-HCl buffer (pH 7.5) and 150 mM NaCl was added. The disrupted VLP preparation was refolded by incubation for 1 h with increasing concentrations of $CaCl_2$ up to a final concentration of 5 mM. VLPs were pelleted by ultracentrifugation and resuspended in 10 mM potassium-MES buffer (pH 6.2). At each step, the VLP structure formation was confirmed by electron

microscopy after negative staining, as described previously.[11] To estimate the amounts of encapsulated plasmid DNA, refolded and purified VLPs were treated with 10 IU benzonase (SIGMA-ALDRICH, Irvin, UK) for 1 h at 20°C to remove DNA on the surfaces of VLPs and disrupted with EGTA (1 mM). Absorbance of the supernatant was measured for detection of plasmid DNA contents.

Density analysis of refolded VLPs

Refolded VLPs were separated on a CsCl equilibrium density gradient and fractioned into 0.2 ml aliquots. HEV-VLPs in each fraction were detected by ELISA as previously described,[10] as well as DNA contents.

Gene transfer in mammalian cells

Four cell lines (NIH/3T3 (mouse), RK13 (rabbit), COS-7 (monkey), HepG2 (human)) were used in transfection experiments. Sterilized coverslips were placed in six-well plates, and 5×10^5 cells per well were seeded in the plates. After overnight culture, cells were washed twice with a medium, and about 1 μg of VLP-encapsulated EGFP expression vector (BD Bioscience Clontech, CA, USA) diluted with 0.5 ml medium was added. After 2 h of incubation at 37°C, VLPs were removed. Cells were then incubated for 48 h at 37°C. At the end of the culture period, cells were removed from the culture medium and washed three times with PBS. Coverslips were then mounted onto microscope slide glasses. Fluorescence of the GFP-expressing cells was observed under a fluorescence microscope.

Immunization

Mice were orally immunized four times with 50 μg protein of HEV-VLP/DNA (pJWNL432) complex or 20 μg naked pJWNL432 DNA in 100 μl of potassium-MES buffer at 1 week intervals.

Immunohistochemical analysis

At 2 days after oral immunization, the mice were killed and tissues were collected. Cryostat sections were air-dried and incubated in 0.5% HIO_4 for 10 min to quench endogenous peroxidase activity. The sections were further pretreated with chicken anti-mouse IgG antibody (Chemicon International, Inc., CA, USA) to prevent nonspecific reactions of a secondary antibody. The sections were then incubated with an HIV env-specific mAb (HIV-1 IIIB gp120 mAb (902)), which was obtained through the AIDS Research and Reference Reagent Program,[31] for 30 min at 37°C. The bound antibodies were visualized with a biotinated secondary antibody, HRP-labeled avidin–biotin complex (ABC-peroxidase staining kit, Elite Vector Lab. Inc., CA, USA) and 3.3′-diaminobenzidine tetrachloride with 0.01% H_2O_2. Sections were slightly counterstained with hematoxylin. An mAb (A1/3D1, ANOGEN, Canada) against hepatitis C virus core, which is same isotype to 902, was used as a control.

ELISA

Serum and fecal samples were collected at 0 (preimmunization), 2, 4, 6 and 12 weeks after the first immunization. Feces were suspended in ice-cold PBS at 200 mg/ml, and the centrifuge supernatant was used as fecal extract. Culture plates (96-well) were coated with purified HEV-VLPs or synthesized oligopeptides (P18) at a concentration of 10 or 100 μg/well, respectively, overnight at 4°C followed by 30 min of blocking with PBS containing 0.1% FBS and 0.05% Tween 20. To determine the anti-HIV env gp120 antibody responses, CV-1 cells were seeded in 96-well plates and infected with recombinant Sendai virus expressing HIV env gp120 of NL432 strain (SeV gp120),[32] and then the plates were incubated at 37°C. At 3 days after infection, plates were washed and fixed with PBS containing 10% formalin for 10 min. Test samples were added to each well and incubated at room temperature for 1 h. For detection of anti-HIV env gp120 antibody, test samples were reacted with wild-type Sendai virus-infected CV-1 cells before addition to the wells to eliminate the nonspecific antibody. Biotin-labeled anti-mouse IgG (Vector, CA, USA) or IgA (CALTAG, CA, USA) was used as the detection antibody. Following 1 h incubation, the plates were washed and further incubated with avidin-HRP (Vector, CA, USA). The reaction was developed using an ABTS substrate (Roch Diagnostic, Mannheim, Germany).

Generation of CTL effector cells

Effector cells were derived from spleen, MLN and PP cells as precursor CTLs. Aliquots of 5×10^6 spleen cells were co-cultured with 2.5×10^6 mitomycin C-treated autologous spleen cells labeled with a peptide at 37°C in a CO_2 incubator. The effector cells generated were harvested after 5 days of culture.

Cytotoxicity assay

Target cells, A20.2J cells (2×10^6), were incubated at 37°C in a 5% CO_2 atmosphere with 10 μg/ml of P18 or control peptide for 16 h. The target cells were then washed and labeled with ^{51}Cr. The ^{51}Cr-labeled target cells were incubated for 5 h with effector cells. Spontaneous release varied from 5 to 10%. Percent lysis was calculated as ((experimental release−spontaneous release)/(100% release−spontaneous release)) × 100. All the experiments were performed at least four times, and each experimental group consisted of five mice.

Blocking of cytolysis

^{51}Cr-labeled target cells (10^6 cells) were preincubated at 4°C for 1 h with anti-H-2 K^d, D^d or L^d mAb (Meiji Institute of Health Science Ltd., Tokyo, Japan) (1 μg/ml), and effector cells were then added. In a separate experiment, effector cells (10^7 cells) were preincubated with anti-CD4 mAb (GK1.5) or anti-CD8 mAb (Lyt2.2) (10 μg/ml) at 4°C for 1 h, and then the labeled target cells were added. Blocking of cytolytic activities by these mAbs was assessed by a 5-h ^{51}Cr release assay.

Statistical analysis

Statistical analysis was performed using Mann–Whitney's U test and Kruskal–Wallis test. Values are expressed as means ± s.d.s. A 95% confidence limit was taken as significant ($P < 0.05$).

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Transfusion-transmitted hepatitis E caused by apparently indigenous hepatitis E virus strain in Hokkaido, Japan

Keiji Matsubayashi, Yasuhiro Nagaoka, Hidekatsu Sakata, Shinichiro Sato, Kanji Fukai, Toshiaki Kato, Kazuaki Takahashi, Shunji Mishiro, Mitsunobu Imai, Naokazu Takeda, and Hisami Ikeda

BACKGROUND: In industrialized countries, sporadic cases of hepatitis E have been reported in individuals who have never been in an endemic area. Hepatitis E virus (HEV) infection commonly occurs via the fecal-oral route but a potential risk of transfusion transmission route has been suggested.

STUDY DESIGN AND METHODS: A 67-year-old Japanese male patient who had never been abroad received a transfusion of blood from 23 voluntary donors and developed acute hepatitis with unknown etiology after transfusion. His blood samples were tested for viral markers of hepatitis viruses.

RESULTS: HAV, HBV, HCV, CMV, and EBV were ruled out as causative agents in this case. The patient's blood sample in the acute phase contained HEV RNA as well as IgM and IgG anti-HEV. HEV RNA was also detected in one of the FFP units transfused. The donor had no history of traveling abroad and had a normal ALT level at the time of donation. The PCR products from the patient and the donor showed complete identity for two distinct regions of HEV within open reading frame 1.

CONCLUSION: The patient was infected with HEV via transfused blood from a volunteer donor. A potential risk of posttransfusion hepatitis E should be considered even in nonendemic countries.

Hepatitis E virus (HEV) is a major cause of epidemic hepatitis that is usually developed as acute hepatitis in endemic areas in Asia, Africa, Central and South America, and the Middle East.[1] Recent evidence indicates that, in industrialized countries, sporadic acute or fulminant hepatitis E occurs in individuals who have no history of traveling to HEV endemic areas[2-10] and that hepatitis E is a zoonotic disease; pigs and other animals appear to be linked to human infection as reservoirs.[11-18] In Japan, HEV infection has been rarely reported and has been considered as an imported infection from endemic areas for a long time. An epidemiologic study with a sensitive ELISA system, however, revealed that 2 to 14 percent of the healthy population in Japan was seropositive for the presence of IgG anti-HEV.[19] Approximately 13 percent of the non-A, -B, and -C acute hepatitis cases in Japan were caused by HEV.[9] Moreover, after the initial discovery and the characterization of indigenous Japanese strain, JRA1, from a patient with non-A, -B, and -C acute hepatitis, who had never been abroad,[7] several indigenous Japanese HEV strains were recovered from patients with acute or fulminant hepatitis of non-A, -B, and -C etiology.[8-10,20] Although the question of when the first HEV strain made inroad remains unsettled, it is likely that heterogeneous strains of HEV

ABBREVIATIONS: HEV = hepatitis E virus; nt = nucleotide(s).

From the Hokkaido Red Cross Blood Center, Sapporo; the Department of Internal Medicine, Nikko Memorial Hospital, Muroran; the Department of Medical Sciences, Toshiba General Hospital, Tokyo; the Department of Microbiology, Kanagawa Prefectural Public Health Laboratory, Chigasaki; and the Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan.

Address reprint requests to: Keiji Matsubayashi, MS, Hokkaido Red Cross Blood Center, 2-2 Yamanote, Nishi-ku, Sapporo, Japan 063-0002; e-mail: kmatsu@hokkaido.bc.jrc.or.jp.

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have already been circulating and HEV has recently been recognized as an important causative agent of sporadic hepatitis of non-A, non-B, non-C aetiology in Japan. In endemic countries where fecal-oral routes of HEV transmission are common, it is suggested that there is a potential risk of transfusion-transmitted hepatitis E, because HEV viremia is known to appear in the early stage of infection and a significantly higher seroprevalence was observed in transfused hemodialysis patients compared to blood donors.[21] In India, where HEV is endemic, two cases of transfusion-transmitted hepatitis E were reported but they were not confirmed by molecular approaches.[22] Here we report a probable case of transfusion-transmitted hepatitis E in Japan, where HEV had been believed not to be endemic. Sequence analysis showed the isolates of both donor and patient appeared to be identical.

MATERIALS AND METHODS

Characteristics of the patient

In July 2002, a 67-year-old Japanese male patient (S.K.), who had never been abroad, received a transfusion of blood products from 23 voluntary donors during open-heart surgery. Although he was discharged 24 days after the operation, he was hospitalized again for acute hepatitis of unknown etiology with elevated levels of ALT and AST and bilirubinemia. He was followed-up for 134 days after the operation and his blood samples were collected periodically and stored below –20°C until testing.

Transfused blood samples

Twenty-three blood products from the 23 voluntary donors, 14 FFP units, 8 RBC units, and 1 PLT unit, were transfused to the patient during the operation and their stored blood samples were examined virologically including for HEV RNA.

The Japanese Red Cross Blood Centers have implemented a storing system of blood samples for every unit of donated blood since September 1996 to assess adverse effects of transfusion. All of the samples are stored below –20°C until testing.[23]

Blood donor samples with elevated ALT levels

There were 559,545 blood donations in Hokkaido from October 2000 through April 2002. Of these, 15,285 (2.7%) were disqualified because of an elevated ALT of greater than 60 IU per L. Of these, 40 had an ALT level of greater than 500 IU per L and tested negative for the presence of HBV and HCV by NAT. Among them, the samples of 18 donors, 16 men and 2 women, were subjected to RT-PCR testing for the presence of HEV RNA. These samples were stored below –20°C until testing.

RT-PCR for HEV RNA detection

Detection of HEV RNA was performed by nested RT-PCR targeting two distinct regions within ORF1. For 365 nucleotides (nt) within the methyltransferase-coding region, corresponding to nt 105 to 469 of JRA1 strain,[7] RT-PCR was carried out as described previously by Takahashi et al.[8] and a template for direct sequencing was prepared by the second-round PCR with the sense degenerate primer M13/HE5-2 (5′-GTTTTCCCAGTCACGACGCCYTKGCGAATGCTGTGG-3′) and a mixture of antisense degenerate primers M13/HE5-3 (5′-CAGGAAACAGCTATGACTCRAARCAGTARGTGCGGTC-3′) and HE5-6 (5′-CAGGAAACAGCTATGACTYAAAACAGTAGGTTCGATC-3′). M13 sequences for direct sequencing are underlined.

To amplify sequences within the hypervariable and proline-rich hinge region, corresponding to nt 2127 to 2464 of JRA1 isolate, seminested RT-PCR was performed as described above with the sense primer HE-V1 (5′-ACCTGGGAGTCAGCCAAT-3′) and the antisense primer HE-V2 (5′-AACCAAGTACACTCAGACTCAAAG-3′) for the first-round PCR and internal sense primer HE-V3 (5′-TATACTCGCACCTGGTCGG-3′) and HE-V2 for the second-round PCR.

Sequence analyses of PCR products

The amplification products were sequenced on both strands with a cycle sequencing kit (PRISM BigDye Terminator, Version 2, Applied Biosystems Japan Ltd, Tokyo, Japan) and a genetic analyzer (Prism Model 3100 or 3700, Applied Biosystems Japan Ltd). The PCR product of a 326-nt region was sequenced with M13 primers, M13/RV (5′-CAGGAAACAGCTATGAC-3′) and M13/M4 (5′-GTTTTCCCAGTCACGAC-3′). For the PCR product of the hypervariable region, the same primers for the second-round PCR were used for sequencing. The sequences determined were analyzed with computer software (GENETYX-Win, Version 5.2, Software Development, Tokyo, Japan). The sequences were aligned together with reported HEV strains with a computer program (CLUSTAL W, Version 1.8).[24] A phylogenetic tree based on the 326-nt region within ORF1 was constructed by the neighbor-joining method[25] and the final tree was obtained by a computer program (TreeView, Version 1.6.6).[26] Bootstrap values were determined by resampling 1000 of the data sets.

The nucleotide sequence data reported in this article will appear in DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB113303 and AB113311 for HRC-SK, AB113304 and AB113312 for HRC-IM, AB113305 for HRC-HE1, AB113306 for HRC-HE2, AB113307 for HRC-HE3, AB113308 for HRC-HE4, AB113309 for HRC-HE5, and AB113310 for HRC-HE6.

ELISA for HEV antibodies

Samples were tested for IgM and IgG antibodies to HEV by ELISA that used virus-like particles as antigen that were produced in baculovirus-infected insect cells.[19]

Assays for viral markers other than HEV

Antibody assays to viruses other than HEV were performed with commercially available kits: anti-HAV IgM (AxSYM HA-M, Version 2.0, Abbott Laboratories, North Chicago, IL), anti-CMV IgM (Celltite SEIKEN Cytomegalo, Denka Seiken Co. Ltd, Tokyo, Japan), anti-EBV IgM and IgG (Diagnostics VCA-Test BML IgG and IgM test, BML, Inc., Tokyo, Japan), anti-HCV (AxSYM HCV, Abbott Laboratories), anti-HBc (AxSYM HBcAb, Abbott Laboratories), and anti-HBs (AxSYM AUSAB, Abbott Laboratories). HBsAg was assayed with AxSYM HBsAg (Abbott Laboratories, North Chicago, IL). HBV DNA and HCV RNA were assayed with NAT probe assays (DNA Probe FR-HBV, REBIOGEN, Inc., Tokyo, Japan; and Amplicor GT HCV Monitor, Roche Diagnostics, Berkeley, CA, respectively), according to the instructions of each company.

RESULTS

Clinical course of the patient

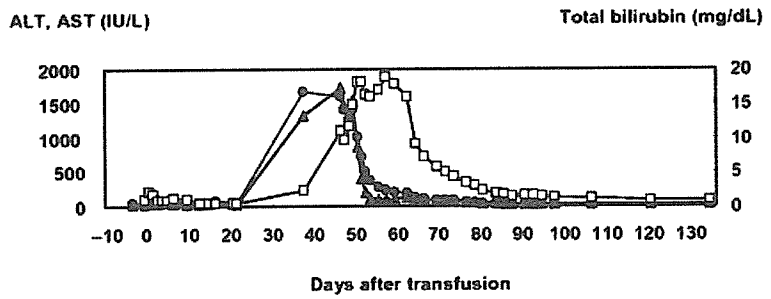
The clinical course of the patient (S.K.) is summarized in Fig. 1. When he was hospitalized again for acute hepatitis, he had an elevated ALT level of 1595 IU per L and an AST level of 1727 IU per L on Day 46 after transfusion; these normalized within 1 month, whereas the total bilirubin level rose to 11.0 mg per dL 2 weeks after the maximum ALT and AST elevation. His clinical state was improved 96 days after the transfusion. The retrospective testing of his blood sample from 4 days before the operation showed that he was negative for the presence of anti-HAV IgM, anti-CMV IgM, anti-EBV IgM, anti-HCV, HCV RNA, HBsAg, and HBV DNA.

In contrast, IgM and IgG class antibodies against HEV were detectable in his plasma sample on Day 37 after transfusion. HEV-RNA was detected from the serum sample of Day 37 and viremia lasted at least until Day 85 post-transfusion. HEV markers were not positive in his blood sample at 4 days before the operation. The IgG anti-HEV continued to be positive for 134 days after transfusion when last tested.

HEV testing of transfused blood

The 23 samples of transfused blood were tested for the presence of HEV RNA to determine whether the HEV was transfusion transmitted. HEV-RNA was detected in one of 23 samples of transfused blood to the patient. The donor I.M. a 24-year-old Japanese woman living in Hokkaido, had a normal ALT level of 10.0 IU per L at the time of donation and IgM- and IgG-class anti-HEV was not detectable (Table 1). FFP from this donor (I.M.) was transfused to the case patient (S.K.). The RBC product from the HEV-positive donation was transfused to another patient, Y.M. A following study revealed that patient Y.M., who had lymphoma showed no sign of hepatitis, clinically, virologically, or serologically of follow-up after transfusion. Neither HEV-RNA nor anti-HEV were detected in his blood 130 days after transfusion.

A blood sample from the HEV-positive donor's (I.M.) previous donation (15 months before the case donation) was available for testing. The sample was negative for the presence of HEV RNA or IgM- and IgG-class anti-HEV and had an ALT level of 8.0 IU per L. Five months after the case donation, the donor had seroconverted with IgM and IgG anti-HEV and HEV RNA was not detectable at that time (Table 1). On interview by telephone, donor I.M. had not been out of Japan during the incubation period and no



Day	−4	37	46	51	66	78	85	93	106	120	134
HEV RNA	−	+	+	+	+	+	+	−	NT	NT	NT
Anti-HEV IgM	−	+	+	+	+	+	+	+	+	+	+
Anti-HEV IgG	−	+	+	+	+	+	+	+	+	+	+

Fig. 1. Clinical course of the patient with hepatitis E and the testing results for HEV. The ALT (●), AST (▲), and total bilirubin levels (□) are also shown.

TABLE 1. Characteristics of the HEV-RNA-positive donor*

	ALT (IU/L)	HEV RNA	Anti-HEV IgM	Anti-HEV IgG
Previous donation (−15 months)	11	−	−	−
Case donation	10	+	−	−
Follow-up exam (+5 months)	8	−	+	+

* + = positive; − = negative.

clinical sign or symptoms of hepatitis during a follow-up of 5 months after the case donation.

HEV sequence study with PCR products from the donor and the patient

The PCR products of donor I.M. and patient S.K. were compared to each other for sequences corresponding to a 326-nt region encoding methyltransferase within the ORF1 of the HEV genome. The sequence (HRC-IM) of the PCR product from the transfused blood that was positive for the presence of HEV RNA showed complete identity with that (HRC-SK) from patient S.K.'s blood at 37 days after transfusion. According to Schlauder and Mushahwar's classification of HEV,[27] these isolates were segregated to genotype IV and were very similar to JKK-Sap and JSY-Sap,[8] which were isolated from hepatitis E patients living in Hokkaido (Fig. 2). JKK-Sap was different by only 1 nt at position nt 261 and JSY-Sap by two nucleotides at the positions nt 261 and nt 330, based on the JKK-Sap sequence, respectively. The amino acid sequences were completely identical for these strains. Furthermore, for the 307-nt proline-rich hinge region of ORF1, the isolates from donor I.M. and patient S.K. showed complete identity.

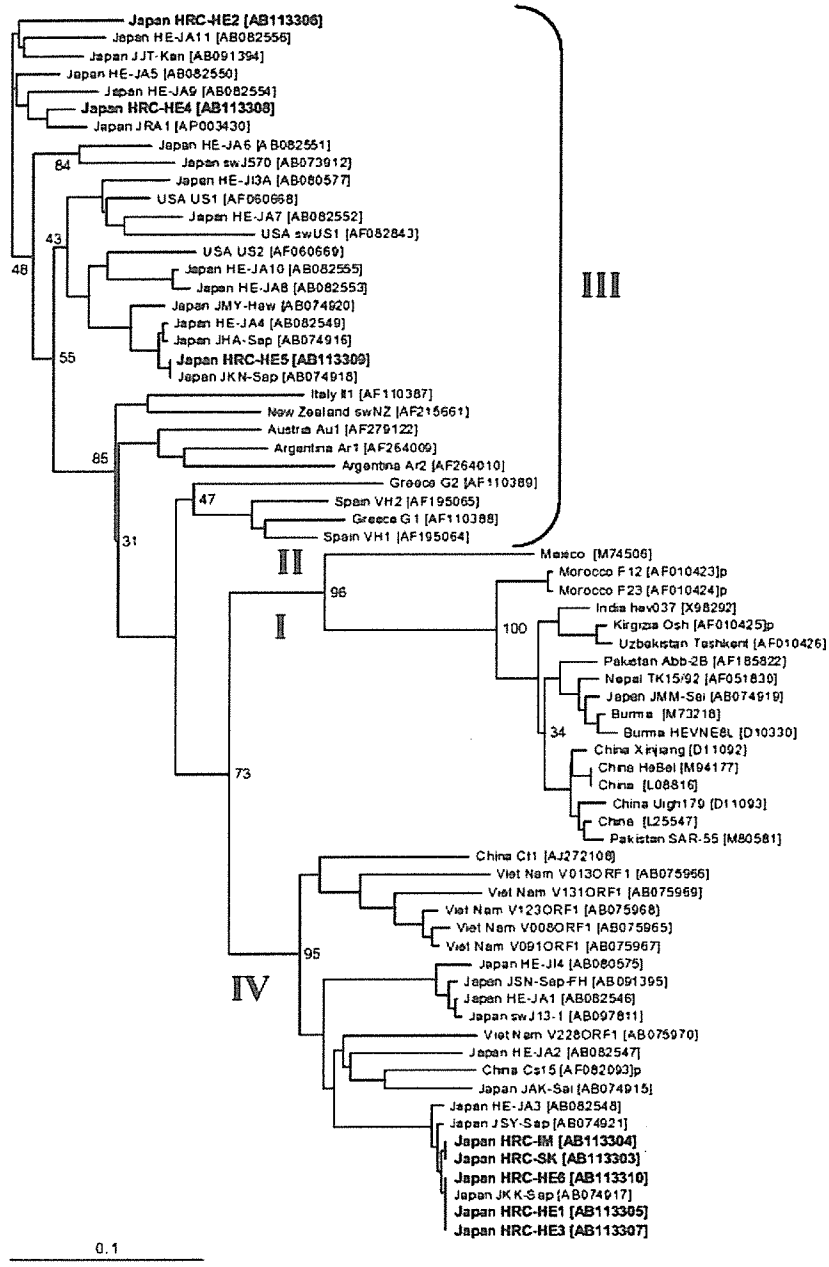


Fig. 2. Phylogenetic tree based on a 326-nt region of ORF1 for HEV strains derived from the case donor HRC-IM and patient HRC-SK and six donors with elevated ALT of greater than 500 IU per L. Accession numbers for the reference sequences are indicated parentheses. The isolated strains in this study are shown in boldface. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings.

Detection and analysis of HEV RNA in donors with elevated ALT

Six of the stored samples from 18 donors with elevated ALT levels higher than 500 IU per L, who were all men and aged 29 to 48 year, were positive for the presence of HEV RNA (Table 2). Phylogenetic analysis based on the 326-nt sequence of the ORF1 indicated that isolates of HRC-HE2, HRC-HE4, and HRC-HE5 were segregated to genotype III, and HRC-HE1, HRC-HE3, and HRC-HE6 as well as HRC-IM, to genotype IV (Fig. 2). All four strains of genotype IV were very closely related to each other with 99.4 to 100 percent identity in this region. HRC-HE1 and HRC-HE3 were completely identical with JKK-Sap. The addresses of HEV-positive donors were not concentrated in a particular area but widely distributed over Hokkaido. Although HRC-HE1 and HRC-HE3 showed identical sequences, the two donors lived in different cities, and there was a time lag of 6 months between their donation dates.

Because some of the donors with a high ALT level were repeat donors, samples from different donations could be

TABLE 2. Characteristics of disqualified donors with elevated ALT of greater 500 IU per L*

Donor	Date of donation	Age (years)	Sex	ALT level (IU/L)	Anti-HEV IgM	Anti-HEV IgG	HEV RNA	HEV strain (genotype)
1	October 2000	45	Male	878	–	–	–	
2	August 2000	29	Male	11	–	–	–	
	December 2000	29		767	+	+	+	HRC-HE1 (IV)
	August 2001	30		12	+	+	–	
	February 2002	31		19	+	+	–	
	May 2002	31		16	–	+	–	
3	December 2000	42	Male	558	–	–	–	
4	January 2001	32	Female	670	–	+	–	
5	March 2001	30	Male	506	+	+	+	HRC-HE2 (III)
6	April 2001	35	Male	1008	–	–	–	
7	April 2001	40	Male	1470	+	+	+	HRC-HE3 (IV)
8	June 2001	33	Female	545	–	–	–	
9	June 2001	36	Male	675	–	–	–	
10	July 2000	46	Male	21	–	–	–	
	July 2001	47		713	+	+	+	HRC-HE4 (III)
11	July 2001	31	Female	748	–	–	–	
12	August 2001	36	Male	1458	–	–	–	
13	August 2001	49	Male	647	–	–	–	
14	October 2001	39	Male	641	–	+	+	HRC-HE5 (III)
15	April 2000	47	Male	17	–	–	–	
	November 2001	48		740	+	+	+	HRC-HE6 (IV)
16	November 2001	40	Male	771	–	–	–	
17	December 2001	56	Male	531	–	–	–	
18	April 2002	33	Female	948	–	–	–	

* + = positive; – = negative.

studied. For all HEV-RNA-positive donors except for Donor 14, anti-HEV IgM was detected in the HEV-RNA-positive donations (Table 2). In the three HEV-infected donors, Donors 2, 10, and 15, ALT levels were normal and neither HEV RNA nor anti-HEV was detected in the previous donations. For Donor 2, HEV RNA was negative and ALT not elevated except for the HEV-RNA-positive donation; the HEV-positive donation had both IgG and IgM antibody to HEV and his anti-HEV IgM was still detectable 13 months after the HEV-positive donation.

DISCUSSION

We report the first case of transfusion-transmitted acute hepatitis E fully investigated by molecular approaches. The HEV-positive blood donor was asymptomatic and resident in Japan, where hepatitis E has been considered not endemic, and her donation was made in an early stage of HEV infection. Fecal-oral transmission is the common route of HEV infection in the outbreaks in endemic areas,[1] whereas little is known about the transmission routes for sporadic hepatitis E cases in industrialized countries. Vertical transmission as well as transfusion transmission has been suggested in endemic areas.[28]

In India, where hepatitis E is endemic, Arankalle and Chobe[22] reported two cases of transfusion-transmitted hepatitis E by means of retrospective analyses. Nevertheless, they were not successful in demonstrating the association of blood transfusion with hepatitis E infection by molecular approaches probably because of degradation of HEV RNA in the specimens during storage.

In our study, specimens from both the donors and the patient before and after the transfusion were available in good condition, which made it possible to determine that the blood transfusion was associated with HEV infection with molecular approaches.

The case patient was positive for both anti-HBc and HBsAg and negative for the presence of HBV DNA before and after transfusion, suggesting that he was not in an active HBV carrier state. Testing results for other five viruses regarding to hepatitis except for HEV showed that they were ruled out as a causative agent to this case. Based on the clinical data, he was diagnosed with acute hepatitis E. The case donor had a seroconversion of anti-HEV and appeared to be asymptomatic for HEV infection. The amplification products of two distinct regions of HEV corresponding to the methyltransferase gene and the hypervariable and proline-rich hinge domain of ORF1 from both the patient and the donor were sequenced, showing complete identity. Therefore, it is highly probable that the transfusion was responsible for the current hepatitis E case. In addition, the onset of the hepatitis was closely associated with the timing of the blood transfusion. The onset of the hepatitis was somewhere between 24 and 46 days after transfusion, which corresponded to the incubation period of 32 days in a case of transmission of HEV to a human volunteer.[29] Nevertheless, the possibility cannot be ruled out that the infection

occurred via other route and the sequence identity of the HEV in the donor and the patient was coincidental. RBCs, derived from the HEV-positive donation, did not appear to cause hepatitis E by transfusion to another patient. The patient had no clinical sign of hepatitis during follow-up after transfusion; neither HEV RNA nor anti-HEV were detected on the 130 days after transfusion but any other sample from the patient was not available for testing. The viral load of HEV in the RBC product could be too low to cause infection.

We also found six HEV-RNA-positive samples among donors with an ALT level of greater 500 IU per L in the same area, Hokkaido, and some of them had strains quite similar to the case strain, HRC-IM and HRC-SK.

Phylogenetic analyses of HEV isolates indicated a cluster of genotype IV indigenous to Hokkaido. The cluster includes highly homogeneous strains of genotype IV with 99.4 to 100 percent nucleotide sequence identities: JKK-Sap and JSY-Sap from hepatitis E patients; HRC-HE1, HRC-HE3, and HRC-HE6 from voluntary blood donors with an ALT level of greater than 500 IU per L; and HRC-IM from the case donor. Of three HEV strains of genotype III isolated in this study, HRC-HE5 showed very similar nucleotide sequence to JKN-Sap and JHA-Sap[8] isolated from acute hepatitis E patients. They all were derived from individuals living in Hokkaido. These results suggest that multiple HEV strains of genotype III and IV indigenous to Hokkaido may exist and are circulating there. It is interesting to note that a recently isolated swine HEV strain in Hokkaido showed 99 percent nucleotides homology over the entire genome with a human HEV strain of genotype IV.[16,17] Moreover, a direct evidence of HEV transmission from animal to human via uncooked deer meat was provided in Japan.[18] These support the idea that hepatitis E is a zoonotic disease and swine and deer are as reservoirs for human infection.

By implementation of sensitive HBV and HCV tests including NAT for donor screening, the residual risk of posttransfusion hepatitis B and C has become minimal.[30-32] Regarding other viruses associated with hepatitis such as HAV, HEV, CMV, and EBV, a specific test for each virus is not performed as routine donor screening in Japan. Although it may not be very effective in the early stage of infection or as a surrogate test for HBV or HCV infection,[33] ALT testing may be helpful in preventing posttransfusion hepatitis caused by other viruses associated with hepatitis. Approximately 8000 (2.3%) units of donated blood are disqualified yearly owing to an elevated ALT level of higher than 60 IU per L in Hokkaido. Forty donors showed ALT levels of higher than 500 IU per L in 1.5 years, of which at least six samples without HBV DNA nor HCV RNA were HEV-positive. It should be noted that the 6 donors were disqualified not by donor interviewing but by ALT testing. This suggests the possibility that asymptomatic HEV infection was present among other blood donors. Although the appropriate cutoff value might be reconsidered, ALT screening should not be discontinued because information about HEV infection is still poor and there is no other screening test to eliminate such asymptomatic HEV-positive donors in Japan.

In conclusion, although transfusion-transmitted HEV is probably much too rare to sustain HEV transmission in industrialized countries, where HEV infection is believed to be nonendemic, it should be taken into account that HEV is spread through uncertain routes, and the potential risk of transfusion-transmitted HEV infection should be considered. Further epidemiologic study is required to understand the current transmission routes of HEV infection.

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Essential Elements of the Capsid Protein for Self-Assembly into Empty Virus-Like Particles of Hepatitis E Virus

Tian-Cheng Li,[1]* Naokazu Takeda,[1] Tatsuo Miyamura,[1] Yoshiharu Matsuura,[2] Joseph C. Y. Wang,[3] Helena Engvall,[3] Lena Hammar,[3] Li Xing,[3] and R. Holland Cheng[3,4]

Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011,[1] and Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Suita-shi, Osaka 565-0871,[2] Japan; Karolinska Institute, Department of Biosciences, 141 57 Huddinge, Sweden[3]; and Department of Molecular and Cellular Biology, University of California, Davis, California 95616[4]

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Hepatitis E virus (HEV) is a noncultivable virus that causes acute liver failure in humans. The virus's major capsid protein is encoded by an open reading frame 2 (ORF2) gene. When the recombinant protein consisting of amino acid (aa) residues 112 to 660 of ORF2 is expressed with a recombinant baculovirus, the protein self-assembles into virus-like particles (VLPs) (T.-C. Li, Y. Yamakawa, K. Suzuki, M. Tatsumi, M. A. Razak, T. Uchida, N. Takeda, and T. Miyamura, J. Virol. 71:7207–7213, 1997). VLPs can be found in the culture medium of infected Tn5 cells but not in that of Sf9 cells, and the major VLPs have lost the C-terminal 52 aa. To investigate the protein requirement for HEV VLP formation, we prepared 14 baculovirus recombinants to express the capsid proteins truncated at the N terminus, the C terminus, or both. The capsid protein consisting of aa residues 112 to 608 formed VLPs in Sf9 cells, suggesting that particle formation is dependent on the modification process of the ORF2 protein. In the present study, electron cryomicroscopy and image processing of VLPs produced in Sf9 and Tn5 cells indicated that they possess the same configurations and structures. Empty VLPs were found in both Tn5 and Sf9 cells infected with the recombinant containing an N-terminal truncation up to aa residue 125 and C-terminal to aa residue 601, demonstrating that the aa residues 126 to 601 are the essential elements required for the initiation of VLP assembly. The recombinant HEV VLPs are potential mucosal vaccine carrier vehicles for the presentation of foreign antigenic epitopes and may also serve as vectors for the delivery of genes to mucosal tissue for DNA vaccination and gene therapy. The results of the present study provide useful information for constructing recombinant HEV VLPs having novel functions.

Hepatitis E virus (HEV), which causes severe acute liver failure, belongs to the genus *Hepevirus* in the family *Hepeviridae* (22). HEV contains an approximately 7.2-kb single-stranded positive-sense RNA molecule (21). The RNA is 3′ polyadenylated and includes three open reading frames (ORF). ORF1, mapped in the 5′ half of the genome, encodes viral nonstructural proteins (7, 12). ORF2, located at the 3′ terminus of the genome, encodes a protein-forming viral capsid (11, 25). ORF3, mapped between ORF1 and ORF2, encodes a 13.5-kDa protein that is associated with the membrane as well as with the cytoskeleton fraction (27). This protein is shown to be phosphorylated by the cellular mitogen-activated protein kinase (6, 8). The ORF3 protein may have a regulatory function (6, 8). Ever since HEV was first discovered in 1980 and visualized by immune electron microscopy in 1983 (2), many efforts have been made, using different expression systems, to express the structural protein (5, 11, 17, 26). It is particularly important to characterize the viral protein because so far no practical cell culture system for growing HEV is available. Only one neutralization epitope has been identified; it maps between amino acids 578 and 607 of the ORF2 protein (pORF2) (18).

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: (81)-42-561-0771. Fax: (81)-42-561-4729. E-mail: litc@nih.go.jp.

The expression of foreign proteins in baculovirus systems opens the prospect of studying HEV capsid assembly, since virus-like particles (VLPs) of pronounced spikes on the surface can be formed with the recombinant protein expressed with this system (11, 25). This VLP is capable of inducing systemic and mucosal immune responses in experimental animals (9). With an oral inoculation of 10 mg of recombinant HEV VLPs, cynomolgus monkeys can develop anti-HEV immunoglobulin M (IgM), IgG, and IgA responses and protect against HEV infection (10). All these data suggest that VLPs are a candidate HEV vaccine.

The VLPs produced from Tn5 cells appear as T=1 icosahedral particles, which are composed of 60 copies of truncated pORF2 (25). The protein contains two distinctive domains: the shell (S) domain forms the semiclosed icosahedral shell, while the protrusion (P) domain interacts with the neighboring proteins to form the protrusion. The projection of T=1 recombinant HEV VLPs appears as spikes decorated with spherical rings (25), which fits with the morphology obtained from negatively stained HEV native virions. The diameter of these VLPs, 27 nm, is less than that reported for partially purified native virions (16). However, VLPs retain the antigenicity of the native HEV virion by designated antigenic sites at the P domain and by the capsid connection at the S domain. The particles appear empty, with no significant RNA-like density inside. The N-terminal region of pORF2 is rich in positively charged amino acid residues and may interact with RNA mol-

TABLE 1. Oligonucleotides used in the construction of baculovirus recombinants

Recombinant baculovirus	Forward primer[a]	Reverse primer[b]
Ac[n111]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n111c52]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**TGCTAGCGCAGAGTGGGGGGCTAAAA
Ac[n111c58]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**GGCTAAAACAGCAACCGCAGAGATGG
Ac[n111c59]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**TAAAACAGCAACCGCAGAGATGGAGA
Ac[n111c60]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**AACAGCAACCGCAGAGATGGAGACGG
Ac[n111c64]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**AGAGATGGAGACGGGACCAGCACCCA
Ac[n111c72]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**ACCCAGGCTAGTGGTGTAAGTGGAAA
Ac[c52]	CAGGATCC**ATG**CGCCCTCGGCCTATTTTGTTGCTGCT	AATCTAGA**CTA**TGCTAGCGCAGAGTGGGGGGCTAAAA
Ac[n123]	AAGGATCC**ATG**GATGTCGACTCTCGCGGCGCCATCTT	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n124]	AAGGATCC**ATG**GTCGACTCTCGCGGCGCCATCTT	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n125]	AAGGATCC**ATG**GACTCTCGCGGCGCCATCTTGCG	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n126]	AAGGATCC**ATG**TCTCGCGGCGCCATCTTGCGCCG	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n130]	CAGGATCC**ATG**ATCTTGCGCCGGCAGTATAATCTATC	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n125c59]	AAGGATCC**ATG**GACTCTCGCGGCGCCATCTTGCG	AATCTAGA**CTA**TAAAACAGCAACCGCAGAGATGGAGA

[a] BamHI (underlined) and an initiation codon (bold) are indicated.
[b] XbaI (underlined) and a stop codon (bold) are indicated.

ecules (21). Thus, the deletion of the N-terminal 111 amino acid (aa) residues and the insufficient volume of the central cavity may lead to the failure of RNA encapsidation (25).

Cell type dependence in the VLP formation of the recombinant capsid protein was observed when aa residues 112 to 660 of ORF2 were expressed with a recombinant baculovirus in two insect cell lines, Tn5 and Sf9. In Tn5 cells, two major bands, having molecular masses of 58 kDa (58K) and 53 kDa (53K), were found in the cell lysate, while a peptide in the VLPs comprising a 53K protein was found in the culture medium. The 53K protein has been designated as either the 50K or 54K protein in previous studies (9, 11). In Sf9 cells, an additional peptide with a size between that of 58K and that of 53K was found in the cell lysate. However, no VLP was recovered from the culture medium. In Tn5 cells, terminal sequencing revealed that 58K and 53K proteins have the same first 15 aa in the N terminus and that a posttranslation cleavage by cellular protease(s) occurred at the pORF2 C termini and converted 58K into 53K. An independent but similar observation was obtained when pORF2 of the Pakistani strain was expressed in Sf9 cells (17) where several immunoactive proteins were detected in the cell lysate, and a 53K protein was secreted into the culture medium, but no VLP was found. Further investigation of pORF2 expression in Sf9 and Tn5 cells may allow us to understand the mechanism underlying the subunit assembly and particle formation of the recombinant HEV capsid.

We analyzed particle formation with pORF2 containing a series of truncated deletions at the N- and/or C-terminal region. In both Sf9 and Tn5 cells, amino acid residues 126 to 601 appeared to form the pORF2 core structure and were capable of self-assembling into VLPs. These results indicated that the cell dependence on particle formation is due to the difference between Sf9 and Tn5 cells in the modification process of pORF2.

MATERIALS AND METHODS

Generation of recombinant baculoviruses and expression of capsid proteins. DNA fragments encoding the N- and/or C-terminal aa-truncated pORF2 were amplified by PCR using plasmid pHEV5134/7161 as a template. Plasmid pHEV5134/7161 containing a full-length genotype I (G1) HEV pORF2 was described previously (11). The primers used in the construction of baculovirus recombinants are shown in Table 1. Amplified DNA fragments were purified by using a QIAGEN PCR purification kit (QIAGEN, Valencia, CA), digested with restriction enzymes, and ligated with baculovirus transfer vector pVL1393 (Pharmingen, San Diego, CA). An insect cell line derived from *Spodoptera frugiperda* (Sf9) (19) (Riken Cell Bank, Tsukuba, Japan) was cotransfected with a linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (Pharmingen), and the transfer vectors were cotransfected by the Lipofectin-mediated method as specified by the manufacturer (Gibco BRL, Gaithersburg, MD). The cells were incubated at 26.5°C in TC-100 medium (Gibco BRL) supplemented with 8% fetal bovine serum and 0.26% Bacto tryptose phosphate broth (Difco Laboratories, Detroit, MI). The proteins in the culture medium and cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blot assay using serum from a patient with acute hepatitis E (11). Each recombinant virus was plaque purified three times. The baculovirus recombinants thus obtained were designated as Ac[n111], Ac[n111c52], Ac[n111c58], Ac[n111c59], Ac[n111c60], Ac[n111c64], Ac[n111c72], Ac[c52], Ac[n123], Ac[n124], Ac[n125], Ac[n126], Ac[n130], and Ac[n125c59]; a schematic diagram is shown in Fig. 1. Both insect Sf9 and Tn5 cells, the latter from a *Trichoplusia ni* insect cell line, BTI-Tn-5B1-4 (Invitrogen, San Diego, CA), were infected with recombinant baculoviruses at a multiplicity of infection of 10 and incubated for 5 days at 26.5°C as previously described (11, 23).

Purification of VLPs. The culture medium was harvested on day 5 after infection. The intact cells, cell debris, and progeny baculoviruses were removed by centrifugation at 10,000 × *g* for 90 min. The supernatant was then spun at 25,000 rpm for 2 h in a Beckman SW28 rotor. The resulting pellet was resuspended in 4.5 ml EX-CELL 405 at 4°C overnight. After mixing with 1.96 g of CsCl, the sample was centrifuged at 35,000 rpm for 24 h at 4°C in a Beckman SW50.1 rotor. The visible white band (at a density of 1.285 g/ml) was harvested by puncturing the tubes with a 21-gauge needle, diluted with EX-CELL 405 medium, and then centrifuged again in a Beckman TLA45 rotor at 45,000 rpm (125,000 × *g*) for 2 h to remove CsCl. The VLPs were placed on a carbon-coated grid, and the proteins were allowed to be absorbed into the grid for 5 min. After being rinsed with distilled water, the sample was stained with a 1% aqueous uranyl acetate solution and examined with a Hitachi H-7000 electron microscope operating at 75 kV.

Terminal amino acid sequence analysis. The VLPs were further purified by 5 to ~30% sucrose gradient centrifugation at 35,000 rpm for 2 h in a Beckman SW50.1 rotor. The visible white band was harvested as described above, diluted with EX-CELL 405, and again centrifuged at 45,000 rpm for 2 h in a Beckman TLA55 rotor to precipitate the VLPs. N-terminal aa microsequencing was carried out using 100 pmol of the protein by Edman automated degradation on an Applied Biosystems model 477 protein sequencer, and C-terminal aa sequencing was performed by Applied Biosystems.

SDS-PAGE and Western blot analysis. Dispersed insect cells were incubated for 20 min at room temperature to allow the cells to attach to culture flasks in TC-100 (Sf9 cells) or EX-CELL 405 (Tn5 cells) medium. The culture medium was removed, and the cells were infected with the recombinant baculoviruses at

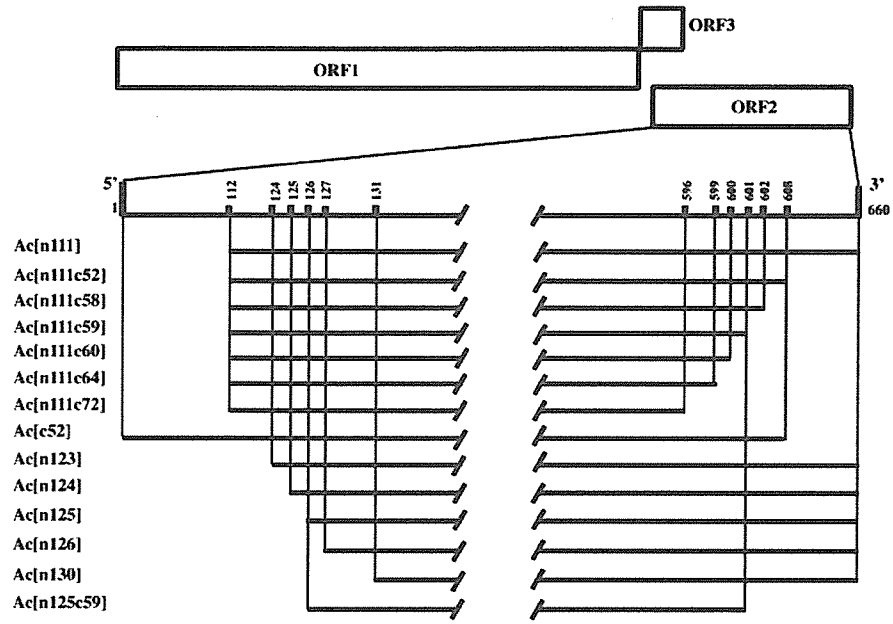

FIG. 1. Genome organization of HEV and schematic diagram of recombinant baculovirus vectors. DNA fragments encoding N- and C-terminal aa-truncated ORF2 were prepared by PCR with the primers listed in Table 1 and were used to construct 14 recombinant baculoviruses. Full-length pORF2 consisted of 660 aa. The N- and C-terminal aa numbers of the truncated protein are indicated.

a multiplicity of infection of 10. Virus adsorption was carried out for 1 h at room temperature, and then the cells were incubated at 26.5°C. The proteins in the cell lysate and in the culture medium were separated by 10% SDS-PAGE and stained with Coomassie blue. For Western blotting, the proteins in the SDS-PAGE gel were electrophoretically transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in 50 mM Tris-HCl (pH 7.4)–150 mM NaCl and reacted with a patient's serum from an acute phase. Human IgG antibody was detected by using alkaline phosphatase-conjugated goat anti-human immunoglobulin (1:1,000 dilution) (DAKO A/S, Copenhagen, Denmark). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate P-toluidine were used as coloring agents (Bio-Rad Laboratories).

Cryo-electron microscopy (cryo-EM) and image reconstruction. A 3-μl drop of purified HEV VLP (~1 mg/ml) was applied onto holey carbon film. After extra solution was wiped away with filter paper, the grid was rapidly plunged into liquid ethane surrounded by liquid nitrogen. Thus embedded in a thin layer of vitrified ice, the specimen was then transferred via a Gatan 626 cryo-transfer system to a Philips CM120 microscope. The specimen was observed at liquid nitrogen temperature and photographed at a magnification of 45,000. Each area was photographed twice, with defocus levels of 1 μm and 3 μm, respectively. The electron dose of each exposure was less than 10 electrons/Å^2. The selected electron micrographs were digitized with a Zeiss scanner at a step size of 14 μm, corresponding to 3.1 Å at the specimen. The images were reconstructed according to icosahedral symmetry with Fourier-Basel procedures (4, 28). Briefly, the particle orientation and center of each image were estimated with the EMPFT program, where the structure of Tn5-produced HEV VLP was used as the initial model (1). The first reconstruction was generated from selected images and used as a model to refine the orientation and center parameters. After itinerant runs of EMPFT, the parameters were stable and appeared unchanged from one EMPFT run to another. The final reconstruction was computed by combining 353 images at a resolution of 23 Å. The surface-rendering map was generated with the NAG Explorer program combined with custom-created modules.

Mass spectrometry. The mass spectrometry experiment was done with a Reflex III mass spectrometer from Bruker, equipped with gridless delayed extraction. The samples were mixed with an equal volume of a saturated solution of sinapinic acid (Sigma Chemical Co., St. Louis, MO) in 33% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid. On the target plate, a thin layer was prepared with a saturated solution of sinapinic acid in ethanol. A sample volume of 0.5 μl was applied to a thin layer of sinapinic acid and allowed to crystallize. Data were acquired in the linear instrument mode. Data were processed and evaluated by XMASS software from Bruker.

RESULTS

C-terminal 52-amino-acid deletion is necessary for formation of VLPs in Sf9 cells. To understand the mechanism underlying VLP formation in Sf9 and Tn5 cells, we prepared a series of baculovirus recombinants expressing pORF2 with different deletions at the N- and/or C-terminal region (Table 1 and Fig. 1). The cell lysate and culture medium of infected insect cells were analyzed by Western blotting. In a previous study, the N-terminal 111 aa-truncated HEV pORF2 was expressed by a recombinant baculovirus, Ac[n111], in both insect cells (11). Two major proteins, ~58K and ~53K, were detected in both cell lysates. The 53K protein was released into cell culture medium and assembled into VLPs in Tn5 cells but not in Sf9 cells (11).

Analysis of the N- and C-terminal aa sequences of the VLPs revealed that the N terminus was at aa residue 112 and the C terminus ended at aa residue 608, indicating that the C-terminal 52 aa of ORF2 were deleted. The protein that forms VLPs contains 497 amino acids (112 to ~608), and its molecular mass was about 53K. An N-terminal 111 aa- and C-terminal 52 aa-truncated construct, Ac[n111c52], was generated, and the protein was expressed in both Sf9 and Tn5 cells. As expected, a single 53K protein was found in both Sf9 and Tn5 cell lysates (Fig. 2, Ac[n111c52] lanes in Sf9 and Tn5). Interestingly, these 53K proteins were released into both culture media as VLPs, as observed by electron microscopy (Fig. 3). The particle appeared empty and homogenous in size. Therefore, C-terminal truncation to aa residue 608 is crucial for particle formation and release into Sf9 cells.

Ac[n111c58] and Ac[n111c59] encode truncated pORF2s with an N-terminal 111-aa deletion and respective C-terminal deletions of 58 and 59 aa. The expressed proteins migrated to

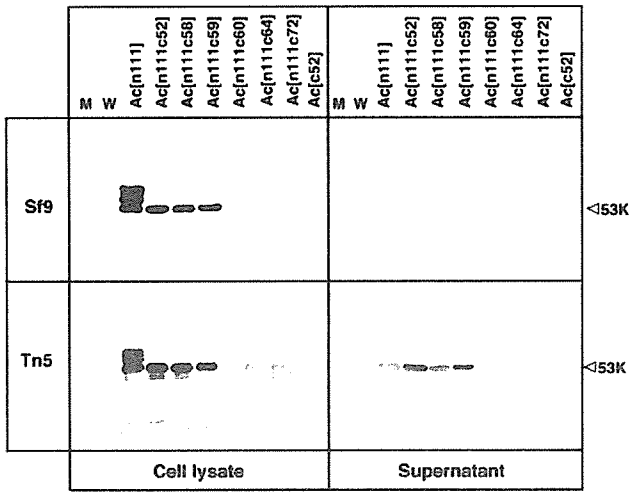

FIG. 2. Western blot assay of truncated pORF2 expressed in Sf9 and Tn5 cells. Eight recombinant baculoviruses, Ac[n111], Ac[n111c52], Ac[n111c58], Ac[n111c59], Ac[n111c60], Ac[n111c64], Ac[n111c72], and Ac[c52], were used to infect the insect cells. Ten microliters of the culture medium (right column) and 5 μl of the cell lysate (left column) were separated by 10% SDS-PAGE, and HEV-specific proteins were detected by Western blot analysis using the serum of a patient with acute hepatitis E. M, molecular weight markers; W, wild-type baculovirus-infected cells.

a position similar to that of 53K and appeared in both cell lysates as well as in the culture medium (Fig. 2); both were also assembled into VLPs (data not shown). In contrast, truncated pORF2 from Ac[n111c60], Ac[n111c64], and Ac[n111c72] was not released into the culture medium to detectable levels, and VLP was not formed even though protein expression remained similar to those of the other constructs (Fig. 2). Instead, a protein with a molecular mass of 42 kDa was detected in both of the cell lysates as well as in the culture medium by Western blot analysis. When pORF2 with a C-terminal 52-aa deletion was expressed with a recombinant baculovirus, Ac[c52], two major proteins, 65K and ~53K, were observed in infected Tn5 and Sf9 cell lysates 5 days postinfection (p.i.). However, these two proteins were not detected in their culture media (Fig. 2, Ac[c52] lanes in Sf9 and Tn5). These results indicated that aa residues before 601 were essential to the formation of VLPs.

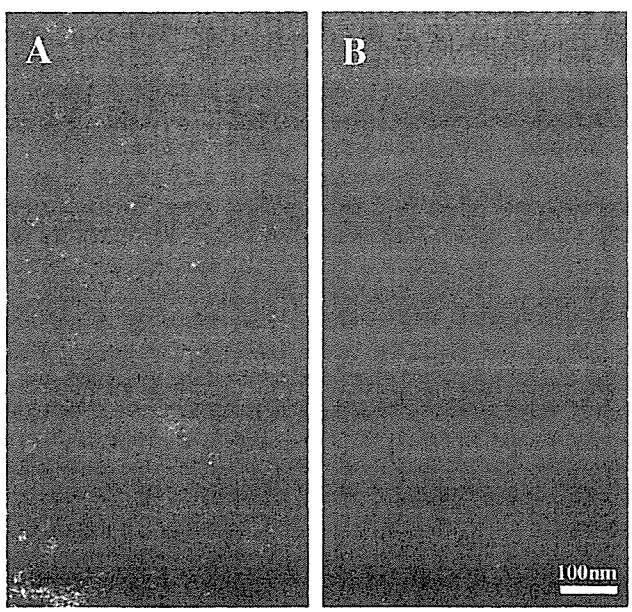

FIG. 3. EM of the HEV VLPs generated in Tn5 (A) and Sf9 (B) cells with recombinant baculovirus Ac[n111c52]. The VLPs were stained with 2% uranyl acetate. Bar, 100 nm.

VLPs produced in Sf9 and Tn5 cells possess the same configurations and structures. The morphology of the VLPs generated in Sf9 cells appeared to be similar to that generated in Tn5 cells, as observed in the negatively stained particles (Fig. 3). To investigate the structural properties of these two released VLPs, we performed cryo-electron microscopy and image processing using VLPs produced in Tn5 cells. The electron cryomicrographs showed that the particle projected as a spiky hollow sphere, indicating that no RNA-like density was packed inside the capsid (Fig. 4A). The image processing was done according to the icosahedral procedure. The rotational symmetry of 522 was applied to reconstruct the final three-dimensional structure. The reconstructed VLP displayed a T=1 surface lattice with protruding density located at each of 30 twofold axes (Fig. 4B). The VLP was composed of 60 copies of pORF2, and the protruding density consisted of dimeric, projecting domains from twofold-related peptides. The particle diameter was 270 Å, measured from the three-dimensional reconstruction. The protein shell was 85 Å thick at the twofold axes. A channel can be observed under each protruding density. The protruding density was about 43 Å high, and the twofold platform was 56 Å in the long axes (data not shown). The threefold-related dimers formed a regular triangle, and the dimer-dimer distance was 76 Å measured from center to center (Fig. 4B). Molecular interactions at the icosahedral threefold region appeared much stronger than those at the fivefold region. There was no significant difference in radial density distribution between Tn5- and Sf9-produced VLPs (Fig. 4C).

We further determined the composition of the particles obtained from Sf9 and Tn5 cells using mass spectrometry (Fig. 5). HEV VLPs produced from Tn5 and Sf9 cells with recombinant baculovirus Ac[n111c52] were analyzed. In both cases, the major density peak was monitored at the position corresponding to a mass of 53 kDa. The peak was symmetrically distributed, and a shoulder tip can be found in both cases. The shoulder tip was about 1 kDa larger than the main density peak. The signals further confirmed that the molecular mass of truncated pORF2 was 53 kDa, disregarding the production cell lines.

Essential N-terminal amino acids for VLP formation. Deletion of the N-terminal 111 residues is necessary for particle formation, which is consistent with our previous observation (11). The subsequent question is how many amino acids can be removed from pORF2 N termini without changing its capability to form VLPs. We made five constructs to express proteins with 123-, 124-, 125-, 126-, and 130-aa deletions at the N terminus by using five recombinant baculoviruses: Ac[n123], Ac[n124], Ac[n125], Ac[n126], and Ac[n130], respectively. As shown in Fig. 6, three proteins, having molecular masses of 58 to 51 kDa, were detected by Western blotting in both cell lysates at 5 days p.i., and the largest bands (58 to ~57K) were

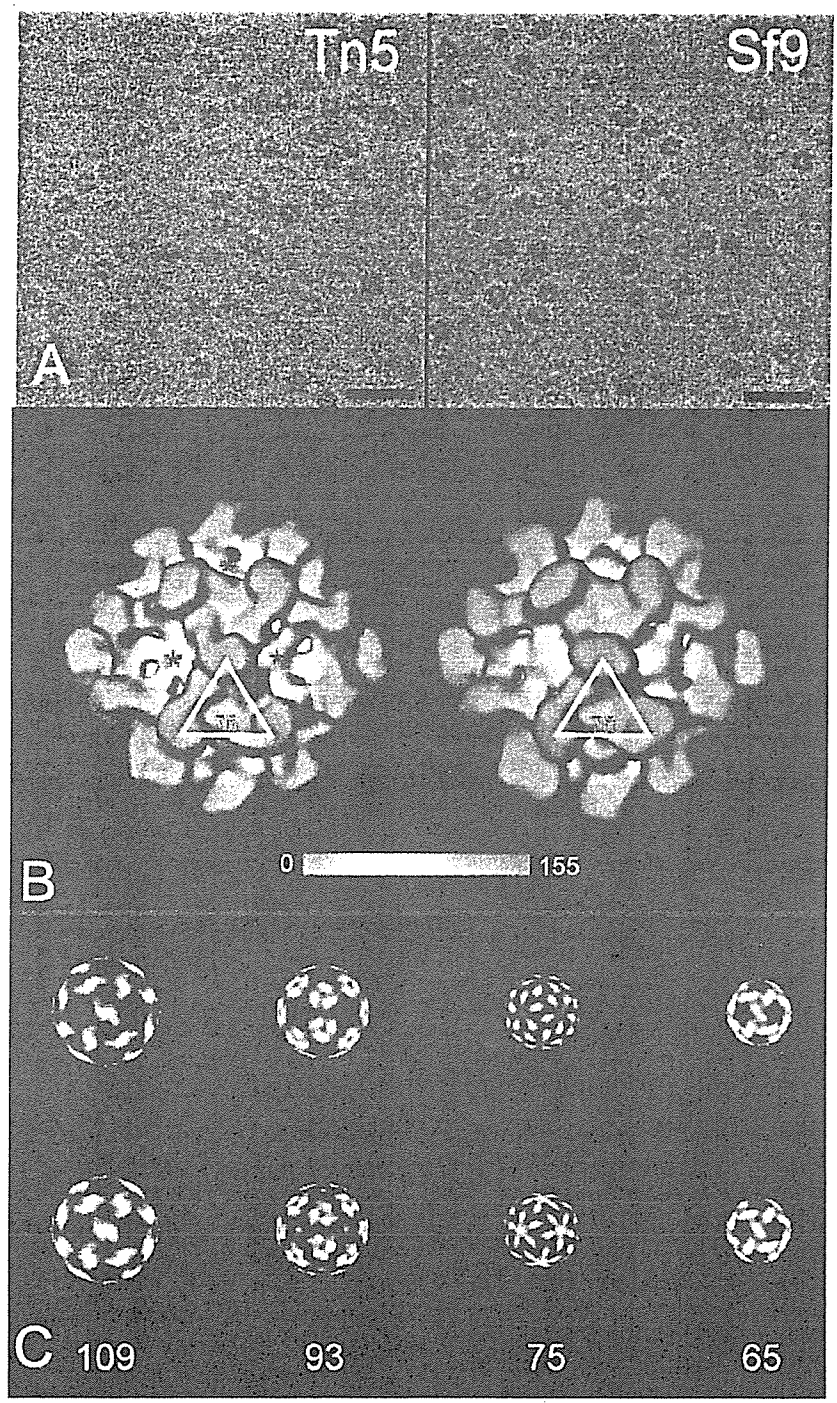

FIG. 4. HEV VLP structures determined by cryo-electron microscopy and image reconstruction. (A) Cryo-electron micrograph of ice-embedded HEV VLPs produced from Tn5 and Sf9 cells. The bar corresponds to 100 nm. (B) Surface-shaded representation of HEV VLP structures viewed along icosahedral twofold axes. VLPs from both Tn5 (left panel) and Sf9 (right panel) cells were color coded according to the radius, as indicated in the scale bar. The adjacent protruding spikes remain at equal distances of 76Å (white lines). The asterisks mark the positions of three adjacent icosahedral fivefold axes. (C) Sequential radial density projections generated from the twofold-oriented density map at corresponding radii. The protein density appears as the light color, while the background density is black.

thought to be the primary translation products encoded by N-terminal 123, 124, 125, 126, and 130 aa-truncated ORF2. In Tn5 cells, a C-terminal 52-aa-deleted product, about 51K protein, was the major protein to be efficiently released into the culture medium, where VLP formation occurred in Ac[n123]-, Ac[n124]-, and Ac[n125]-infected Tn5 cells (data not shown). Although the 51K protein was released into the culture medium, no VLP formation occurred in Ac[n126]- or Ac[n130]-

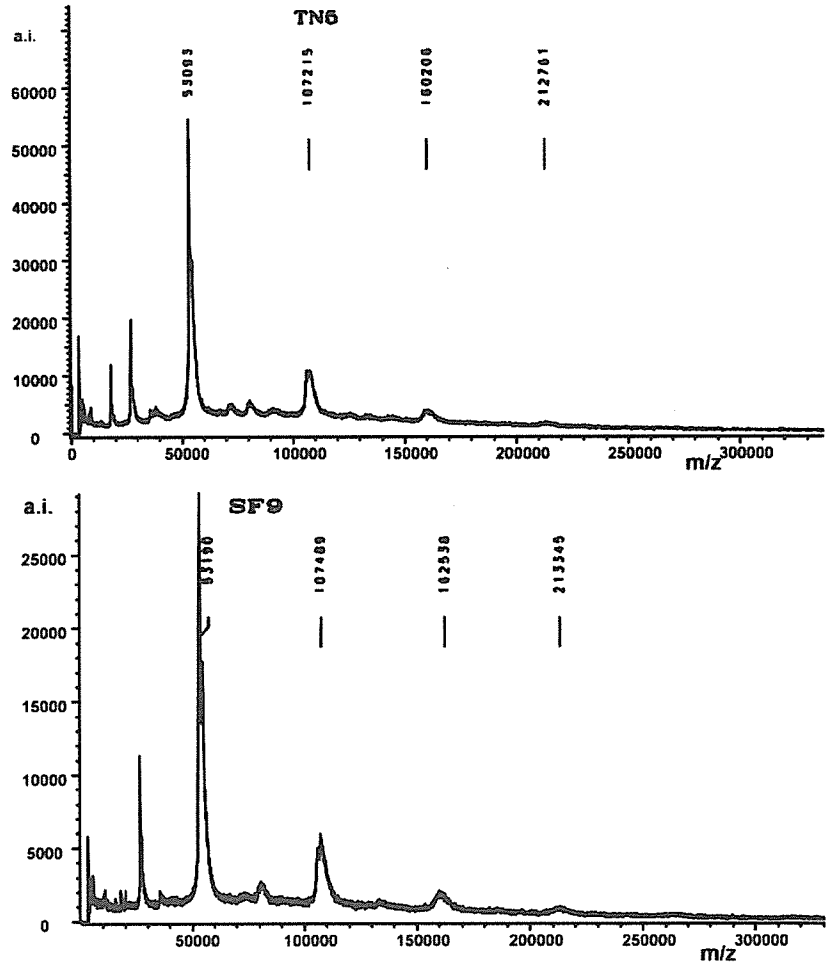

FIG. 5. Mass spectra from purified HEV VLPs displayed as the counts of isotope abundance (a.i.) versus mass/charge values (*m/z*). HEV VLPs produced from Tn5 (top panel) and Sf9 (bottom panel) cells with recombinant baculovirus Ac[n111c52] gave consistent mass spectra in which the abundant elements show similar *m/z* values at 53,000, 107,000, and 160,000.

infected Tn5 cells. In contrast, the 51K protein was not released into the culture medium in infected Sf9 cells (Fig. 6). These results demonstrated that aa residues after 125 were essential to the formation of VLPs.

When Ac[n125c59], an N-terminal 125 aa- and C-terminal 59 aa-truncated recombinant baculovirus, was expressed in Sf9 and Tn5 cells, the 51K protein was detected in both cell lysates and the culture media, where VLP formation occurred in both insect cell types (Fig. 6). This confirmed our observation that a C-terminal deletion of 52 to 59 amino acids was required for particle formation when Sf9 cells were used.

DISCUSSION

HEV is enigmatic due to the virus's inability to grow in conventional cell culture. Large quantities of the HEV capsid protein carrying antigenicity and immunogenicity comparable to those of the native virion have been generated for a long time, because the capsid protein is a key molecule for the diagnosis of hepatitis E as well as for vaccine development.

We previously found that when an N-terminal 111 aa-truncated ORF2 protein was expressed in Tn5 and Sf9 cells, two major peptides, having molecular masses of 58 and 53 kDa, were generated in both cells, and only the 53-kDa protein generated in Tn5 cells was released into culture medium and self-assembled into VLPs (11). The 58K protein presented the primary translation product, and the 53K protein is a processing product from the 58K protein. In this study, we examined the difference between Tn5 and Sf9 cells in HEV ORF2 gene expression and found that when a recombinant baculovirus (Ac[n111c52]) harboring a construct of the C-terminal 52-aa deletion was used, no difference between Sf9 and Tn5 cells in protein translation and particle formation was found. The observation that Ac[n111] failed to produce VLPs in Sf9 cells raised a question about the posttranslation modification in insect cells. In Tn5 cells, the levels of protein expression by Ac[n111] and Ac[n111c52] appeared to be similar. Therefore, it is likely that the 58K protein was incorrectly processed in Sf9 cells, thus affecting VLP assembly.

In addition, when Sf9 insect cells were infected with Ac[n111], the expressed proteins were localized in the cyto-

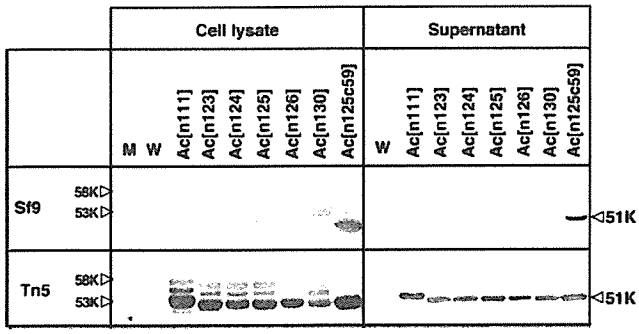

FIG. 6. Expression of N-terminally truncated pORF2 in Sf9 and Tn5 cells infected with Ac[n123], Ac[n124], Ac[n125], Ac[n126], Ac[n130], and Ac[n125c59]. A Western blot assay was carried out as described in the legend to Fig. 2. Ac[n111] was included for the expression of the 58K and 53K proteins. M, molecular weight markers; W, wild-type baculovirus-infected cells.

plasm and observed as inclusion-like bodies (one to four structures per cell) by EM (25). In contrast, when Sf9 cells were infected with Ac[n111c52], there were no inclusion-like bodies (data not shown), and the expressed proteins were localized evenly in the cytoplasm. Concomitantly, expressed protein was poorly detected in the culture medium from Ac[n111]-infected Sf9 cells at 3 days p.i., whereas a large amount of the 53K protein was detected in the culture medium from Ac[n111c52]-infected Sf9 cells. These findings suggest that the C-terminal aa of ORF2 might affect the localization, and subsequently the release, of the capsid protein from the insect cells. However, we do not yet know whether the VLPs form before release in infected cells or after release in culture medium.

The presence of Leu601 in pORF2 is important for the formation of HEV VLPs. A protein with a longer (580 to 610) deletion of aa residues was aberrant in protein folding; this may reduce the ORF2 homo-oligomerization (24). The prediction of the secondary structure based on protein sequence suggests two β-strand motifs in the region between aa 580 and 601 (580 to ~589 and 593 to ~601). The failure in the particle assembly with Ac[n111c60] is due to incomplete formation of this β-strand motif. Although aa 111 to 601 and aa 111 to 602 formed VLPs, the yield of each of these was about 10 to 20% of the yields of aa 111 to 660 (data not shown). This is in contrast to the fact that the levels of protein expression inside the cells were similar in these constructs. This observation further confirmed that stability of the C-terminal β-strand motif is essential for VLP assembly.

The N-terminal 111-aa-deletion was found to be essential for cellular membrane dissociation of pORF2 expressed in insect cells (17, 24). We extended the N-terminal deletion up to Val125 without altering the ability to form HEV VLPs (Fig. 6). The ORF2 protein exhibits two-domain folding (25), with a domain organization similar to those of the norovirus (NV) capsid protein (15) and the tomato bushy stunt virus capsid protein (14). The N-terminal aa residues 112 to 125 may be the arm region extending from the S domain into the particle interior. In NV, the N-terminal region appeared to serve as a switch controlling the S domain configuration in the assembly process (3). Removal of the first 20 amino acids did not affect NV-like particle self-assembly, but a longer deletion at the N-terminal region did (3). Thus, residues 112 to 125 are putatively located in the HEV virion interior and may regulate VLP assembly.

Tn5 and Sf9 are insect cell lines that are commonly used in recombinant protein expression. The Tn5 cell is becoming more and more popular because it yields higher quantities of tissue factor than Sf9. Under optimum conditions, Tn5 cells produce 28-fold more secreted soluble tissue factor than Sf9 cells on a per-cell basis (23). In this paper, we report the difference between Tn5 and Sf9 cells in a protein synthesis system. The ORF2 protein underwent posttranslation cleavage, which is crucial for HEV VLP assembly. Although the HEV virion assembly mechanism remains unclear, our data indicate that the region consisting of ORF2 residues 126 to 601 is the kernel element for the monomer-monomer interaction and thus initiates VLP assembly.

Recombinant HEV VLPs themselves can be candidates for parenteral as well as oral hepatitis E vaccines (9, 10), and these VLPs have potential as mucosal vaccine carrier vehicles for the presentation of foreign antigenic epitopes through oral administration (13). Furthermore, HEV VLPs can be a vector for gene delivery to mucosal tissue for the purposes of DNA vaccination and gene therapy (20). The results of the present study provide the basic tool to construct VLPs having novel functions.

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