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Challenges in creating a vaccine to prevent hepatitis E

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Summary

Recombinant hepatitis E virus capsid protein (HEV CP) assembles orally immunogenic virus-like particles (VLP) when expressed in an insect cell system. We used plant expression cassettes, pHEV101 and pHEV110, for transformation of potato to express HEV CP, and 10 independent transgenic lines of HEV101 and 6 lines of HEV110 were obtained. ELISA for HEV CP was performed on tuber extracts. Accumulation of HEV CP in tubers varied from about 5 to 30 μg/g fresh tuber depending on the transgenic plant line. We further compared the expression levels with the yield of tubers for each line. Tuber yield varied less than expression levels, and ranged from about 600 to 1000 g per pot. Although Western blot showed that apparently intact HEV CP accumulated, we observed very limited assembly of virus-like particles in potato tubers. Oral immunization of mice with transgenic potatoes failed to elicit detectable anti-CP antibody response in serum, suggesting that VLP assembly is a key factor in orally delivered HEV CP vaccines.
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Keywords: Hepatitis E; HEV; VLP; Transgenic potato

1. Introduction

Hepatitis E virus (HEV) is a causative agent of hepatitis E that occurs in many developing countries [1], and this virus is currently classified into a tentative genus, "Hepatitis E-like viruses." HEV is transmitted mainly by the fecal-oral route, and large epidemics due to this virus are often associated with contaminated water [2,3]. Hepatitis E has been formerly known as an enterically transmitted non-A, non-B hepatitis [4]. The fact that HEV can survive in the intestinal tract suggests that the virus is relatively stable to acid and mild alkaline conditions.

HEV contains a single-stranded positive-sense approximately 7.5 kb RNA molecule that is 3′ polyadenylated and includes three open reading frames (ORFs). ORF1, mapped in the 5′ half of the genome, is thought to encode viral nonstructural proteins. ORF2, located at the 3′-terminus of the genome, encodes a 72 kDa protein for the putative viral capsid. ORF3, with unknown function, is mapped between ORF1 and ORF2 [5]. In the absence of an appropriate cell culture for HEV propagation, research has focused on the expression of the ORF2 protein in heterologous systems. Recently, virus-like particles (VLP) of recombinant hepatitis E virus (rHEV) were produced by using a baculovirus system carrying an N-terminally truncated *ORF2* gene of the Burma strain [6]. Thus, rHEV VLP were formed in Tn5 cells and could be collected from the culture supernatant.

In order to evaluate the potential of rHEV VLP as an oral immunogen, we analyzed the immune responses in mice and monkeys after oral administration [7,8]. The animals were orally inoculated with purified rHEV VLP without adjuvant. ELISA indicated that oral immunization with rHEV VLP induced immune responses in both mice and monkeys. In addition, the monkeys were completely protected from infection when challenge was carried out with native HEV, suggesting that rHEV VLPs are a potential mucosal vaccine for HEV infection.

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For expression and delivery of recombinant subunit vaccine antigens including Norwalk virus capsid protein (NVCP), transgenic plants have been created [9]. Clinical trials using potatoes expressing NVCP showed very promising results, with 19 of 20 volunteers showing immune responses against NVCP delivered by ingestion of raw transgenic potatoes containing approximately 500 μg antigen per dose [10]. Previous studies with NVCP expression in tobacco and potato cells demonstrated that subunits assembled to form VLP very similar to those obtained with baculovirus-infected insect cell expression, although up to 75% of the antigen was present as monomers or partially assembled aggregates [9]. VLP assembly may be important for obtaining stability against acid and protease-mediated degradation in the stomach, as well as for presentation of conformation-dependent epitopes that may be needed for effective virus neutralization.

In this study, we expanded our effort to create transgenic plants that express HEV capsid proteins (HEV CP).

2. Materials and methods

2.1. Preparation and purification of rHEV VLP

The molecular cloning and construction of a recombinant baculovirus Ac5480/7126 harboring the HEV capsid protein gene lacking 111 amino acids at the N-terminal were described previously [6]. The rHEV VLP were prepared using Tn5 cells infected with Ac5480/7126 at a multiplicity of infection of 10. Following 7 days of incubation at 26.5 °C, intact cells and cell debris were removed from the culture medium, and the rHEV VLP were concentrated by centrifugation and purified by isopycnic binding in CsCl gradient. A visible band containing rHEV VLP was collected, and the rHEV VLP were diluted and pelleted by centrifugation.

2.2. Western blot assay

Leaf samples were extracted by FastPrep (speed 5, 30 s) in 4 ml/g leaf of 50 mM sodium phosphate pH 6.6, 50 mM NaCl, 50 mM sodium ascorbate, 1 mM EDTA, 1 mM PMSF, 0.1% Triton X-100, and clarified for 2 min at 4 °C in microcentrifuge. Aliquots were subjected to SDS-PAGE, blotted to PVDF membrane, and probed with guinea pig anti-HEV (1:1000), visualized with ECL+ (anti-guinea pig IgG-HRP 1:5000) on STORM scanner.

2.3. Antigen-capture ELISA

Potato leaf or tuber extracts were prepared as described above in Section 2.2. Microtiter plates were coated with 50 μl per well of rabbit anti-HEV serum diluted 1:10,000 in carbonate/bicarbonate coating buffer overnight at 4 °C. Insect cell-derived HEV VLP reference standard was diluted in PBST/1% dry milk at 100 ng/ml and two-fold dilutions down to 3.125 ng/ml. Leaf or tuber extracts were diluted 25- and 50-fold in PBST/1% dry milk. The reference standards and plant extracts were loaded at 50 μl per well and incubated at 37 °C for 1 h. Wells were washed with PBST and then probed with guinea pig anti-HEV serum diluted 1:5000, followed by goat anti-guinea pig IgG-HRP conjugate (Sigma) diluted 1:5000. Color was developed using TMB substrate solution for 5 min.

2.4. Construction of plant expression vector

Intermediate plant expression cassettes were constructed using a vector pIBT210 [11]. Since two truncated forms

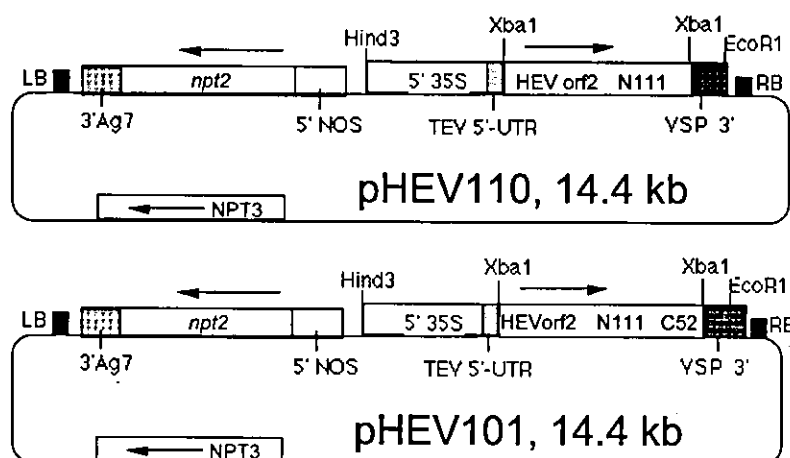

Fig. 1. Plant transformation vectors for expression of HEV CP. Binary T-DNA plasmid vectors, pHEV101 and pHEV110, for expression of HEV CP, p54 and p58, are shown. They contain left border (LB) and right border (RB) sequence motifs that delineate the DNA to be transferred (T-DNA) and integrated into nuclear chromosomal DNA. Within the T-DNA borders lies a selectable marker (*ntp2*), which confers resistance to the antibiotic kanamycin, and will allow specific regeneration of transformed plants. Also included are expression cassettes for HEV CP, which are driven by the constitutive CaMV 35S promoter linked to the tobacco etch virus (TEV) 5′-UTR, which acts as a translational enhancer, and terminated by the soybean VSP 3′ end [9].

of HEV ORF2 appeared to yield VLP assembly in insect cells (ΔN111 and ΔN111/ΔC52), we inserted these into pIBT210. Then, the expression cassettes were transferred into a binary vector (pGPTV-Kan) for use in *Agrobacterium*-mediated delivery of foreign DNA into plant cells. These constructs, pHEV101 (ΔN111/ΔC52) and pHEV110 (ΔN111), are shown in Fig. 1.

3. Results

3.1. Characterization of rHEV

The capsid proteins of HEV with its N-terminal 111 amino acids truncated were expressed with a recombinant baculovirus in insect cells, where the capsid proteins self-assembled into VLP [6]. The rHEV VLP were purified by centrifugation and characterized by SDS-PAGE and Western blot assay, where a major protein band with a molecular weight of 54 kDa was observed. The particles possess antigenicity similar to that of authentic HEV particles and consequently they appear to be a good antigen for the sensitive detection of HEV-specific IgG and IgM antibodies [12]. Furthermore, the VLP may be the most promising candidate for an HEV vaccine owing to its potent immunogenicity [7,8]. Therefore, we used the same construct to express HEV CP in the transgenic plant.

3.2. Coding sequence analysis

We first examined the coding sequence for the HEV CP to determine whether the nucleotide sequence should be altered for optimization of plant expression. Codon use is fairly favorable to both dicot and monocot plants. Of 660 total codons, 3.6% are monocot-unfavorable and 12.8% are dicot-unfavorable, defined as either making up less than 10% of codon choice for that amino acid or less than one third the frequency of the most popular codon for that amino acid, inclusive.

3.3. Expression in potato plants

We used pHEV101 and pHEV110 for transformation of potato "Desiree" as described [9,13]. After regeneration of multiple independent kanamycin-resistant lines, we screened leaf samples by ELISA for HEV CP expression. Expression levels ranged up to approximately 0.33% total soluble protein, which is similar to the levels we obtained for NVCP [9]. There was no apparent difference in expression from either construct pHEV101 (ΔN111/ΔC52 coding sequence) and pHEV110 (ΔN111 coding sequence) as the range and maximal expression were similar for both.

We selected the best lines for transplant to the greenhouse and after 2 months growth we assayed leaves for expression of HEV CP by ELISA. We observed that the antigen

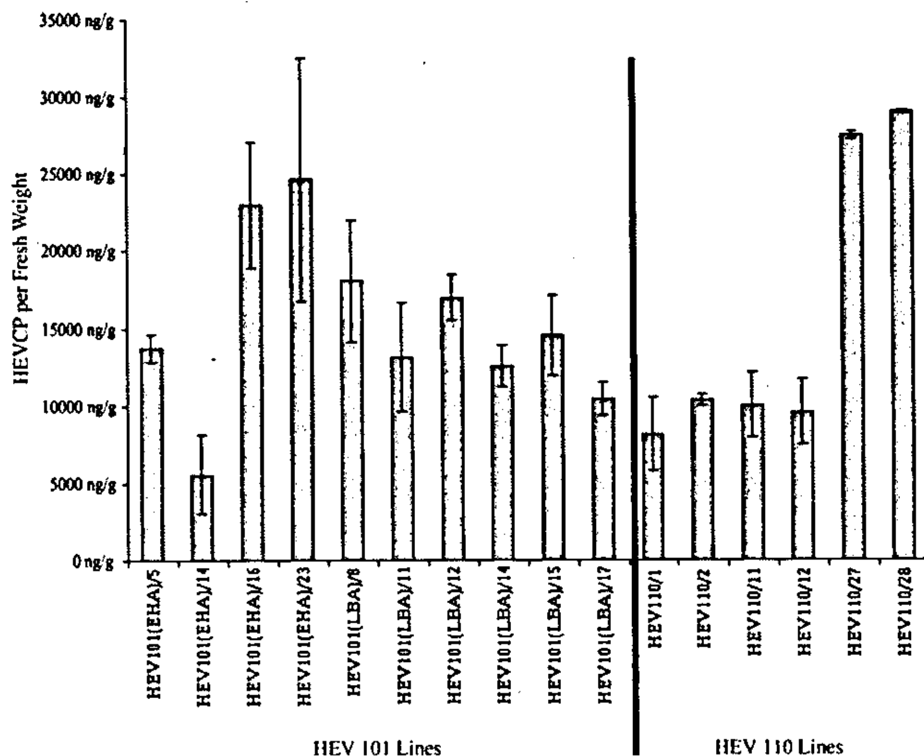

Fig. 2. Expression of HEV CP in tubers of transgenic potato lines. HEV101 (10 lines) or HEV110 (6 lines) tubers were extracted and assayed by ELISA for HEV CP. Error bars indicate standard error for three different tubers from the same line.

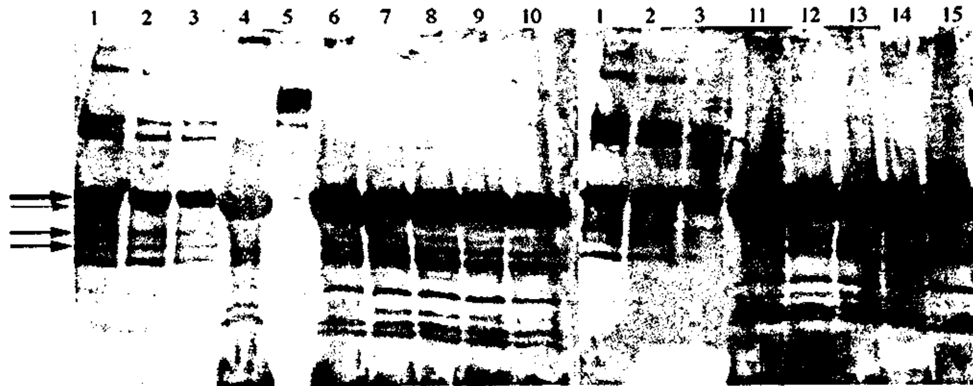

Fig. 3. Western blot of HEV CP expressed in potato leaves. Leaf samples were extracted by FastPrep and clarified by centrifugation. Aliquots were subjected to SDS-PAGE, blotted to PVDF membrane, and probed with guinea pig anti-HEV, visualized with ECL+ on STORM scanner. Lanes are: (1) 50 ng HEV VLP from baculovirus-infected insect cells, (2) 25 ng HEV VLP, (3) 12.5 ng HEV VLP, (4) untransformed Desirée extract, (5) BioRad Kaleidoscope Molecular Weight Marker, (6) HEV101(EHA)/5, (7) HEV101(EHA)/12, (8) HEV101(EHA)/16, (9) HEV101(EHA)/17, (10) HEV101(LBA)/11, (11) HEV101(LBA)/14, (12) HEV110/1, (13) HEV110/2, (14) HEV110/11 and (15) HEV110/12. Bold arrow indicates major band of VLP sample that corresponds to the 54 kDa HEV CP; thin arrows indicate products of proteolysis.

expression on a total protein basis was reduced four- to five-fold compared to that in tissue-cultured plantlets. This is not unexpected, since soil-grown plants in natural light have higher levels of total leaf protein. Further, it is possible that the recombinant antigen is less stable in the soil-grown plants.

3.4. HEV CP expression in potato tubers

Ten independent transgenic lines of HEV101 and six lines of HEV110 were grown to maturity in the greenhouse and tubers were harvested and washed. ELISA for HEV CP was performed on tuber extracts as described for leaf extracts. Accumulation of HEV CP in tubers varied from about 5 to 30 μg/g fresh tuber, depending on the transgenic plant line (Fig. 2). This compares well with the expression of NVCP in potato tubers [9] and is better than expression of *E. coli* LT-B protein in potato [13]. We further compared the expression levels with the yield of tubers for each line. Tuber yield varied less than expression levels, and ranged from about 600 to 1000 g per pot. We used these data to select lines HEV101-16, HEV101-23, HEV110-27, and HEV110-28 for highest yields of recombinant protein.

Western blot of transgenic potato leaf extract showed that most of the recombinant HEV CP accumulated as 54 kDa, similar to the insect cell-derived antigen (Fig. 3). Some apparent proteolytic products of lower M_r were observed in both insect cell- and potato-derived material. HEV CP in plants transformed with either HEV101 or HEV110 showed similar patterns, with no qualitative or quantitative differences apparent. Failure to detect a larger protein for the single-truncation HEV101 (ΔN111) than that observed for the double truncation HEV110 (ΔN111/ΔC52) suggests that the ΔC52 truncation may occur in planta via an endogenous protease.

4. Discussion

HEV CP has been expressed in baculovirus-infected insect cell system and shown to assemble VLP [6,14]. The VLP have several advantages for the mucosal immunogen as follows: (1) rHEV VLP are composed of a single protein assembled into particles without nucleic acid. (2) rHEV VLP are easy to prepare and purify in a large quantities, approximately 1 mg per 2×10^7 insect cells. (3) rHEV VLP are antigenically similar to the native virion. (4) rHEV VLP are highly immunogenic in experimental animals when injected parenterally.

Our goal is to create transgenic plants that express HEV CP in edible tissues as VLP, in order to obtain an economical oral vaccine. It is likely that the success of oral delivery using VLP from insect cells is due to the particulate structure of the antigen, which contributed either to enhanced resistance to degradation in the gut or to enhanced uptake into the gut immune system.

In our studies with potato expressing HEV CP, we found very few VLPs, with the great majority of ELISA-positive antigen remaining near the top of a sucrose gradient (data not shown). Oral immunization of mice with potatoes expressing HEV CP failed to elicit detectable antibody responses in serum (data not shown). We extracted fecal pellets on day 18 after oral immunization on day 17 to evaluate the content of ELISA-reactive HEV capsid protein. Substantial antigen (3–4% of the dose) was present in pellets of mice that were fed HEV CP potato or gavaged with insect cell-derived VLP. Thus, the potato cells probably provided some protection to the soluble HEV CP present in potato tubers, and perhaps even limited uptake of antigen that may have been present as VLP. Since orally delivered insect cell-derived VLPs stimulated antibody responses and protected monkeys against HEV challenge [8], it is likely that poor VLP assembly in potato

was a major factor in the lack of oral immunogenicity of potato-derived HEV CP in mice. Future studies should focus on the optimization of VLP assembly in plant tissues, which may involve alternative plant host systems, and/or tissue and subcellular targeting of antigen.

Acknowledgements

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

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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).

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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

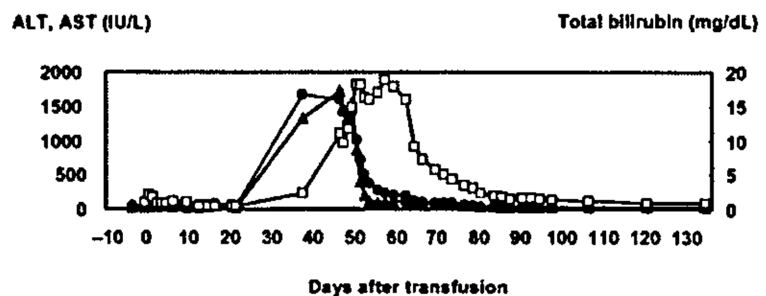
The clinical course of the patient (S.K.) is summarized in Fig. 1. When he was hospitalized again for acute hepatitis, he had an elevated ALT level of 1595 IU per L and an AST level of 1727 IU per L on Day 46 after transfusion; these normalized within 1 month, whereas the total bilirubin level rose to 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 posttransfusion. 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.

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.

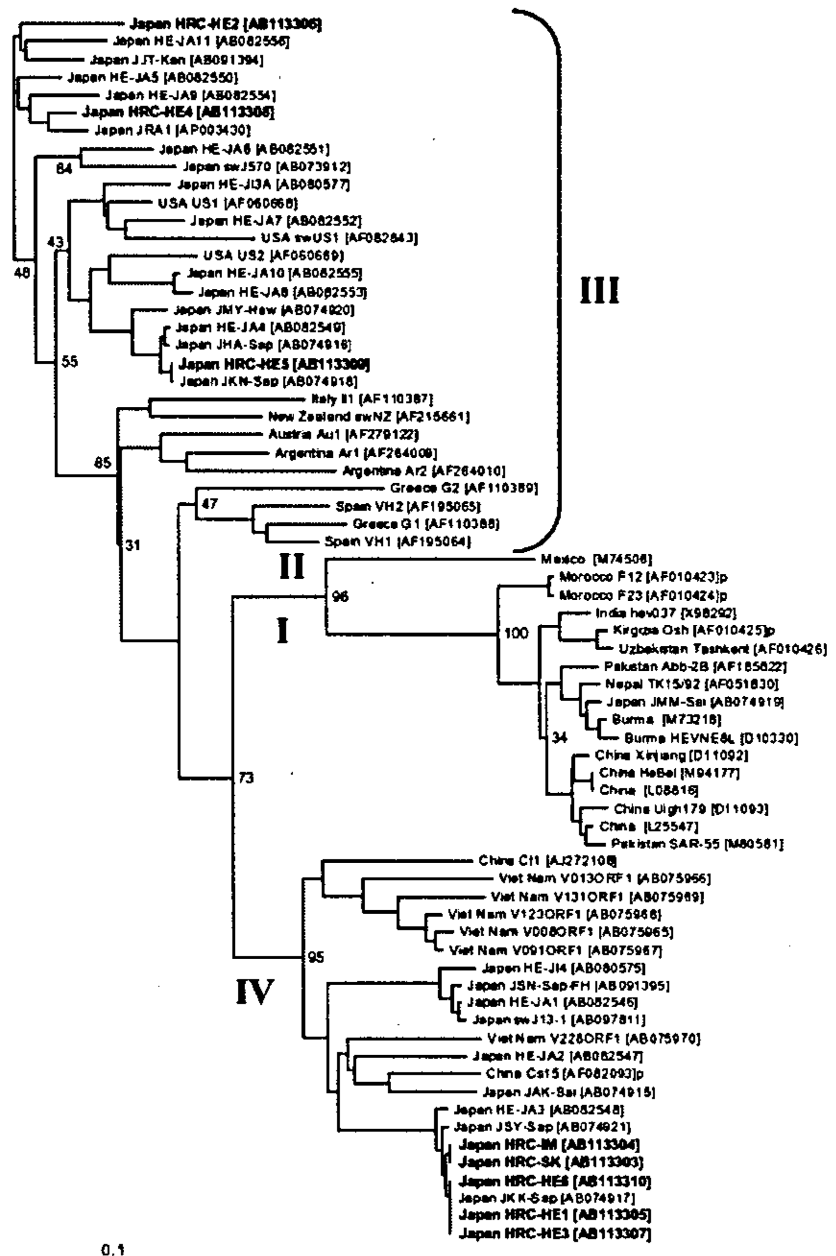


Fig. 2. Phylogenetic tree based on a 326-nt region of ORF1 for HEV strains derived from the case donor HRC-IM and patient HRC-SK and six donors with elevated ALT of greater than 500 IU per L. Accession numbers for the reference sequences are indicated parentheses. The isolated strains in this study are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

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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RESEARCH ARTICLE

DNA vaccine-encapsulated virus-like particles derived from an orally transmissible virus stimulate mucosal and systemic immune responses by oral administration

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Delivery of foreign genes to the digestive tract mucosa by oral administration of nonreplicating gene transfer vectors would be a very useful method for vaccination and gene therapy. However, there have been few reports on suitable vectors. In the present study, we found that plasmid DNA can be packaged in vitro into a virus-like particle (VLP) composed of open reading frame 2 of hepatitis E virus, which is an orally transmissible virus, and that these VLPs can deliver this foreign DNA to the intestinal mucosa in vivo. The delivery of plasmid DNA to the mucosa of the small intestine was confirmed by the results of immunohistochemical analyses using an expression plasmid encoding human immunodeficiency virus env (HIV env) gp120. After oral administration of VLPs loaded with HIV env cDNA, significant levels of specific IgG and IgA to HIV env in fecal extracts and sera were found. Moreover, mice used in this study exhibited cytotoxic T-lymphocyte responses specific to HIV env in the spleen, Payer's patches and mesenteric lymph nodes. These findings suggest that VLPs derived from orally transmissible viruses can be used as vectors for delivery of genes to mucosal tissue by oral administration for the purpose of DNA vaccination and gene therapy.
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Keywords: *VLP; oral DNA vaccine; CTL; HIV; mucosal immunity*

Introduction

The successful outcome of novel gene therapies and DNA vaccinations largely depends on the development of effective delivery systems.[1] In human applications, both the efficacy and safety of any delivery system used for gene transfer are major concerns. It has been shown that tissue-specific gene transfer by a viral vector could be achieved naturally and effectively through cell specificity of the virus receptors.[2] However, there is a risk of vector toxicity through viral infection of the host cells. Also, the limited sizes of transgenes often present a serious obstacle. Nonviral vectors, such as liposomes, are safer but do not have a cell-specific targeting component and have limited transduction both *in vitro* and *in vivo*. This limitation has been partly overcome by the development of molecular conjugates consisting of cell-specific ligands that confer cell specificity to nonviral vectors.[3,4]

The development of a system for delivering genes to or conferring immunity to mucosal tissue by oral administration would provide a convenient means for effective treatment or prevention of various human diseases, including cancers, infectious diseases and immunological disorders.[5] Since many pathogenic viruses and bacteria establish their initial infections through the mucosal surface, vaccine strategies that can stimulate mucosal immunity have been widely studied (reviewed in Ogra *et al*[6]). However, there are several difficulties in oral immunization with nonreplicating molecules, such as low pH in the stomach, the presence of proteolytic enzymes in the digestive tract and the presence of physical as well as biochemical barriers associated with the mucosal surface itself.[6]

Among the various nonreplicating molecules, a virus-like particle (VLP), an empty particle with a structure similar to that of an authentic virus particle, offers the possibility of a new approach for vaccine development.[7] It is expected that the VLP structure will provide resistance to severe environments in the digestive tracts and enable specific binding to the mucosal surface if an appropriate VLP is chosen.[8] However, VLPs can induce immune responses to themselves, and this is a problem for using VLPs as a vaccine vector to carry foreign DNA. A system using polyoma virus VP1 VLPs as a carrier of DNA by intranasal administration has been reported.[9] These VLPs work as an adjuvant, since DNA vaccine can induce immune responses by intranasal administration without VLPs. Hepatitis E virus (HEV) is an unclassified calicivirus-like, positive-strand RNA virus that causes

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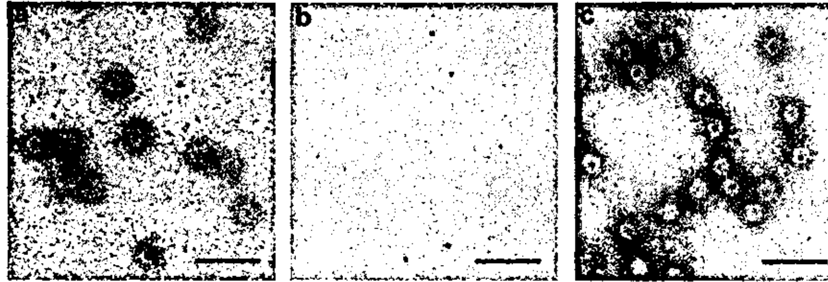

Figure 1 Electron micrographs of HEV-VLPs: (a) purified HEV-VLPs before treatment; (b) disassembled HEV-VLPs after treatment of VLPs with EGTA and DTT; and (c) refolded HEV-VLPs in the presence of $CaCl_2$, DMSO and DNA. Bars represent 50 nm.

human acute hepatitis by fecal–oral transmission. HEV first infects epithelial cells of the small intestine and then reaches the liver through the portal vein. It has recently been reported that overexpression of a part of open reading frame 2 (ORF2) in a baculovirus expression system results in the assembly of this protein into a VLP.[10] We have also reported that VLPs carrying foreign epitopes elicit strong mucosal and systemic immune responses to both the VLPs and exogenous epitopes without the requirement of any kind of adjuvant when orally administered to mice.[11]

Since infection with human immunodeficiency virus (HIV) most likely occurs through exposure of mucosal tissue to the virus, HIV-specific immune responses at mucosal sites are critical for the initial control of infection. Therefore, a nonreplicating vaccine vector that elicits mucosal immunity by oral administration would be a powerful HIV vaccine. In the present study, we found that unrelated plasmid constructs can be encapsulated into HEV-VLPs and delivered to the intestinal mucosa by oral administration. HIV DNA vaccine-loaded HEV-VLPs can elicit mucosal and systemic cellular as well as humoral immune responses by oral administration.

Results

In vitro refolding of VLPs

The HEV-VLPs produced by a recombinant baculovirus system were disassembled by the removal of calcium ions (Figure 1b). When calcium ions were supplemented to the disrupted VLPs in the presence of plasmid DNA, the DNA was encapsulated into the refolded VLPs (Figure 1c). No significant morphological difference due to the VLP disassembling–refolding process was observed under an electron microscope.

Density shifts of VLPs and amount of plasmid DNA after DNA encapsulation

Plasmid DNA encapsulation in the refolded VLPs was confirmed by CsCl equilibrium gradient centrifugation. VLP density is greater when loaded with a DNA plasmid. A heavier density gradient peak was present only when DNA was incorporated into the VLPs (Figure 2d). A single lighter density peak was produced for VLPs alone (Figure 2a), refolded VLPs (Figure 2b) and intact VLPs in the presence of plasmid DNA (Figure 2c). Despite the various sizes of plasmid DNA used for encapsulation, the amounts of plasmid in VLPs were almost the same (17–19 μg per 50 μg of HEV-VLPs) (Figure 2e and f). A solution with a high concentration of plasmid DNA showed high viscosity, and VLPs including DNA were not obtained for general use in experiments. Based on these results, we used this amount (1 mg/ml) as the optimal concentration (data not shown).

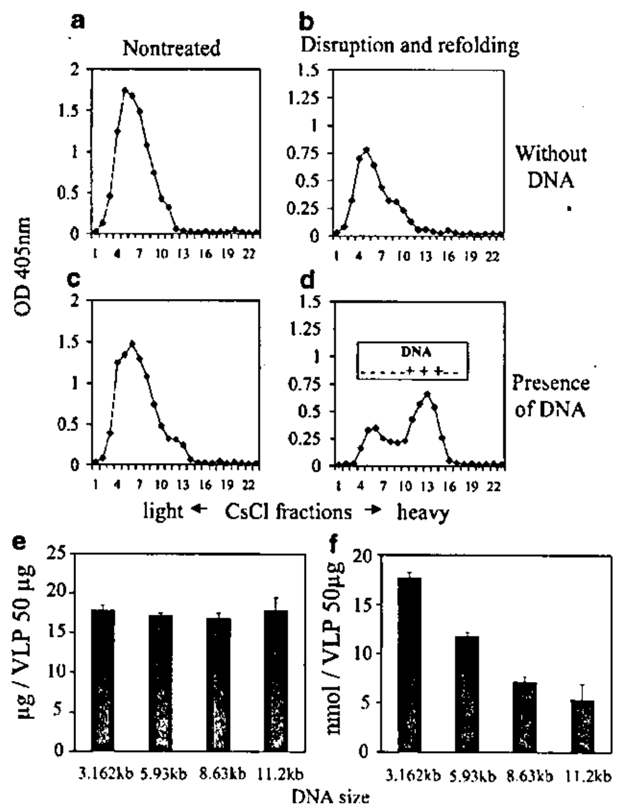

Figure 2 CsCl gradient profiles of intact and refolded VLPs. No DNA added: (a) intact; (b) refolded. DNA added: (c) intact; (d) refolded. The amount of DNA encapsulated in VLPs is expressed as μg (e) and molality (f) per 50 μg VLP protein.

Gene transfer by HEV-VLPs

Initially, four cell lines derived from mice, rabbits, monkeys and humans were studied for their ability to

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

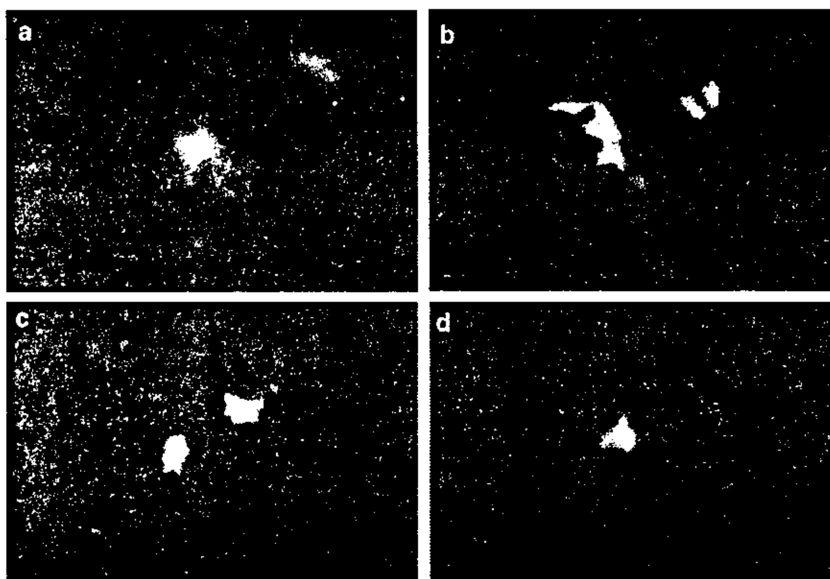

Figure 3 Expression of GFP in cells transfected with plasmid DNA encapsulated in HEV-VLPs: (a) NIH/3T3 cells (mouse); (b) RK-13 cells (rabbit); (c) COS-7 cells (monkey); and (d) HepG2 cells (human).

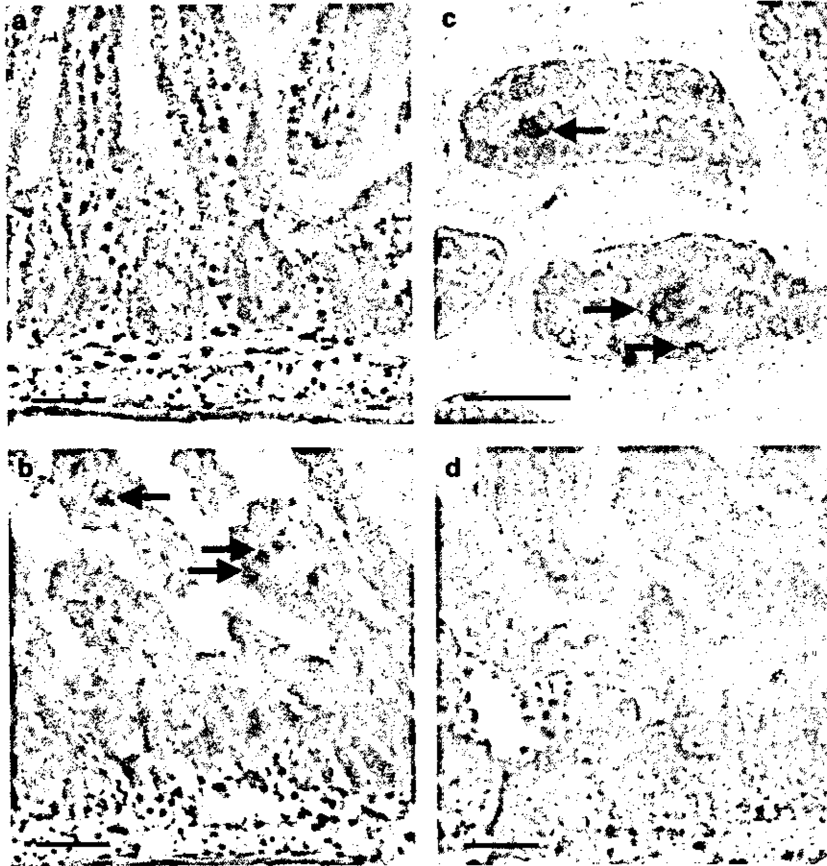

Figure 4 *Immunostaining of serial sections of small intestine tissue from mice 2 days after oral administration of pJWNL432-encapsulated VLPs. HIV env proteins were observed in epithelial cells (arrow) (b, c), and control mAb did not show any positive reactions (d). Control mice were also administered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

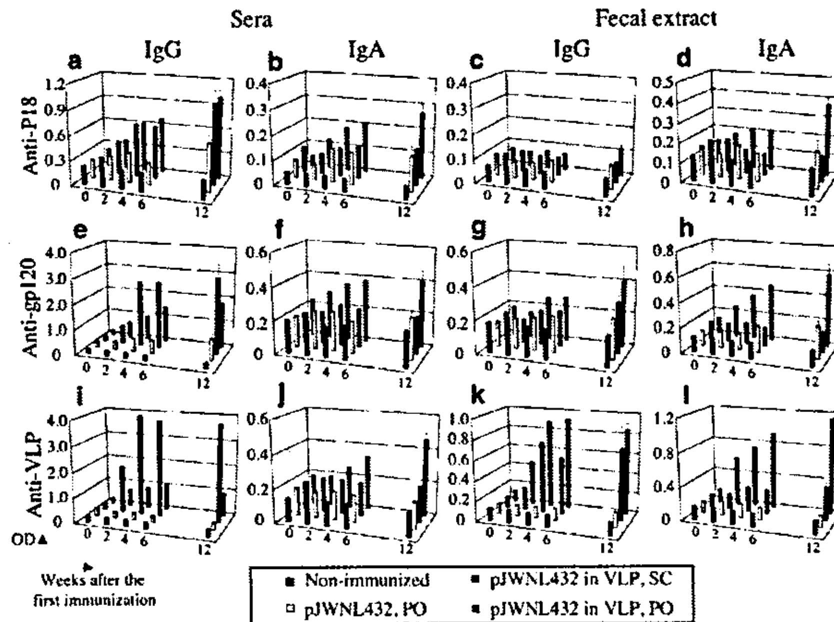

Figure 5 *IgG (a, c, e ,g, i and k) and IgA (b, d, f, h, j and l) levels in sera (a, b, e, f, i and j) and fecal extracts (c, d, g, h, k and l) of immunized mice. Mice were orally (■) or subcutaneously (□) administered pJWNL432 encapsulated in VLP or naked (■). Symbols indicate HIV env-specific antibody 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.*

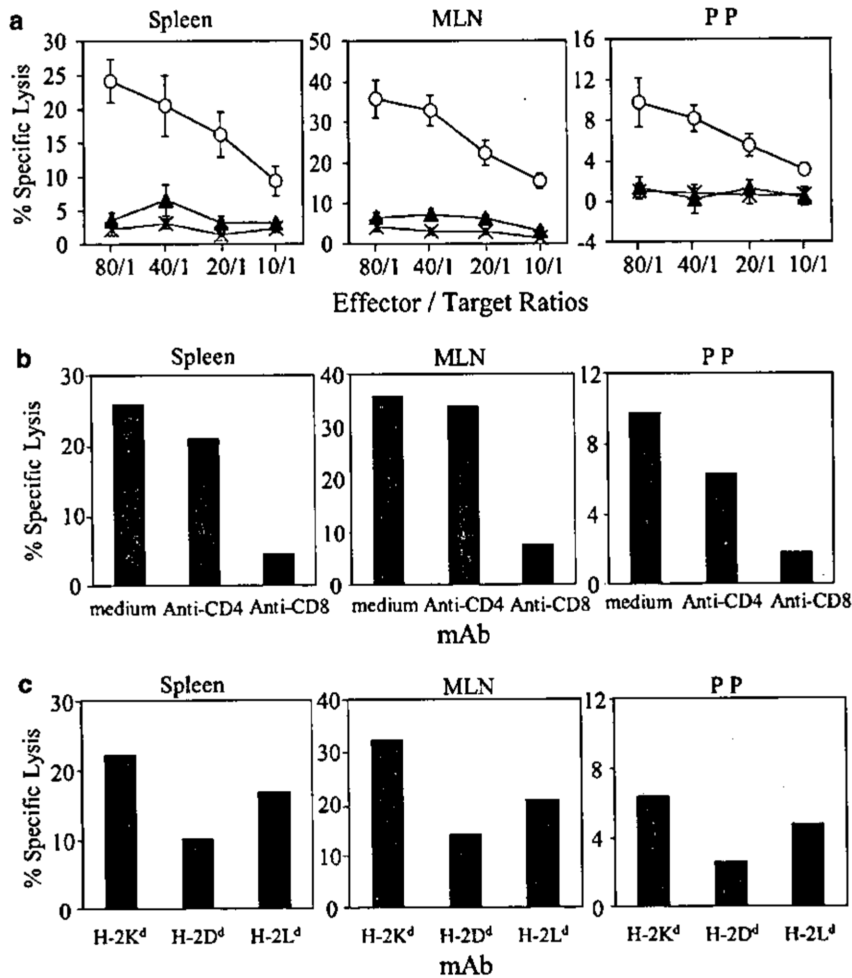

Figure 6 Spleen, MLN and PP cells from mice orally administered pJWNL432-encapsulated VLPs elicited CTL. (a) Mice were orally administered pJWNL432 encapsulated in VLPs (circles) or naked (triangles). Results for nonimmunized controls are also shown (x). (b) Effector cells obtained from the spleen, MLN 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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