netic analysis of Autographa californica nuclear polyhedrosis virus attachment to different insect cell lines. J. Gen. Virol. **73**, 3185-3194.

41) Yap C-C., Ishii K., Aoki Y., Aizaki H., Tani H., Shimizu H., Ueno Y., Miyamura T., and Matsuura Y. (1997). A hybrid baculovirus-T 7 RNA polymerase system for recovery of an infectious virus from cDNA. Virology **231**, 182-191.

Virology 293, 273–280 (2002)
doi:10.1006/viro.2001.1240, available online at http://www.idealibrary.com on IDEAL®

# Chimeric Recombinant Hepatitis E Virus-like Particles as an Oral Vaccine Vehicle Presenting Foreign Epitopes

Masahiro Niikura,* Shiki Takamura,† Gisen Kim,† Satoru Kawai,‡ Masayuki Saijo,* Shigeru Morikawa,* Ichiro Kurane,* Tian-Cheng Li,§ Naokazu Takeda,§ and Yasuhiro Yasutomi†,[1]

*Department of Virology 1, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan; †Department of Bioregulation, Mie University School of Medicine, Tsu, Mie, Japan; ‡Department of Medical Zoology, Dokkyo University School of Medicine, Mibu, Tochigi, Japan; and §Department of Virology 2, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan

*Received July 9, 2001; returned to author for revision August 16, 2001; accepted October 11, 2001*

Many viral and bacterial pathogens establish infections through mucosal surfaces in their initial stage. However, only a few nonreplicating molecules successfully induce strong mucosal immune reaction without the addition of adjuvants by oral administration. To overcome this difficulty, we investigated whether hepatitis E virus-like particles (HEV-VLPs) could be utilized as a carrier molecule for foreign antigenic epitopes and to stimulate mucosal immunity without the need for adjuvants. To accomplish this goal, we incorporated a B cell epitope tag, consisting of 11 amino acids at the C-terminal of HEV-VLP. The chimeric VLP showed morphology similar to that of the mature HEV virion and VLP. The inserted epitope was reactive with a specific monoclonal antibody in the VLP form, suggesting that it was exposed on the surface of the VLP. After oral administration without adjuvant, this chimeric HEV induced significant levels of specific IgG and IgA to both the inserted epitope and HEV-VLP in intestinal secretions. These humoral immune responses were observed as early as 2 weeks after the first immunization. These results suggest the potential of HEV-VLP as a mucosal vaccine carrier vehicle for the presentation of antigenic epitopes through oral administration. © 2002 Elsevier Science (USA)

*Key Words:* oral vaccine; mucosal immunity; hepatitis E virus; virus-like particle.

## INTRODUCTION

Hepatitis E virus (HEV) is a calicivirus-like, unclassified positive-strand RNA virus that causes acute hepatitis in humans by fecal–oral transmission (Berke and Matson, 2000; Reyes *et al.*, 1999; Wong *et al.*, 1980). Though no *in vitro* culture system to amplify HEV has been developed, overexpression of a part of open reading frame 2 (ORF2) in the baculovirus expression system allows this protein to assemble into virus-like particles (VLPs) (Li *et al.*, 1997b). Formation of this VLP occurs only when the N-terminal of ORF2, where the potential signal sequence is encoded, was deleted from the expression construct (Li *et al.*, 1997b; Tam *et al.*, 1991). Further, it was reported that an additional endogenous cleavage of 52 amino acids at the C-terminal was necessary for the assembly of VLP (Li *et al.*, 1997b; Xing *et al.*, 1999; T. C. Li, unpublished data). HEV-VLP appears as an empty particle that is slightly smaller than the mature HEV particle (Li *et al.*, 1997b; Xing *et al.*, 1999). Recently, it was found that the VLP elicits strong immune responses when administered orally or intraperitoneally into mice (Li *et al.*, 2001).

Many pathogenic viruses and bacteria establish their initial infections through mucosal surfaces. Consequently, vaccine strategies that can stimulate mucosal immunity have been widely studied (Bergmann and Waldman, 1988; Ulrich *et al.*, 1998). Recent research demonstrated that mucosal immunity involves a systemic network of mucus throughout a body and plays a crucial part of the defense mechanisms against infection (Boyaka *et al.*, 1999; Czerkinsky *et al.*, 1999).

Stimulation of mucosal immunity through oral administration of vaccines is beneficial in terms of its convenience (Morrow *et al.*, 1999). However, oral immunization with nonreplicating molecules faces several difficulties, including the low pH in the stomach, proteolytic enzymes in the digestive tract, and physical as well as biochemical barriers associated with the mucosal surface itself (Morrow *et al.*, 1999). Once these barriers are overcome, oral immunization by nonreplicating molecules can stimulate mucosal immunity through the specialized epithelial M cells and the intestinal lymphoid organs (Neutra, 1999). It is believed that the particulate form itself and the affinity for the mucosal surface mediate the antigenicity of immunogens in the case of oral administration (Bergmann and Waldman, 1988; de Aizpurua and Russell-Jones, 1988; Estes *et al.*, 2000), although the precise

[1] To whom correspondence and reprint requests should be addressed at Department of Bioregulation, Mie University School of Medicine, 2-174 Edobashi, Tsu, 514-8507 Mie, Japan. Fax: +81-59-231-5225. E-mail: yasutomi@doc.medic.mie-u.ac.jp.

0042-6822/02 $35.00
© 2002 Elsevier Science (USA)
All rights reserved.

mechanisms and critical parameters are yet to be elucidated.

Mucosal immunization with VLPs is a relatively new approach for vaccine development among nonreplicating molecules (Medina and Guzman, 2000). The advantages of VLPs as epitope carriers include the following: (1) multiple repetitive structures of identical subunits augment the antigenicity of the inserted epitopes, (2) the presence of helper T-cell (Th) epitopes on VLP molecules provides stronger immunogenicity, (3) VLPs may stimulate cytotoxic T-cell responses as well as humoral immune responses, and (4) specific binding to the mucosal surface is expected, by choosing an appropriate VLP (Allsopp *et al.,* 1996; Ulrich *et al.,* 1998). Oral administration of VLPs usually results in a weaker immune response than can be achieved through nasal administration (Sedlik *et al.,* 1999). Therefore, for oral immunization, it is often necessary to add mucosal adjuvants, such as cholera toxin, to the VLPs in order to induce significant mucosal immunity (Balmelli *et al.,* 1998; Brennan *et al.,* 1999; Fooks *et al.,* 1998; Modelska *et al.,* 1998; Mrsny *et al.,* 1999; O'Neal *et al.,* 1997; Yuan *et al.,* 1998). However, these adjuvants can cause severe side effects (Boyaka *et al.,* 1999). Ideally VLPs should stimulate good immune responses without the need for adjuvant. A VLP of Norwalk virus, a calicivirus, is one of the few successful oral mucosal vaccines that do not require an adjuvant (Ball *et al.,* 1998, 1999). Although the Norwalk virus-VLP showed encouraging results for induction of Norwalk virus-specific antibody in phase 1 trials, a chimeric VLP has not yet been reported. Successful oral mucosal vaccination without an adjuvant has also been reported using a chimeric plant virus particle carrying two rabies virus epitopes (Modelska *et al.,* 1998). This chimeric virus is, however, replication-competent in plants and may be more difficult than nonreplicating VLPs in control of leakage to the environment.

In the present study, we investigated the potential of an HEV-VLP as an oral vaccine vehicle that presents foreign immunogenic epitopes and stimulates mucosal immunity without the addition of any kind of adjuvant. Since an HEV-VLP could induce immune responses by the oral route (Li *et al.,* 2001), it was highly probable that the chimeric HEV-VLP could induce immune response to inserted foreign epitopes as well. We report that an HEV-VLP can accommodate at least a B cell epitope consisting of 11 amino acids and elicit significant levels of specific antibody to the inserted epitope in intestinal fluids.

## RESULTS

### Formation of chimeric VLP containing a foreign epitope

To investigate whether HEV-VLP can accommodate an antigenic epitope, we examined six insertion sites, including four internal positions and both the N- and the C-termini of dORF2. Utilizing any of the insertion sites, the chimeric dORF2 was expressed at almost equal levels in the cell lysates (Fig. 1A). The antigenicity of the tag epitope was maintained in all cases, as evidenced by Western blot analysis (Fig. 1B). Even at the C-terminal, where the native HEV amino acid sequence is cleaved, the tag was not cleaved off from dORF2 (Fig. 1B, lane C). Among these chimeras, only the N- and C-terminal insertions resulted in the chimeric dORF2 being released into the culture supernatant at significant amounts (Figs. 2C and 2D), although small amounts were released when the insertions were made at either site 3 or 4. These results indicate that internal insertions somehow disturbed the release of dORF2 into the culture supernatants.

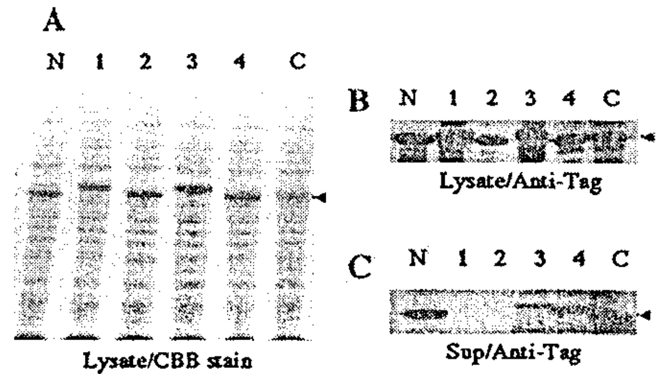


FIG. 1. Expression of chimeric dORF2. (A) Expression in the cell lysates was examined by Coomasie brilliant blue staining. (B) Antigenicity of the tag epitope in the cell lysates was confirmed by Western blotting with the anti-tag antibody. (C) Presence of each chimeric dORF2 in the cell supernatant (8 $\mu$l) was examined by Western blotting with the anti-tag antibody. The insertion site for each chimera is indicated at the top of the panel. N, N-terminal; 1 to 4, sites 1 to 4, respectively; C, C-terminal. Arrowhead on the right of each panel indicates the position of the chimeric dORF2.

We attempted to purify the chimeric VLP from the supernatant of Tn5 cells expressing chimeric dORF2 with the tag at either the C- or the N-terminus. Since the released chimeric dORF2 with the tag insertion at either site 3 or 4 could not be precipitated by the initial ultracentrifugation (data not shown), we did not make any further attempt to use them. As shown in Fig. 2A, the chimeric VLP with the tag at the C-terminal (VLP-52C) was purified by ultracentrifugation through CsCl as previously described for the HEV-VLP (Li *et al.,* 1997b). The VLP-52C was slightly larger than the HEV-VLP without the tag (Fig. 2A). This purified VLP-52C retained the antigenicity of HEV as well as the intact tag epitope, as shown by the reactivity of specific antibodies (Figs. 2B and 2C). In contrast, the chimeric dORF2 with the tag at the N-terminal (VLP-52N) did not form a visible band in the CsCl gradient, although it was pelleted from the supernatant by ultracentrifugation (data not shown).

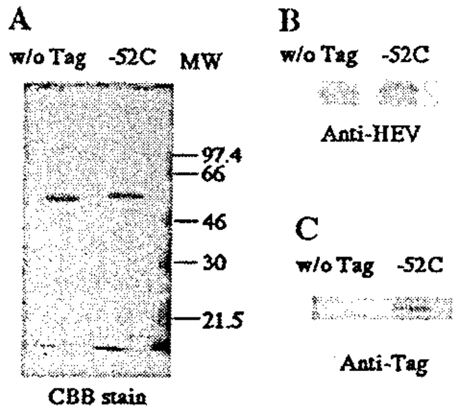


FIG. 2. Purification of the chimeric VLP. Purified VLP-52C was analyzed for its purity (A) and reactivity to anti-HEV (B) and anti-tag antibodies (C). (A) Equal amounts (0.3 μg) of purified VLP-52C (−52C) and VLP without tag (w/o Tag) were separated on SDS-PAGE and stained by Coomasie brilliant blue staining. Positions of molecular weight markers are indicated on the right of the panel. (B and C) Equal amounts (0.1 μg) of VLP-52C (−52C) and VLP without tag (w/o Tag) were analyzed by Western blotting using anti-HEV (B) and anti-tag (C) antibodies, respectively.

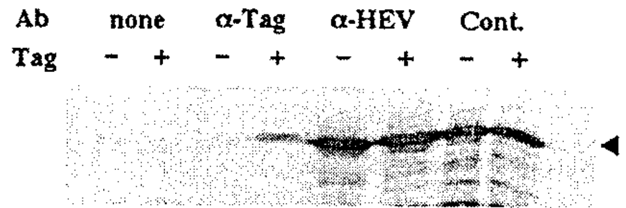


FIG. 4. Surface exposure of the tag epitope on VLP-52C. Surface exposure of the tag epitope on intact VLP-52C was examined by immunoprecipitation with the anti-tag antibody. Antibodies used are indicated at the top of panel. none, negative control without antibody; α-Tag, anti-tag antibody; α-HEV, anti-HEV antibody; cont., purified VLP-52C and VLP without tag were run as controls. Second row indicates VLP either with (+) or without the tag (−).

## Characterization of the chimeric VLP

By electron microscopic observation, the VLP-52C was approximately 25 nm in diameter and indistinguishable from the VLP without the tag (Fig. 3). Utilizing two methods, we confirmed that the inserted epitope tag was exposed on the surface. The intact VLP-52C was immunoprecipitated with the anti-tag antibody, while the anti-HEV antibody immunoprecipitated both VLP-52C and the VLP without the tag (Fig. 4). Furthermore, the anti-tag antibody specifically reacted with the intact VLP-52C in an enzyme-linked immunosorbent assay (ELISA) (data not shown). These results suggested that the tag epitope was exposed on the surface of VLP-52C.

## Antibody response to VLP-52C

Mice were immunized with 50 μg of purified VLP-52C by the oral route four times at 2-week intervals. Specific IgG antibodies to the tag, as well as to HEV, were detected in the intestinal fluids as early as 2 weeks postimmunization (wpi) (Fig. 5A). The IgG levels in the intestinal fluids kept increasing until the termination of the experiments. Specific IgA to both the tag and HEV also appeared in the intestinal fluids from 2 wpi, paralleling the IgG levels (Fig. 5B). The IgA levels kept increasing until termination of the experiment as well. As expected, the control mice immunized with VLP without the tag developed IgG and IgA only to HEV.

In sera, specific IgG antibody levels to both the tag and HEV showed slightly higher optical density (OD) values than the nonimmunized controls, but never reached significant levels, as it did in intestinal fluids (Fig. 5C). The specific IgA levels in sera were also low, although the OD values were again higher than the nonimmunized controls (Fig. 5D). The control mice immunized with VLP without the tag showed low OD values similar to HEV.

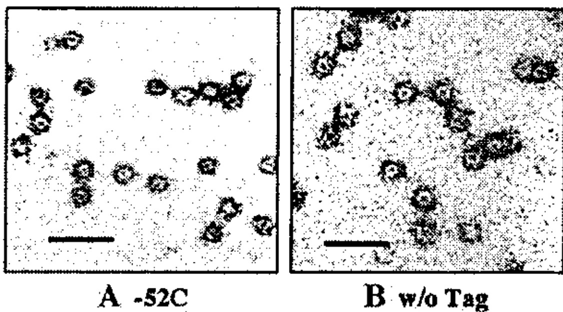


FIG. 3. Electron micrograph of VLP-52C. VLP-52C (A) and VLP without the tag (B) were observed under electron microscopy after negative staining at a magnification of ×60,000. Inserted bar indicates 100 nm.

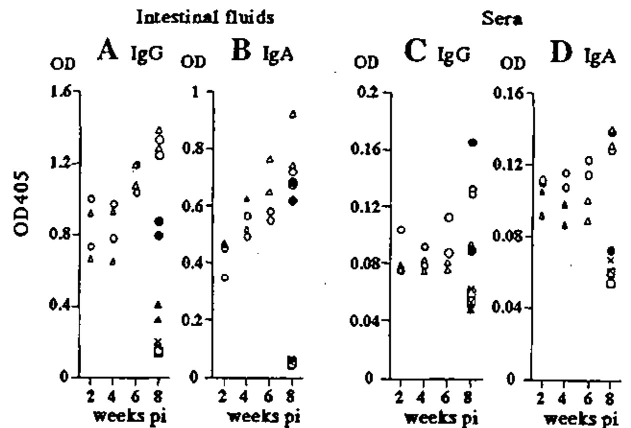


FIG. 5. IgG (A and C) and IgA (B and D) levels in intestinal fluids (A and B) and sera (C and D) of orally immunized mice. Circles and triangles indicate HEV-specific and the tag epitope-specific antibody levels, respectively, in individual mice. Two immunized mice were sacrificed at each time point (2, 4, 6, and 8 weeks p.i.). Specific antibody levels to HEV and the tag epitope of control mice immunized with VLP without the tag (closed circles and closed triangles, respectively) and background levels of antibody to HEV and the tag epitope of nonimmunized mice (squares and crosses, respectively) are also shown. Antibody levels are indicated as $OD_{405}$ in ELISA when sera and intestinal fluids were diluted at 1:100 and 1:2, respectively.

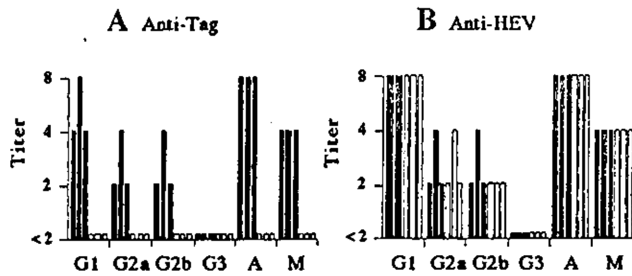


FIG. 6. Isotypes of antibodies specific to the tag epitope (A) and HEV (B) in intestinal fluids in orally immunized mice sacrificed at 10 wpi. IgA (A), IgM (M), and IgG subclass (G1, G2a, G2b, and G3) levels were examined by ELISA using isotype-specific secondary antibodies and shown as end-point titers. Solid and open bars indicate antibody levels of each mouse immunized with the chimeric VLP and VLP without tag epitope insertion, respectively.

The specific antibodies in the intestinal fluids were analyzed for their isotypes at 10 wpi. At this time point, average OD values and SD for IgG and IgA in the intestinal fluids were 1.02 ± 0.22 and 0.64 ± 0.038, and 0.96 ± 0.086 and 0.66 ± 0.040, respectively, to HEV and the tag in three mice immunized with the chimeric VLP. In the control mice immunized with VLP without the tag, the average OD values and SD for IgG and IgA were 0.88 ± 0.047 and 0.64 ± 0.027, and 0.11 ± 0.024 and 0.084 ± 0.013, respectively, to HEV and the tag. All subclasses of IgGs except IgG3, IgM, and IgA to HEV were evident in all mice (Fig. 6B). All mice failed to develop IgG3 to both the tag and HEV above the detectable level (Figs. 6A and 6B). In the control mice immunized with VLP without the tag, very similar HEV-specific antibody reactions to those with the chimeric VLP were shown (Fig. 6B), while no detectable level of any isotype antibody specific to the tag was observed (Fig. 6A), as expected.

## DISCUSSION

We have successfully generated chimeric VLPs based on HEV dORF2. With electron microscopy, the chimeric VLP was indistinguishable from the VLP without an inserted foreign epitope. This chimeric VLP induced antibodies in the intestinal fluids to both the inserted tag epitope and HEV-VLP. No adjuvant was required to induce significant antibody levels, suggesting that this chimeric VLP may be useful as an oral mucosal vaccine vehicle.

The precise mechanisms involved in the HEV virion formation are not yet clear. It was reported that intact ORF2 is expressed as a membrane glycoprotein when artificially expressed in mammalian cells *in vitro* (Jameel *et al.*, 1996; Zafrullah *et al.*, 1999), most probably because the N-terminal amino acid sequence serves as a signal peptide. On the other hand, the intact ORF2 expression in insect cells resulted in various sizes of proteins with cleavages at both the N- and the C-termini; the 53-kDa polypeptides were secreted in the culture supernatant (McAtee *et al.*, 1996a,b; Robinson *et al.*, 1998). It was also reported that only after cleavage of the C-terminal, which resulted in molecular mass reduction to 54 kDa, ORF2 participates in the VLP formation (Li *et al.*, 1997b; Xing *et al.*, 1999). The added tag 52 amino acids upstream from the C-terminal where dORF2 was normally cleaved in insect cells was not cleaved off in the infected cells during the generation of the chimeric VLP. This is most likely due to the alteration of the amino acid sequence recognized by the proteolytic enzyme involved in the C-terminal modification of HEV-VLP. The successful addition of extra amino acid sequences to the C-terminal of dORF2 suggests that the presence of extra amino acids at the C-terminal is not crucial for preventing dORF2 from being incorporated into the VLP form. Rather, the amino acid sequences encoded by the HEV ORF2 genome prevented the formation of VLP.

The results of immunoprecipitation and ELISA using intact chimeric VLPs suggest that the tag epitope is exposed on the surface of the HEV-VLP. The successful induction of antibodies to the tag may also support the hypothesis that the tag is exposed on the surface, since an internally localized B-cell epitope in chimeric parvovirus-VLP failed to induce a specific antibody response (Rueda *et al.*, 2000). Furthermore, this hypothesis is consistent with the three-dimensional analysis of the Norwalk virus-VLP particle, in which the C-terminal is exposed to the VLP surface (Prasad *et al.*, 1999). The instability of VLP-52N during the CsCl purification may also be explained by the internal location of the N-terminal in the Norwalk virus-VLP. Altered morphology after CsCl purification was reported with a chimeric parovirus-VLP with an epitope insertion inside of the VLP (Rueda *et al.*, 2000). Alternatively, it is also possible VLP-52N might have aggregated but did not form VLP in the supernatants. Internal insertions of the tag epitope into dORF2 abolished the VLP formation. The insertion sites 2, 3, and 4 were within or fairly close to the reported antigenic region (Khudyakov *et al.*, 1993, 1994, 1999), though the localization of these B cell epitopes in ORF2 is somewhat controversial (Li *et al.*, 1997a). Considering that B-cell epitopes are generally hydrophilic and most likely exposed to the VLP surface, B-cell epitope regions may not be directly involved in the protein–protein interactions to form the VLP. Our unsuccessful insertions into internal sites suggest that the integrity of internal regions must be maintained for proper protein folding and VLP formation. To find potential internal insertion sites, a precise three-dimensional structural map of the HEV-VLP may be necessary.

The induction of antibody by oral administration to the tag indicates that the tag amino acid sequence survived the harsh conditions in the digestive tract, even though it seemed exposed on the VLP surface. The inserted tag epitope induced an antibody response strongly biased for intestinal fluids. Similarly biased induction of antibod-

ies in intestinal secretion was observed by oral immunization with chimeric plant virus particles (Modelska *et al.*, 1998). Considering the marginal specific antibody levels in sera, the dose (50 μg) used in these experiments may not have been sufficient to induce significant serum antibody. To evaluate the potential of chimeric HEV-VLP to induce systemic serum antibodies, further experiments are necessary. Contrary to our observations with the chimeric HEV-VLP, the induction of IgA response after oral delivery required a higher concentration of antigen than that required to induce serum IgG with Norwalk virus-VLP (Ball *et al.*, 1998). In addition, the intestinal IgA response occurred later than the serum IgG response. Even in that report, doses exceeding 200 μg were necessary to induce consistent serum IgG. Taken together, the chimeric HEV-VLP is a very strong stimulator for mucosal immunity when administered orally. Recent reports showed that a parvovirus-VLP (Sedlik *et al.*, 1999) and a papillomavirus-VLP (Liu *et al.*, 1998) can induce specific CTL to the inserted T cell epitope by mucosal immunization without adjuvant. We are now investigating whether the HEV-VLP also induces specific CTL responses by using chimeric VLPs with CTL epitopes instead of the tag.

The keys for successful oral stimulation of the immune response are to increase the uptake of carrier molecules by M cells utilizing a particulate form and to ensure specific binding with the intestinal epithelium (Bergmann and Waldman, 1998; de Aizpurua and Russell-Jones, 1988; Estes *et al.*, 2000). These keys may have worked in our experimental model as well. Recent findings showed that HEV infects many animal species including rodents (Favorov *et al.*, 2000; Maneerat *et al.*, 1996). Therefore, it is possible that the chimeric HEV-VLP specifically bound to the intestinal epithelium of the inoculated mice. On the other hand, as HEV naturally infects hosts by the oral–fecal route, it is highly possible that the VLP retained the particulate structure in the digestive tract including the acidic environment in the stomach. In fact, the HEV-VLP was shown to be stable in an acidic environment (Xing *et al.*, 1999). These characteristics are advantageous for the HEV-VLP to be an oral vaccine vehicle. The chimeric HEV-VLP molecules would probably elicit similar strong immune responses in other host species including human as well.

We have examined secretory IgA levels only in the intestinal secretion. However, it has become apparent that IgA responses on different mucosal surfaces were achieved simultaneously, despite the initial stimulation of a single mucosal site (Boyaka *et al.*, 1999; Mestecky and McGhee, 1987). Therefore, it is probable that oral administration of chimeric HEV-VLP stimulates IgA responses simultaneously on distant mucosal surfaces as well. This phenomenon significantly extends the potential use of chimeric HEV-VLP as an oral vaccine vehicle.

TABLE 1

Oligonucleotides Used in This Study

| Oligonucleotide | Sequence (5′ to 3′) |
|---|---|
| HEVBacBg | CGCAGATCTATGGCGGTCGCTCCAGCCC |
| HEV52Pr | CTGCAGCTATGCTAGCGCAGAGTG |
| Htg5 (0) | CAGCCTGAACTCGCTCCAGAGGA |
| Htg5 (+1) | GCCAGCCTGAACTCGCTCCAGAGGA |
| Htg3 (0) | ATCTTCCGGATCCTCTGGAGCGAG |
| Htg3 (GA) | TCATCTTCCGGATCCTCTGGAGCGAG |
| Htg3 (GG) | CCATCTTCCGGATCCTCTGGAGCGAG |
| Tag (−52) | CTGCAGCTAATCTTCGGGGTCCTCCGGGGCGAGCT-CAGGCTGTGCTAGCGCAGAGTGG |
| BglTag | AGATCTATGGCGCAGCCTGAACTCGCTCCAGAGGA-TCCAGAAGATGCGGTCGCTCCAGCCCATGAC |

## MATERIALS AND METHODS

### Cells, animals, and viruses

Sf9 cells were maintained in TC100 medium (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS), 5% tryptose phosphate broth (Difco, Detroit, MI), and kanamycin. Tn5 cells were maintained in Excell 405 serum-free medium (JRH, Lenexa, KS). BALB/c mice (4 weeks old, female) were purchased from Clea Japan (Tokyo, Japan). Recombinant baculoviruses were plaque-purified twice and amplified on Sf9 cells.

### Antibodies

The rabbit antiserum to HEV-VLP was previously described (Li *et al.*, 1997b). The monoclonal antibody recognizing a B-cell tag epitope on glycoprotein D of herpes simplex virus was purchased from Novagen (Madison, WI). This antibody recognizes the amino acid sequence, QPELAPEDPED.

### Construction of recombinant baculoviruses

pVL5480/7126, the baculovirus transfer vector which includes a portion of the ORF2 from HEV (dORF2), was described previously (Li *et al.*, 1997b). To insert the tag sequence within dORF2, oligonucleotides that encode the tag amino acid sequence were synthesized as shown in Table 1. A pair of oligonucleotides was annealed and end-filled with the Klenow enzyme. The filled, double-stranded oligonucleotides were ligated into the dORF2 that had previously been digested by a restriction endonuclease and blunted with either the Klenow enzyme or T4 DNA polymerase. The restriction sites used for insertion sites 1 to 4 were *Hin*dIII, *Sac*II, *Bss*HII, and *Sac*II sites at nucleotide positions 5679, 6245, 6664, and 6773, respectively. For each site, oligonucleotide pairs of Htg5(0) and Htg3(GA), Htg5(+1) and Htg3(0), Htg5(0) and Htg3(GG), and Htg5(+1) and Htg3(0), respectively, were used. After the tag insertion, the chimeric dORF2 genes were amplified by PCR using the primers HEVBacBg and

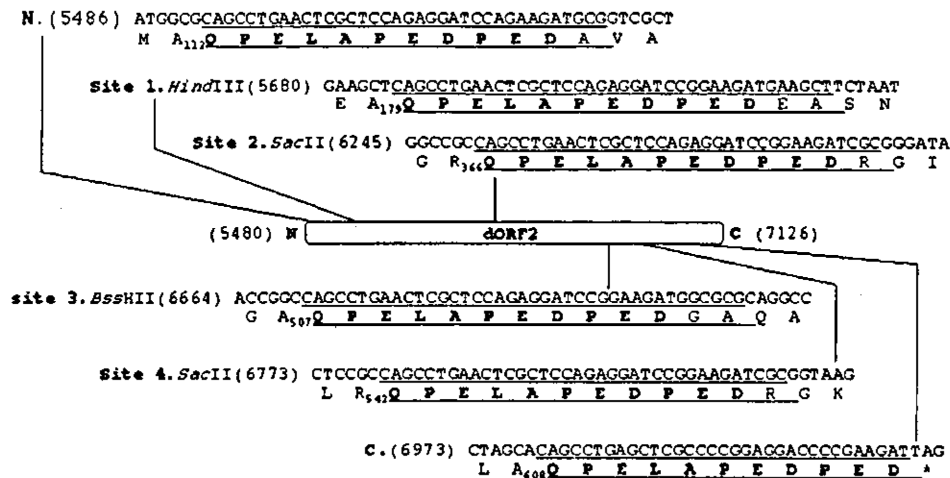


FIG. 7. Schematic diagram and sequences around the tag epitope insertion sites in dORF2. Upper rows show nucleotide sequences and lower rows show the corresponding amino acid sequences. Amino acid numbers relative to the full-length ORF2 are indicated next to the amino acid before the inserted amino acids. Nucleotide numbers referring to the HEV genome are in parentheses. Inserted sequences are underlined. The tag epitope amino acid sequence is in boldface type.

HEV52Pr. The amplified fragments were cloned into pGEMT Easy (Promega, Madison, WI), double-digested by *Bgl*II and *Pst*I, and then inserted back into pVL1393 (Invitrogen, Groningen, The Netherlands) previously digested by *Bam*HI and *Pst*I. To add the epitope tag amino acid sequence at either the N- or the C-terminal of dORF2 in pVL5480/7126, the dORF2 gene was PCR amplified with the primer pair BglTag and HEV52Pr or the primer pair HEVBacBg and Tag (−52), respectively (Table 1). The amplified fragments were cloned into pGEMT Easy and then ligated to the *Bam*HI/*Pst*I–digested pVL1393 as *Bgl*II/*Pst*I fragments. The C-terminal tag was added at a position 52 amino acids upstream from the translational terminal. This site was chosen because the last 52 amino acids at the C-terminal of ORF2 are cleaved off during the formation of VLP. The nucleotide sequences around the inserted tag were determined by an automated sequencer (ABI, Foster City, CA) and are schematically shown in Fig. 7. The plasmid containing the chimeric dORF2 was cotransfected with baculovirus DNA and the recombinant baculovirus was generated as described previously (Li *et al.*, 1997b).

### Production and purification of VLP

The production and purification of HEV-VLP were performed as described (Li *et al.*, 1997b; Xing *et al.*, 1999). Briefly, Tn5 cells were infected with the recombinant baculoviruses at a m.o.i. of >5 and cultured for 6 to 7 days. The supernatant was harvested and centrifuged at low speed, and the recombinant baculoviruses in the supernatant were pelleted by ultracentrifugation at 10,000 *g* for 30 min at 4°C. The VLP in the supernatant was collected by a further ultracentrifugation at 100,000 *g* for 2 h at 4°C. The pellet containing VLP was then resuspended in 10 mM potassium-MES buffer (pH 6.2) and further purified on a CsCl equilibrium density gradient. The purified VLP was pelleted and resuspended in ExCell 405. The purified VLP was kept at 4°C or frozen at −20°C. The correct VLP structural formation was confirmed by observing a purified preparation with an electron microscope, as described previously (Li *et al.*, 1997b).

### SDS–PAGE, Western blotting, and immunoprecipitation

SDS–PAGE was performed on 10% acrylamide gels using denaturing conditions. Cell lysates were prepared in SDS–PAGE sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% 2-mercaptoethanol, 10% glycerol). Briefly, Tn5 cells ($10^6$ cells in a 35-mm dish) were infected and incubated for 2 days. After the infected cells were harvested and washed with PBS, the cells were lysed in 100 $\mu$l sample buffer. The same volume of the lysates was analyzed for the comparison of the expression level. Protein staining was performed in 0.25% Coomasie brilliant blue in methanol:water:acetic acid (9:9:2). When the protein release in culture supernatants was analyzed, supernatants were mixed with equal volumes of 2× SDS–PAGE sample buffer and analyzed by Western blots. For Western blotting, the separated proteins were transferred to nylon membranes (Millipore, Bedford, MA) in a semidry system (ATTO, Tokyo, Japan) by a standard method. The transferred proteins were probed with either the anti-HEV or the anti-tag antibodies and horseradish peroxidase (HRPO)-labeled anti-rabbit IgG (Zymed, San Francisco, CA) or anti-mouse IgG (H + L, Zymed), respectively. The reaction was visualized with POD substrate (Wako, Osaka, Japan). For immunoprecipitation, the purified VLP was biotinylated by EZ-Link reagent (Pierce, Rockford, IL) in phosphate-buffered saline (PBS, pH 7.2) as recom-

mended by the manufacturer. The biotin-labeled VLP (1 μg) was precipitated with 1 μl of either the anti-HEV or anti-tag antibodies with 10 μl Protein G–Sepharose (Amersham Pharmacia, Little Chalfont, CA) in PBS at 4°C. After extensive washing with PBS, the immunoprecipitates were analyzed by SDS–PAGE and visualized by avidin–HRPO (Zymed) and POD substrate after being blotted to nylon membranes.

### Enzyme-linked immunosorbent assay

Purified VLP without tag or synthesized oligopeptides with the tag sequence (QPELAPEDPED) were coated in multiwell plates at a concentration of 10 or 100 μg/well in 100 μl PBS, respectively, overnight at 4°C followed by 30 min of blocking with nonfat milk. Test samples were added to the wells and incubated at room temperature (RT) for 1 h. After being washed, the reacted antibodies were detected using the HRPO-labeled goat anti-mouse IgG (H + L) and ABTS substrate (Roche Diagnostics, Mannheim, Germany). To evaluate the amounts of each Ig isotype, one of the biotin-labeled secondary antibodies (see below) avidin–HRPO (Prozyme, San Leandro, CA), and ABTS substrate were used. Secondary antibodies used were anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM (Serotec, Raleigh, NC), and IgA (CALTAG, San Francisco, CA). The IgG and IgA antibody levels were expressed as optical densities (OD) at dilutions of either 1:2 or 1:100 for intestinal fluids or sera, respectively. For the analyses of antibody isotypes, twofold serial dilutions of intestinal fluids were made and examined in the ELISA. Each isotype level was expressed as the reciprocal of the highest dilution that showed OD values above cut-off values. The cut-off values were set at the average OD + 0.1 of nonimmunized control mice at each dilution. They were calculated for each isotype and antigen.

### Immunization and preparation of intestinal secretion

Mice were orally immunized four times with 50 μg of VLP with or without the tag in 100 μl of PBS at 2-week intervals. The chimeric VLP-immunized mice were sacrificed at 2, 4, 6, 8, and 10 weeks after the first immunization and the antibody levels in the intestinal secretions and sera were determined. Two mice were sacrificed at 2, 4, 6, and 8 wpi, respectively, and three were sacrificed at 10 wpi. Four nonimmunized mice and five immunized with VLP without the tag were included as controls and sacrificed at either 8 or 10 wpi. The intestinal secretions were collected as follows. Small intestines were removed and filled with 2 ml of PBS containing 50 mM EDTA and 1 mM PMSF and left for 10 min at RT. The contents were then transferred to a test tube, vigorously vortexed, and then centrifuged for 10 min at 650 *g* at 4°C. The supernatant was transferred to another tube and PMSF was added to a final concentration of 1 mM. The solution was again mixed by vortexing and centrifuged at 13,000 *g* for 20 min at 4°C. The supernatant was mixed with PMSF and sodium azide to a final concentration of 1 mM and 0.01%, respectively, and incubated for 15 min at 4°C. FBS was added to the secretion at a final concentration of 5%, and the solution was centrifuged for an additional 20 min at 13,000 *g* at 4°C. The supernatants were stored as intestinal fluids along with the sera at −30°C until simultaneously analyzed.

## ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid from the Ministry of Health, Labor, and Welfare of Japan, the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Mie Medical Research Foundation.

## REFERENCES

Allsopp, C. E., Plebanski, M., Gilbert, S., Sinden, R. E., Harris, S., Frankel, G., Dougan, G., Hioe, C., Nixon, D., Paoletti, E., Layton, G., and Hill, A. V. (1996). Comparison of numerous delivery systems for the induction of cytotoxic T lymphocytes by immunization. *Eur. J. Immunol.* 26, 1951–1959.

Ball, J. M., Graham, D. Y., Opekun, A. R., Gilger, M. A., Guerrero, R. A., and Estes, M. K. (1999). Recombinant Norwalk virus-like particles given orally to volunteers: Phase I study. *Gastroenterology* **117**, 40–48.

Ball, J. M., Hardy, M. E., Atmar, R. L., Conner, M. E., and Estes, M. K. (1998). Oral immunization with recombinant Norwalk virus-like particles induces a systemic and mucosal immune response in mice. *J. Virol.* **72**, 1345–1353.

Balmelli, C., Roden, R., Potts, A., Schiller, J., De Grandi, P., and Nardelli-Haefliger, D. (1998). Nasal immunization of mice with human papillomavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. *J. Virol.* **72**, 8220–8229.

Bergmann, K. C., and Waldman, R. H. (1988). Stimulation of secretory antibody following oral administration of antigen. *Rev. Infect. Dis.* **10**, 939–950.

Berke, T., and Matson, D. O. (2000). Reclassification of the *Caliciviridae* into distinct genera and exclusion of hepatitis E virus from the family on the basis of comparative phylogenetic analysis. *Arch. Virol.* **145**, 1421–1436.

Boyaka, P. N., Marinaro, M., Vancott, J. L., Takahashi, I., Fujihashi, K., Yamamoto, M. F., van Ginkel, W., Jackson, R. J., Kiyono, H., and McGhee, J. R. (1999). Strategies for mucosal vaccine development. *Am. J. Trop. Med. Hyg.* 60(Suppl.), 35–45.

Brennan, F. R., Bellaby, T., Helliwell, S. M., Jones, T. D., Kamstrup, S., Dalsgaard, K., Flock, J. I., and Hamilton, W. D. (1999). Chimeric plant virus particles administered nasally or orally induce systemic and mucosal immune responses in mice. *J. Virol.* **73**, 930–938.

Czerkinsky, C., Anjuere, F., McGhee, J. R., George-Chandy, A., Holmgren, J., Kieny, M. P., Fujiyashi, K., Mestecky, J. P., Pierrefite-Carle, V., Rask, C., and Sun, J. C. (1999). Mucosal immunity and tolerance: Relevance to vaccine development. *Immunol. Rev.* **170**, 197–222.

de Aizpurua, H. J., and Russell-Jones, G. J. (1988). Oral vaccination. Identification of classes of proteins that provoke an immune response upon oral feeding. *J. Exp. Med.* **167**, 440–451.

Estes, M. K., Ball, J. M., Guerrero, R. A., Opekun, A. R., Gilger, M. A., Pacheco, S. S., and Graham, D. Y. (2000). Norwalk virus vaccines: Challenges and progress. *J. Infect. Dis.* **181**(Suppl. 2), S367–S373.

Favorov, M. O., Kosoy, M. Y., Tsarev, S. A., Childs, J. E., and Margolis, H. S. (2000). Prevalence of antibody to hepatitis F virus among rodents in the United States. *J. Infect. Dis.* **181**, 449–455.

Fooks, A. R., Jeevarajah, D., Lee, J., Warnes, A., Niewiesk, S., ter Meulen,

V., Stephenson, J. R., and Clegg, J. C. (1998). Oral or parenteral administration of replication-deficient adenoviruses expressing the measles virus haemagglutinin and fusion proteins: Protective immune responses in rodents. *J. Gen. Virol.* 79, 1027–1031.

Jameel, S., Zafrullah, M., Ozdener, M. H., and Panda, S. K. (1996). Expression in animal cells and characterization of the hepatitis E virus structural proteins. *J. Virol.* 70, 207–216.

Khudyakov, Y. E., Favorov, M. O., Jue, D. L., Hine, T. K., and Fields, H. A. (1994). Immunodominant antigenic regions in a structural protein of the hepatitis E virus. *Virology* 198, 390–393.

Khudyakov, Y. E., Khudyakova, N. S., Fields, H. A., Jue, D., Starling, C., Favorov, M. O., Krawczynski, K., Polish, L., Mast, E., and Margolis, H. (1993). Epitope mapping in proteins of hepatitis E virus. *Virology* **194**, 89–96.

Khudyakov, Y. E., Lopareva, E. N., Jue, D. L., Crews, T. K., Thyagarajan, S. P., and Fields, H. A. (1999). Antigenic domains of the open reading frame 2-encoded protein of hepatitis E virus. *J. Clin. Microbiol.* **37**, 2863–2871.

Li, F., Torresi, J., Locarnini, S. A., Zhuang, H., Zhu, W., Guo, X., and Anderson, D. A. (1997a). Amino-terminal epitopes are exposed when full-length open reading frame 2 of hepatitis E virus is expressed in *Escherichia coli*, but carboxy-terminal epitopes are masked. *J. Med. Virol.* 52, 289–300.

Li, T. C., Yamakawa, Y., Suzuki, K., Tatsumi, M., Razak, M. A., Uchida, T., Takeda, N., and Miyamura, T. (1997b). Expression and self-assembly of empty virus-like particles of hepatitis E virus. *J. Virol.* **71**, 7207–7213.

Li, T. C., Takeda, N., and Miyamura, T. (2001). Oral administration of hepatitis E virus-like particles induces a systemic and mucosal immune response in mice. *Vaccine* 19, 3476–3484.

Liu, X. S., Abdul-Jabbar, I., Qi, Y. M., Frazer, I. H., and Zhou, J. (1998). Mucosal immunisation with papillomavirus virus-like particles elicits systemic and mucosal immunity in mice. *Virology* 252, 39–45.

Maneerat, Y. M., Clayson, E. T., Myint, K. S. A., Young, G. D., and Innis, B. L. (1996). Experimental infection of the laboratory rat with the hepatitis E virus. *J. Med. Virol.* **48**, 121–128.

McAtee, C. P., Zhang, Y., Yarbrough, P. O., Bird, T., and Fuerst, T. R. (1996a). Purification of a soluble hepatitis E open reading frame 2-derived protein with unique antigenic properties. *Protein Expr. Purif.* **8**, 262–270.

McAtee, C. P., Zhang, Y., Yarbrough, P. O., Fuerst, T. R., Stone, K. L., Samander, S., and Williams, K. R. (1996b). Purification and characterization of a recombinant hepatitis E protein vaccine candidate by liquid chromatography–mass spectrometry. *J. Chromatogr. B Biomed. Appl.* **685**, 91–104.

Medina, E., and Guzman, C. A. (2000). Modulation of immune responses following antigen administration by mucosal route. *FEMS Immunol. Med. Microbiol.* 27, 305–311.

Mestecky, J., and McGhee, J. R. (1987). Immunoglobulin A (IgA): Molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv. Immunol.* 40, 153–245.

Modelska, A., Dietzschold, B., Sleysh, N., Fu, Z. F., Steplewski, K., Hooper, D. C., Koprowski, H., and Yusibov, V. (1998). Immunization against rabies with plant-derived antigen. *Proc. Natl. Acad. Sci. USA* **95**, 2481–2485.

Morrow, C. D., Novak, M. J., Ansardi, D. C., Porter, D. C., and Moldoveanu, Z. (1999). Recombinant viruses as vectors for mucosal immunity. *In* "Current Topics in Microbiology and Immunology, Vol. 236, Defense of mucosal surfaces: Pathogenesis, Immunity and Vaccines" (J.-P. Kraehenbuhl and M. R. Neutra, Eds.) pp. 255–273. Springer-Verlag, Berlin.

Mrsny, R. J., Daugherty, A. L., Fryling, C. M., and FitzGerald, D. J. (1999). Mucosal administration of a chimera composed of *Pseudomonas* exotoxin and the gp120 V3 loop sequence of HIV-1 induces both salivary and serum antibody responses. *Vaccine* 17, 1425–1433.

Neutra, M. R. (1999). M cells in antigen sampling in mucosal tissues. *In* "Current Topics in Microbiology and Immunology, Vol. 236, Defense of Mucosal Surfaces: Pathogenesis, Immunity and Vaccines" (J.-P. Kraehenbuhl and M. R. Neutra, Eds.), pp. 17–32. Springer-Verlag, Berlin.

O'Neal, C. M., Crawford, S. E., Estes, M. K., and Conner, M. E. (1997). Rotavirus virus-like particles administered mucosally induce protective immunity. *J. Virol.* **71**, 8707–8717.

Prasad, B. V., Hardy, M. E., Dokland, T., Bella, J., Rossmann, M. G., and Estes, M. K. (1999). X-ray crystallographic structure of the Norwalk virus capsid. *Science* 286, 287–290.

Reyes, G. R., Purdy, M. A., Kim, J. P., Luk, K. C., Young, L. M., Fry, K. E., and Bradley, D. W. (1990). Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science* **247**, 1335–1339.

Robinson, R. A., Burgess, W. H., Emerson, S. U., Leibowitz, R. S., Sosnovtseva, S. A., Tsarev, S., and Purcell, R. H. (1998). Structural characterization of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. *Protein Expr. Purif.* 12, 75–84.

Rueda, P., Martinez-Torrecuadrada, J. L., Sarraseca, J., Sedlik, C., del Barrio, M., Hurtado, A., Leclerc, C., and Casal, J. L. (2000). Engineering parvovirus-like particles for the induction of B cell, CD4+ and CTL response. *Vaccine* 18, 325–332.

Sedlik, C., Dridi, A., Deriaud, E., Saron, M. F., Rueda, P., Sarraseca, J., Casal, J. I., and Leclerc, C. (1999). Intranasal delivery of recombinant parvovirus-like particles elicits cytotoxic T-cell and neutralizing antibody responses. *J. Virol.* 73, 2739–2744.

Tam, A. W., Smith, M. M., Guerra, M. E., Huang, C. C., Bradley, D. W., Fry, K. E., and Reyes, G. R. (1991). Hepatitis E virus (HEV): Molecular cloning and sequencing of the full-length viral genome. *Virology* **185**, 120–131.

Ulrich, R., Nassal, M., Meisel, H., and Kruger, D. H. (1998). Core particles of hepatitis B virus as carrier for foreign epitopes. *Adv. Virus. Res.* **50**, 141–182.

Wong, D. C., Purcell, R. H., Sreenivasan, M. A., Prasad, S. R., and Pavri, K. M. (1980). Epidemic and endemic hepatitis in India: Evidence for a non-A, non-B hepatitis virus aetiology. *Lancet* 2, 876–879.

Xing, L., Kato, K., Li, T., Takeda, N., Miyamura, T., Hammar, L., and Cheng, R. H. (1999). Recombinant hepatitis E capsid protein self-assembles into a dual-domain T = 1 particle presenting native virus epitopes. *Virology* 265, 35–45.

Yuan, L., Kang, S. K., Ward, L. A., To, T. L., and Saif, L. J. (1998). Antibody-secreting cell responses and protective immunity assessed in gnotobiotic pigs inoculated orally or intramuscularly with inactivated human rotavirus. *J. Virol.* 72, 330–338.

Zafrullah, M., Ozdener, M. H., Kumar, R., Panda, S. K., and Jameel, S. (1999). Mutational analysis of glycosylation, membrane translocation, and cell surface expression of the hepatitis E virus ORF2 protein. *J. Virol.* **73**, 4074–4082.