Vol. 71, No. 10

Expression and Self-Assembly of Empty Virus-Like Particles of Hepatitis E Virus

TIAN-CHENG LI,[1] YOSHIO YAMAKAWA,[2] KENJI SUZUKI,[2] MASASHI TATSUMI,[3]
MOHAMMED A. AZEEZ RAZAK,[1]† TOSHIKAZU UCHIDA,[4]
NAOKAZU TAKEDA,[1] AND TATSUO MIYAMURA[1]*

*Department of Virology II,[1] Department of Biochemistry and Cell Biology,[2] and Department of
Veterinary Science,[3] National Institute of Infectious Diseases,‡ Shinjuku, Tokyo 162, and
Department of Pathology, School of Medicine, Nihon University, Tokyo 173,[4] Japan*

Hepatitis E virus (HEV) is a pathogenic agent that causes fecally-orally transmitted acute hepatitis. The genome, a single-stranded positive-sense RNA, encodes three forward open reading frames (ORFs), in which an approximately 2-kb structural protein is located in the 3′ end. To produce HEV-like particles the structural protein, with its N terminus truncated (amino acid residues 112 to 660 of ORF2), was expressed in insect Tn5 cells by a recombinant baculovirus. In addition to the primary translation product with a molecular mass of 58 kDa, a large amount of a further-processed molecule with a molecular mass of 50 kDa was generated and efficiently released into the culture medium. Electron microscopic observation of the culture medium revealed that the 50-kDa protein self-assembled to form empty virus-like particles (VLPs). The buoyant density of the VLPs in CsCl was 1.285 g/cm³ and their diameter was 23.7 nm, a little smaller than the 27 nm of native HEV particles secreted into the bile or stools of experimentally infected monkeys. The yield of the VLPs was 1 mg per 10⁷ cells as a purified form. 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 immunoglobulin G (IgG) and IgM antibodies. Furthermore, the VLP may be the most promising candidate yet for an HEV vaccine, owing to its potent immunogenicity.

Hepatitis E virus (HEV), a member of the family *Caliciviridae*, is one of the causative agents of viral acute hepatitis formerly known as enterically transmitted non-A, non-B hepatitis. Like hepatitis A virus, HEV is transmitted mainly by the fecal-oral route and large epidemics due to this virus are often associated with contaminated water (19, 25, 36). HEV contains a single-stranded positive-sense RNA molecule of approximately 7.5 kb 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 71-kDa protein for the putative viral capsid. ORF3, with unknown function, is mapped between ORF1 and ORF2 (3, 4, 6, 14, 29, 30, 38). Ever since HEV was first recognized, in 1980, and visualized by immunoelectron microscopy (IEM), in 1983, many efforts have been made to express the structural protein as empty virus-like particles (VLPs) (11, 31, 32). This is particularly important because no practical cell culture system to allow the growth of HEV has been developed. In fact, the structural proteins have been produced by several expression systems, such as *Escherichia coli* (21, 24, 26), insect cells (13, 32), and yeast and mammalian cells (31). However, in neither the full-length nor a truncated form of the structural protein has an efficient system for generating considerable amounts of viral particles suitable for virological characterization or for practical use in diagnosis been developed.

In this paper, we describe the efficient expression of N-terminally truncated HEV capsid proteins derived from a Myanmar (Burmese) strain isolated in 1986 (34). The viral proteins, expressed by a recombinant baculovirus in insect Tn5 cells, were shown to be self-assembled and to form VLPs which were efficiently released into the culture medium. To our knowledge, this is the first report of high-level expression and effective formation of VLPs of HEV. According to the results of an enzyme-linked immunosorbent assay (ELISA), the particle possesses antigenicity similar to that of an authentic HEV particle and functions as an antigen to detect HEV-specific immunoglobulin G (IgG) and IgM responses. Furthermore, this molecule may be the most promising candidate yet for an HEV vaccine, owing to its potent immunogenicity.

MATERIALS AND METHODS

Construction of recombinant transfer vectors. Preparation of a pool of acute-phase stool specimens from patients with sporadic non-A, non-B hepatitis in Myanmar in 1986 and injection into rhesus monkeys (*Macaca mulatta*) via the intravenous route were described previously (34). Three additional passages, a total of six, were carried out, and then the bile was collected from a monkey at 7, 10, and 15 days after inoculation; the bile specimens were designated bMM41-1, bMM41-2, and bMM41-3, respectively. The total RNA was extracted with RNAzol (Biotecx Laboratories, Inc., Houston, Tex.) from bMM41-1, which contained a large number of HEV virions. The poly(A)-containing RNA was purified with Oligotex-dT30(Super) (Roche Diagnostic Systems, Tokyo, Japan) according to the manufacturer's protocols and converted into cDNA as described previously (28). Amplification of the entire ORF2 by PCR was performed with primers HEV-D2 (5′-TGGGTTCGCGAC CATGCGCCCTCG-3′) and HEV-U2 (5′-CAACAGAAAGAAGGGGGGCA CAAG-3′). The PCR products were cloned into a TA cloning vector, pCRII (Invitrogen, San Diego, Calif.), to generate pHEV5134/7161. This was digested with *Nru*I and *Xba*I, and the resultant 2-kb fragment was ligated with a transfer vector, pVL1393 (Pharmingen, San Diego, Calif.) which was digested with *Sma*I and *Xba*I, to produce pVL5147/7126 (Fig. 1). DNA fragments with deletions of the N-terminal 111 amino acids (aa) encoded by ORF2 were amplified by PCR with two primers, HEV-D13 (5′-AAGGATCC<u>ATG</u>GCGGTCGCTCCAGCCCATGACACCCCGC CAGT-3′) and HEV-U14 (5′-GGTCTAGACTATAACTCCCGAGTTTTACCCA

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku, Tokyo 162, Japan. Phone: (81)-3-5285-1111. Fax: (81)-3-5285-1161. E-mail: tmiyam@nih.go.jp.
† Present address: Department of Electron Microscopy, Medical Research Institute, Colombo, Sri Lanka.
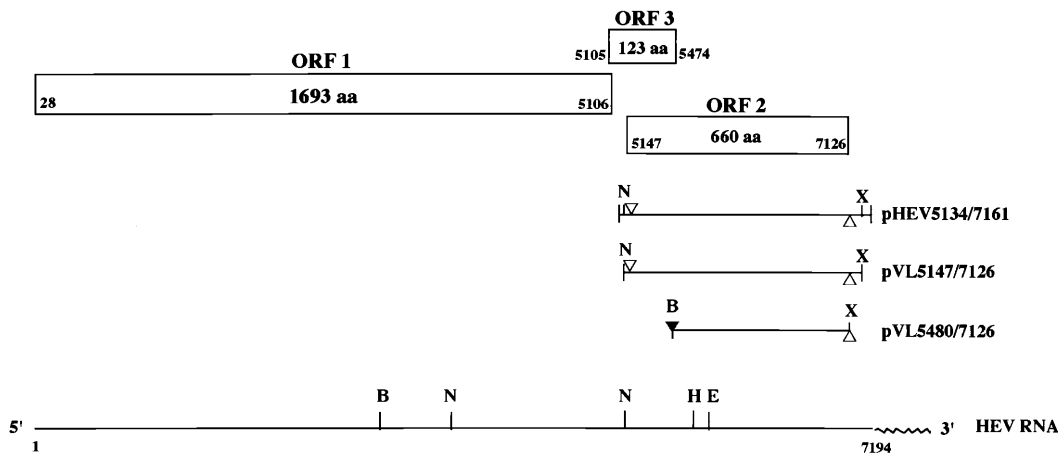‡ Formerly National Institute of Health.

FIG. 1. Genome organization of HEV and schematic diagram of two baculovirus transfer vectors. A DNA fragment encoding HEV nucleotides 5134 to 7161 was amplified by PCR and subcloned into plasmid pCRII to generate pHEV5134/7161. The orientation was determined by restriction enzyme digestion and sequence analyses. The 2.1-kb *Nru*I-*Xba*I fragment that contains the entire ORF2, a part of the 3′ noncoding region, and a sequence from the pCRII multicloning site was isolated and ligated with the baculovirus transfer vector pVL1393 to produce pVL5147/7126. The 1.6-kb DNA fragment encoding a 111-aa-truncated ORF2 (HEV nucleotide residues 5480 to 7126) was prepared by PCR with primers HEV-D13 and HEV-U14 (see Materials and Methods), which contain *Bam*HI and *Xba*I sites, respectively. This was digested with the restriction enzymes and ligated into pVL1393 to generate pVL5480/7126. Symbols and abbreviations: ▽, initiation codon; △, termination codon; ▼, newly introduced initiation codon; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nru*I; X, *Xba*I.

CCTTCATCTT-3′), with the cDNA as a template. The fragments flanked by restriction enzyme sites *Bam*HI and *Xba*I were ligated into the transfer vector, pVL1393, to yield plasmid pVL5480/7126. A methionine codon (underlined) for translation initiation was introduced into the forward HEV-D13 primer between the *Bam*HI site and aa residue 112, alanine (Fig. 1).

Generation of recombinant baculoviruses and expression of capsid proteins. Sf9 cells (Riken Cell Bank, Tsukuba, Japan) derived from an insect, *Spodoptera frugiperda* (27), were cotransfected with linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (Pharmingen) and either pVL5147/7126 or pVL5480/7126 by the lipofectin-mediated method, as specified by the manufacturer (GIBCO BRL, Gaithersburg, Md.). The cells were incubated at 26.5°C in TC-100 medium (GIBCO) supplemented with 8% fetal bovine serum and 0.26% Bacto tryptose phosphate broth (Difco Laboratories, Detroit, Mich.). The proteins in the culture medium and the cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blot assay (17) with either serum (sMT) from a patient (patient B in reference 33) infected with hepatitis E or serum (sKFG) from a patient who developed severe icteric disease soon after entering Japan from Bangladesh in 1995. Each recombinant virus was plaque purified three times (27). Baculovirus recombinants thus obtained were designated Ac5147/7126 for the whole ORF2 and Ac5480/7126 for ORF2 truncated at the N terminus. In addition to Sf9 cells, we used an insect cell line from *Trichoplusia ni*, BTL-Tn 5B1-4 (Tn5) (Invitrogen), for large-scale expression (35). Tn5 cells were infected with recombinant baculoviruses at a multiplicity of infection (MOI) of 10 and incubated in Ex-cell 405 medium (JRH Biosciences, Lenexa, Kans.) for 5 to 6 days at 26.5°C until an extensive cytopathic effect appeared.

Purification of VLPs. The culture medium was harvested, centrifuged at 1,000 × *g* for 15 min, and further centrifuged at 10,000 × *g* for 30 min to remove progeny baculoviruses. The VLPs in the supernatant were concentrated by incubation with 8% polyethylene glycol 6000 in the presence of 0.4 M NaCl at 0°C overnight followed by centrifugation at 10,000 × *g* for 30 min. The VLPs were resuspended in phosphate-buffered saline (PBS) (pH 7.5), layered onto 1 ml each of 10% (wt/wt) and 20% (wt/wt) sucrose solution, and centrifuged at 100,000 × *g* for 2 h at 4°C in a Beckman SW50.1 rotor. The VLPs, discernible as a white band between the two sucrose solutions, were collected, diluted with 10 mM PBS, pH 7.2, and recovered by centrifugation at 100,000 × *g* for 2 h. The yield of the purified VLPs usually amounted to 1 mg per 10⁷ Tn5 cells. The VLPs, if necessary, were further purified by CsCl equilibrium density gradient centrifugation. The VLPs were examined with an electron microscope (EM) and used as antigens for the antibody ELISA and for immunization of animals. Protein concentration was measured with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) by using bovine serum albumin as a standard. N-terminal amino acid microsequencing was carried out with 100 pmol of protein by Edman automated degradation on an Applied Biosystems model 477 protein sequencer.

Antigenicity and immunogenicity tests. IEM was carried out with a Hitachi H-7000 electron microscope (Hitachi Koki Co., Ltd., Tokyo, Japan) working at 75 kV. Approximately 100 ng of the VLPs in 100 μl of PBS was mixed with the same volume of diluted sKFG (1:400) and incubated at 37°C for 1 h. The antigen-antibody complex was precipitated by centrifugation at 100,000 × *g* for 2 h at 4°C, and the pellet was examined with an EM. The purified VLPs (1 μg/ml,

100 μl/well) were used to coat 96-well polystyrene microtitration plates (Immulon 2; Dynatech Industries, Inc., Chantilly, Va.) and an antibody ELISA to detect anti-HEV IgM and IgG was carried out as described by Jiang et al. (17). A reading of >0.14 was considered to be positive. Hyperimmune sera to the VLPs were obtained from guinea pigs and rabbits. The animals were immunized by one subcutaneous injection of the purified VLPs, at 250 μg per guinea pig and 500 μg per rabbit, in Freund's complete adjuvant. After 1 month, the animals received two or three booster injections of a half dose in Freund's incomplete adjuvant at intervals of 1 week. The animals were bled 1 week after the last booster injection. Microplates coated with the rabbit hyperimmune and preimmune sera (1:5,000) were used to capture the HEV antigens in the bile or stool specimens. The hyperimmune serum from the guinea pigs (1:5,000) was used as the detector antibody. When the ratio of the reading of the hyperimmune serum to that of the preimmune serum was >2 and the difference between the readings was >0.1, the sample was considered to be positive.

RESULTS

Expression of HEV capsid proteins in insect cells. First, the entire ORF2 was expressed by using Ac5147/7126 in the two insect cell lines, Sf9 and Tn5. Three major proteins with molecular masses of 72, 58, and 50 kDa were found in both cell lysates at 5 days postinfection (p.i.) (Fig. 2, ORF2). A protein band with a molecular mass of 72 kDa is thought to be the primary translation product encoded by ORF2, and its molecular mass is in agreement with that calculated from the amino acid sequence (71 kDa). The 72- and 58-kDa proteins were each seen at 1 day p.i., and the level of expression reached the maximum at 4 days p.i. All three of these proteins were immunoreactive with sMT and sKFG but not with serum from a healthy individual, indicating that they are HEV specific. The 72-kDa protein, as previously indicated by others (11–13), appeared to be tightly cell associated and was difficult to dissolve unless a high concentration of a chaotropic agent such as 8 M urea was used. The 58- and 50-kDa proteins were accumulated in the cells in the later stage of the infection. Although a trace amount of 50-kDa protein was occasionally found in the culture medium at 5 days p.i., most of the 58- and 50-kDa proteins were also tightly cell associated. None of these proteins self-assembled into VLPs. These findings are basically consistent with the results previously described (31, 37).

Second, in an attempt to express the capsid protein as VLPs, we prepared several mutant baculoviruses to express ORF2 proteins modified at both the N and C termini (see Discus-
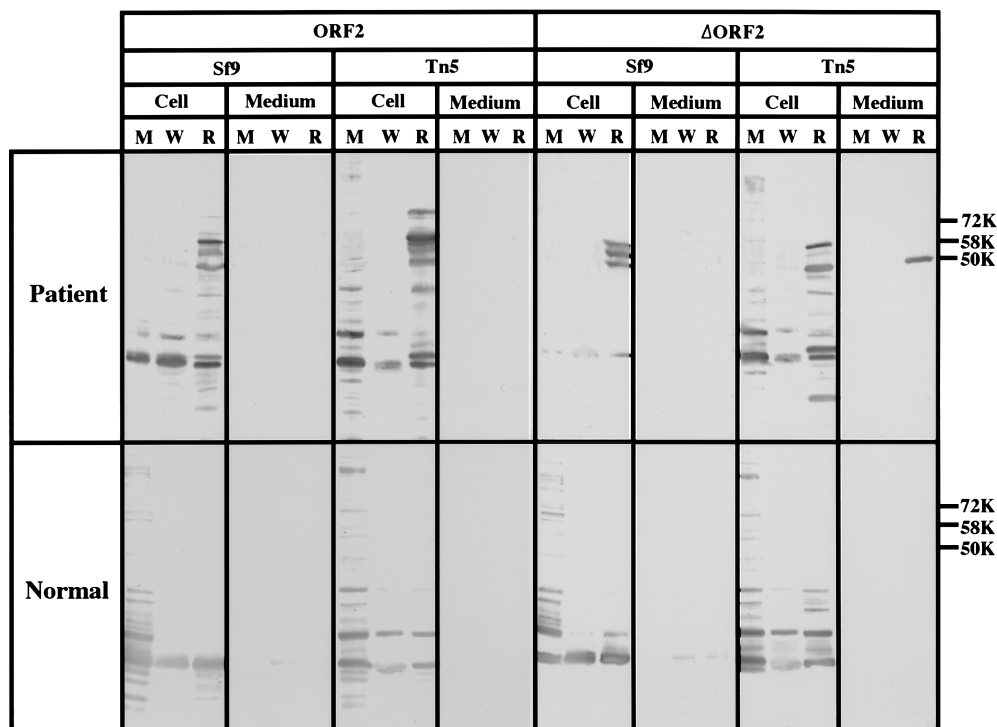
FIG. 2. Western blot assay of the entire (ORF2) and truncated (ΔORF2) ORF2 proteins expressed in Sf9 and Tn5 cells. The insect cells (5×10^6 cells) were incubated for 20 min at room temperature to allow the cells to attach to 25-cm² culture flasks in 5 ml of TC-100 (Sf9 cells) or Ex-cell 405 (Tn5 cells) medium. The culture medium was removed, and the cells were infected with the recombinant baculoviruses pAc5147/7126 (ORF2) and pAc5480/7126 (ΔORF2) at an MOI of 10. Virus adsorption was carried out for 1 h at room temperature, and then the cells were incubated for 5 days at 26.5°C. Five microliters of the culture medium and 3 μl of the cell lysate were separated by SDS–10% PAGE, and HEV-specific proteins were detected by Western blot analysis with sKFG (Patient) (identical results were obtained with sMT) and serum from a healthy individual (Normal). M, mock-infected; W, wild-type baculovirus infected; R, recombinant baculovirus infected.

sion). One of the baculovirus recombinants, Ac5480/7126, that contains an N-terminally 111-aa-truncated ORF2 enabled us to achieve our purpose (Fig. 2, ΔORF2). Both Sf9 and Tn5 cells were infected with the virus at an MOI of 10 and incubated for 5 days. Two or three major immunoreactive proteins, including the 58- and 50-kDa proteins, were found in both cell lysates. These two proteins were detectable in the recombinant virus-infected cells, but not in mock-infected or wild-type baculovirus-infected cells, and were indistinguishable from the 58- and 50-kDa proteins found in the cells expressing the whole ORF2 sequence. The molecular mass of the 58-kDa protein was in agreement with that (59 kDa) calculated from aa residues 112 to 660 of ORF2. There was no difference between these two cells in expression, location, and tight cell association, as far as the 58-kDa protein was concerned. However, production of the 50-kDa protein differed markedly in these two cell lines. In Sf9 cells the 50-kDa protein was almost completely retained in the cells and was hard to find in the culture medium. In contrast, in Tn5 cells a large amount of the 50-kDa protein was found in the culture medium. This protein was detectable only with the serum from a hepatitis E patient (not with the normal serum).

The 50-kDa protein forms empty VLPs. The culture medium of the virus-infected Tn5 cells at 5 days p.i. was clarified by centrifugation and incubated with polyethylene glycol 6000 to see whether or not the 50-kDa protein is precipitable. After being incubated overnight on ice, the solution was centrifuged for 15 min at 10,000 × g and the supernatant and the precipitate were analyzed by SDS-PAGE. The 50-kDa proteins were recovered exclusively from the large precipitate, suggesting that the proteins were aggregated or self-assembled to form

empty particles. To investigate possible particle formation by the 50-kDa molecules, the culture medium was centrifuged to remove progeny baculoviruses and directly examined with an EM (data not shown). Many round, empty, virion-like particles were observed. Most were in an aggregated form. The median size was 23.7 nm in diameter. This is a little smaller than the 27 nm of native HEV particles secreted into the bile (our observation) or the stools (2, 5, 7, 8) of experimentally infected monkeys.

Characterization of VLPs. The buoyant density of the VLPs was determined to be 1.285 g/cm³ in a CsCl gradient. Although the buoyant density of native HEV particles in the stool extract from a human volunteer was reported to be 1.35 g/cm³ and 1.39 to 1.40 g/cm³ in CsCl (5, 25), the density of the native empty virus particle is still unknown. The kinetic experiment showed that the 50-kDa protein was released into the medium at 4 days p.i. and accumulated gradually for at least another 6 days (Fig. 3). The N-terminal 15-aa sequence of the 50-kDa protein was identical to aa residues 112 to 126 of ORF2. This sequence is also the same as that of the N terminus of the primary translation product of the 58-kDa protein predicted from the plasmid construction. This indicates that the 50-kDa protein was generated from the 58-kDa protein by a posttranslational modification, probably by cleavage(s) at its C terminus.

The intracellular 50-kDa protein of infected Tn5 cells also contains the same N-terminal amino acid sequence, and it was recovered as a soluble form after three cycles of freezing-thawing. This suggested that the particle formation occurred in the cells and that the physicochemical properties of the 50-kDa proteins were the same irrespective of whether they were the intracellular or extracellular form.
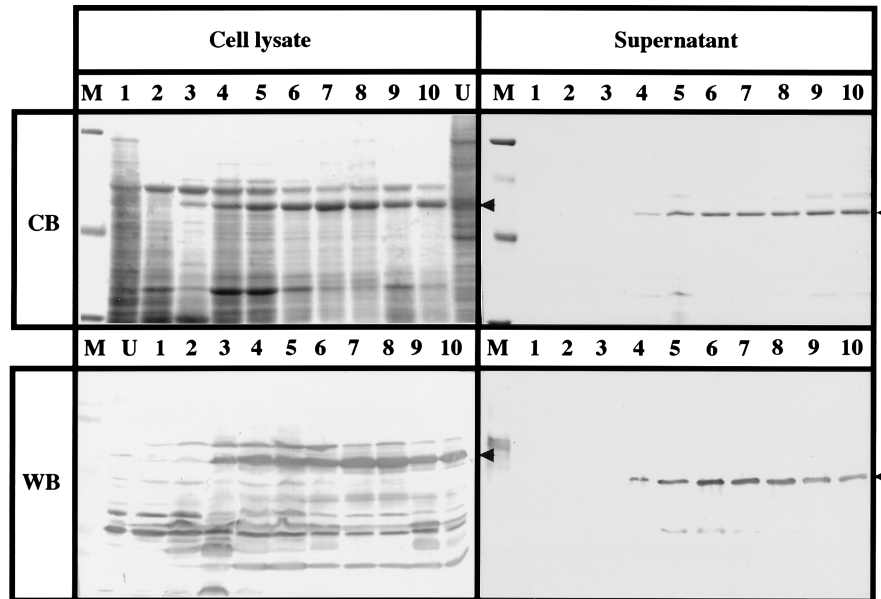
FIG. 3. Time course of expression of the 50-kDa protein. Tn5 cells grown in a 25-cm² flask were infected with baculovirus recombinant Ac5480/7126 at an MOI of 10 and were incubated at 26.5°C in 5 ml of Ex-cell 405 medium. At the indicated times, 5 μl of culture medium and the equivalent amount of cell lysate were analyzed by SDS–10% PAGE. Protein bands were visualized by Coomassie brilliant blue staining (CB) or analyzed by Western blot assay with sKFG (WB). The culture medium (right panel) and cell lysates (left panel) were analyzed separately. M, molecular weight marker; U, uninfected; lanes 1 through 10, 1 to 10 days p.i., respectively; arrowheads, the 50-kDa protein.

Antigenicity of VLPs. To determine their antigenicity, the VLPs were mixed and incubated with patient serum and the resultant antigen-antibody complexes were examined under an EM. As shown in Fig. 4C, the VLPs were coated with hepatitis E-specific antibodies, resulting in massive aggregations, whereas no aggregation was observed when the VLPs were incubated with serum from a healthy individual (Fig. 4B), indicating that the VLPs share antigenicity with authentic HEV. To further examine the antigenic specificity, the VLPs were used as the antigen to coat microplates to detect HEV-specific antibodies elicited in an experimentally infected monkey, as shown in Fig. 5A. A significant increase of both IgM and IgG antibody responses was observed during the clinical course of acute hepatitis. The IgM antibody appeared soon after the infection, reached a peak at 4 weeks, and immediately decreased. No IgM was detected after 12 weeks. The IgG response rapidly
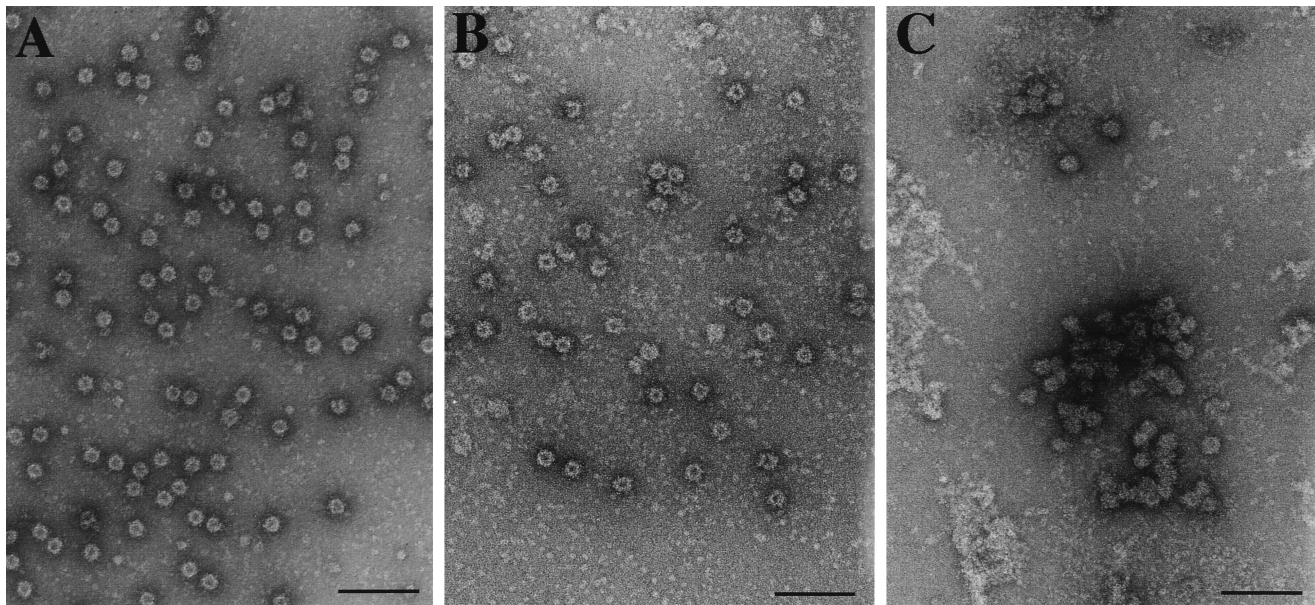

FIG. 4. Antibody-coated aggregation of VLPs as observed by IEM. One hundred microliters of purified VLPs (10 ng/ml) was incubated with the same volume of serum (1:1,600) at 37°C for 60 min. Immune complexes were precipitated by centrifugation at 100,000 × g for 2 h at 4°C in a Beckman TL100.2 rotor. The pellets were resuspended in distilled water and stained with uranyl acetate and then examined under an EM. (A) purified VLPs. (B) VLPs incubated with serum from a healthy individual. (C) VLPs incubated with serum (sKFG) from a hepatitis E patient. Bar = 100 nm.
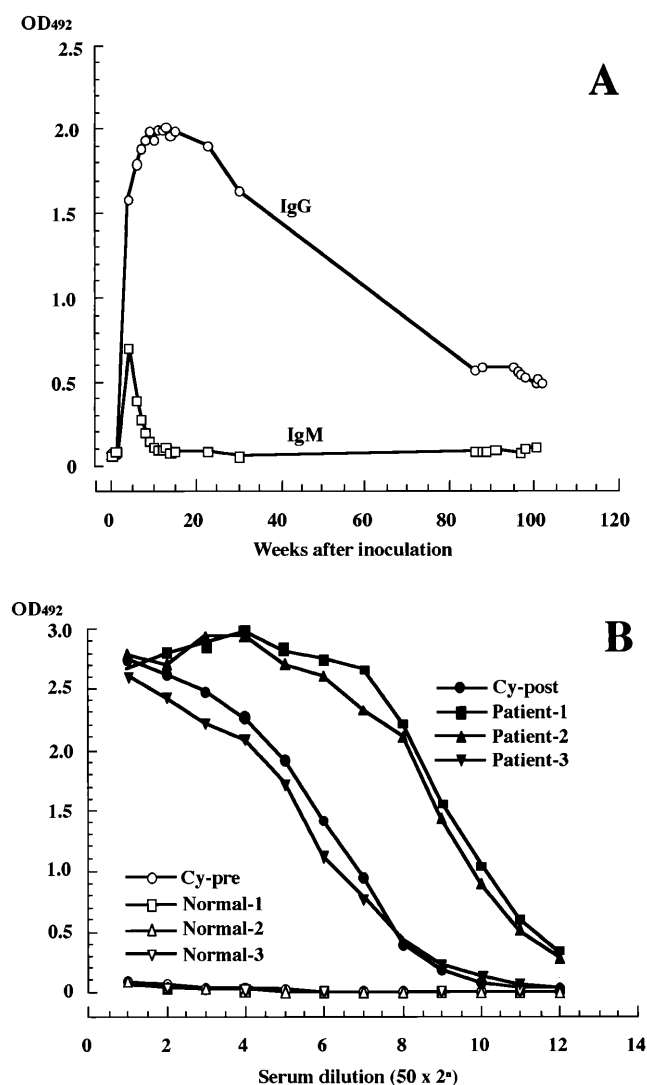
FIG. 5. (A) Detection of anti-HEV antibodies in monkey serum by antibody ELISA using VLPs. Sera obtained periodically from an experimentally infected cynomolgus monkey (CY-2 in reference 34) were examined for IgM and IgG antibodies. (B) Anti-HEV IgG responses in patients with hepatitis E. Twofold dilutions of sera from patients in the acute phase (Patient-1 to Patient-3) and from healthy individuals (Normal-1 to Normal-3) were used to detect IgG antibodies. Preimmune (Cy-pre) and postimmune (Cy-post) sera from an experimentally infected cynomolgus monkey were included as controls. OD_{492}, optical density at 492 nm.

increased and reached a peak at 13 weeks after inoculation. The IgG titer decreased gradually, but the level, as measured by antibody ELISA, remained at an optical density of over 0.5 for at least 2 years. These results indicate that the VLPs possess at least some antigenicity similar to that of native HEV and are capable of serving as an antigen to detect HEV-specific antibodies. In order to confirm these results, the VLPs were used to detect antibodies elicited in patients with hepatitis E. Serial dilutions of sera were subjected to the antibody ELISA, and the IgG response was measured (Fig. 5B). A significant increase of reactivity was observed in the sera from patients but not in those from healthy individuals.

Immunogenicity of VLPs. Antibodies to the VLPs were prepared in rabbits and guinea pigs, as described in Materials and Methods. After injection four times, the animals had high

levels of IgG antibodies and the titer reached as high as $1:10^6$ as determined by the antibody ELISA. To determine the immunogenicity of the VLPs the hyperimmune serum from a rabbit was used to coat microplates and capture HEV antigens in the bile from HEV-infected monkeys or the stool specimens from patients with hepatitis E. The HEV antigen thus captured was detected by the hyperimmune serum from a guinea pig. Titers of 20 to 80 were obtained for the PCR- and EM-positive specimens but not for the negative samples. However bMM41-3, which showed the highest titer, at 320, was EM negative (Table 1). These results demonstrated that the VLPs were immunogenic and were able to elicit antibodies capable of binding to native HEV antigens.

DISCUSSION

Expression of viral structural proteins by baculovirus recombinants has long been used to generate VLPs of various DNA and RNA viruses. VLPs produced by this system usually retain the immunogenic and physicochemical properties of the native virions (9, 10, 16–18, 20, 22). As observed in this study, as well as previously (31, 37), the entire ORF2 sequence was efficiently expressed in insect cells. However, no VLPs were detected (Fig. 2). To determine the possible function of the HEV proteins encoded in ORF1 and ORF3 in self-assembly, we carried out experiments in which Ac5147/7126-infected Tn5 cells were superinfected with baculovirus recombinants that efficiently express the entire ORF1 and/or ORF3 protein. However, neither stimulation of the expression of the 72-, 58-, and 50-kDa proteins nor release of the 50-kDa protein into the medium was observed (data not shown). In contrast, the ORF2 protein truncated at its N terminus was shown to self-assemble and form the VLPs, providing direct evidence that ORF2 indeed encodes the structural protein of HEV.

Two conditions are necessary for efficient particle formation. First, the N-terminal 111 aa, including the 5′ distal leader sequence, should be deleted. Deletion of the N-terminal 15 aa was not sufficient. However, addition of approximately 30 aa derived from the pVL1393 vector to the C terminus of the truncated protein did not disrupt particle formation. In Tn5 cells, the 50-kDa protein was released into the medium while the 58-kDa protein was tightly cell associated, although these two proteins shared the same amino acid sequences at their N termini. These findings indicated that the truncated proteins

TABLE 1. Antigen ELISA to detect HEV in bile from experimentally infected monkeys or in stool specimens from patients with hepatitis E

Specimen	Titer[a]	Result of:	
		EM	PCR[b]
VLPs[c]	1,280	+	ND[d]
Bile[e]	<10	−	−
Stool[f]	<10	−	ND
bMM41-2[g]	80	+	ND
bMM41-3[g]	320	−	ND
Stool[h]	80	ND	+
Stool[h]	20	ND	+

[a] Highest dilution of the specimen.
[b] Performed as described previously (2).
[c] 10 ng.
[d] ND, not done.
[e] From a normal rhesus monkey.
[f] From a healthy individual.
[g] Bile from an infected monkey after passages 2 and 3, respectively.
[h] From patients in China with epidemic hepatitis E (4).

should be further processed through a posttranslational C-terminal deletion mediated by host cellular protease(s) before or during self-assembly. In concert with our observation, the 55-kDa protein, a proteolytically processed ORF2 protein with its 111 aa from the N terminus of a Pakistani strain, has been shown to lack another 51 aa at its C terminus (31). Whether or not this 55-kDa and our 50-kDa protein are the same molecule is unknown.

Second, expression in Tn5, but not Sf9, cells was crucial. Sf9 cells were used exclusively for expression in the previous studies. Sf9 was indeed capable of generating both 58- and 50-kDa molecules whose N-terminal amino acid sequences are identical to that of the VLPs; however, no VLPs were formed. Although morphological changes of these two cell lines after virus infection seem to proceed similarly, as observed by optical or electron microscopy, our preliminary experiments suggested that the viability of the infected cells is an important factor for VLP formation. The biochemical basis of the prolonged viability of Tn5 cells compared with Sf9 cells and the mechanism for particle formation specific to Tn5 cells remain unknown. Release of the VLPs into the culture medium has a great advantage for obtaining a large amount of highly purified VLPs, allowing further characterization of the molecule.

Obviously the size of the VLPs (23.7 nm) is smaller than that of the authentic native HEV virion (27 nm), and the smaller HEV virions have not been observed in the bile or stools from patients with hepatitis E or from experimentally infected monkeys (25). The formation of the smaller particles described in this study is probably due to assembly of both N- and C-terminally truncated ORF2 proteins, but there is currently no collective view to explain the relationship between the processing of the capsid protein and particle formation. Recently Jameel and coworkers reported that the ORF2 protein is synthesized as an 82-kDa precursor, which is then processed into a 74-kDa mature protein through signal sequence cleavage and glycosylated into an 88-kDa protein (15). Our Western blot analysis of the bile and the stools from experimentally infected monkeys identified two major proteins with molecular masses of 50 and 58 kDa. In this context, it would be interesting to see whether or not these proteins are glycosylated. The size of our VLPs seems to be different from those found in previous studies by others, in which VLPs of 30 and 18 nm in diameter were observed by IEM using a fraction with a density of 1.30 g/ml, whereas partial purification yielded particles of approximately 20 nm (32, 37).

Nevertheless, the formation of the VLPs described here is very important for seroepidemiological studies. The VLPs appeared to retain the antigenicity of the native virion, as demonstrated by IEM with serum samples from patients with hepatitis E (Fig. 4). The antibody ELISA using the VLPs as antigen appears to be specific and sensitive enough to detect anti-HEV IgG as well as IgM in human and experimentally infected monkey sera (Fig. 5A). All these results demonstrate that VLPs are usable agents for diagnosis and may allow us to study many non-A, non-B, non-C acute hepatitis cases which have been suspected to be caused by HEV but for which obtaining proof has been hampered by the lack of reliable reagents. The virtually unlimited supply of VLPs by the baculovirus recombinant is capable of overcoming such problems. Our preliminary seroepidemiological study using VLPs indicates that the prevalence of antibody to HEV in areas in which HEV is nonepidemic is much higher than previously suspected (unpublished data). Although the neutralization test with authentic HEV particles is necessary to determine immunogenicity (23), our results indicate that the VLPs are capable of producing antibodies that specifically bind to native HEV an-

tigen. Currently only one serotype has been found in HEV. Our data suggest that the VLPs may be the most promising molecules yet for a recombinant vaccine against HEV infection.

REFERENCES

1. **Arankalle, V. A., L. P. Chobe, J. Jha, M. S. Chadha, K. Banerjee, M. O. Favorov, T. Kalinina, and H. Fields.** 1993. Aetiology acute sporadic non-A, non-B viral hepatitis in India. J. Med. Virol. **40:**121–125.
2. **Arankalle, V. A., J. Ticehurst, M. A. Sreenivasan, A. Z. Kapikian, H. Popper, K. M. Pavri, and R. H. Purcell.** 1988. Aetiological association of a virus-like particle with enterically transmitted non-A, non-B hepatitis. Lancet **i:**550–554.
3. **Aye, T. T., T. Uchida, X. Ma, F. Iida, T. Shikata, M. Ichikawa, T. Rikihisa, and K. M. Win.** 1993. Sequence and gene structure of the hepatitis E virus isolated from Myanmar. Virus Genes **7:**95–110.
4. **Aye, T. T., T. Uchida, X.-Z. Ma, F. Iida, T. Shikata, H. Zhuang, and K. M. Win.** 1992. Complete nucleotide sequence of a hepatitis E virus isolated from the Xinjiang epidemic (1986–1988) of China. Nucleic Acids Res. **20:**3512.
5. **Balayan, M. S., A. G. Andjaparidze, S. S. Savinskaya, E. S. Ketiladze, D. M. Braginsky, A. P. Savinov, and V. F. Poleschuk.** 1983. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology **20:**23–31.
6. **Bi, S.-L., M. A. Purdy, K. A. McCaustland, H. S. Margolis, and D. W. Bradley.** 1993. The sequence of hepatitis E virus isolated directly from a single source during an outbreak in China. Virus Res. **28:**233–247.
7. **Bradley, D. W., and M. S. Balayan.** 1988. Virus of enterically transmitted non-A, non-B hepatitis. Lancet **i:**819.
8. **Bradley, D. W., K. Krawczynski, E. H. Cook, Jr., K. A. McCaustland, C. D. Humphrey, J. E. Spelbring, H. Myint, and J. E. Maynard.** 1987. Enterically transmitted non-A, non-B hepatitis: serial passage of disease in cynomolgus macaques and tamarins and recovery of disease-associated 27- to 34-nm virus like particles. Proc. Natl. Acad. Sci. USA **84:**6277–6281.
9. **Brown, C. S., J. W. Van Lent, J. M. Vlak, and W. J. Spaan.** 1991. Assembly of empty capsids by using baculovirus recombinants expressing human parvovirus B19 structural proteins. J. Virol. **65:**2702–2706.
10. **Dingle, K. E., P. R. Lambden, E. O. Caul, and I. N. Clarke.** 1995. Human enteric *Caliciviridae*: the complete genome sequence and expression of VLPs from a genetic group II small round structured virus. J. Gen. Virol. **76:**2349–2355.
11. **Fuerst, T. R., P. O. Yarbough, Y. Zhang, P. McAtee, A. Tam, J. Lifson, K. McCaustland, J. Spellbring, D. Bradley, H. S. Margolis, M. Francotte, N. Garcon, M. Slaoui, J. P. Prieels, and K. Krawczynski.** 1996. Prevention of hepatitis E using a novel ORF-2 subunit vaccine, p. 384–392. *In* Y. Buisson, P. Coursaget, and M. Kane (ed.), Enterically-transmitted hepatitis viruses. La Simarre, Tours, France.
12. **He, J., W. Ching, P. Yarbough, H. Wang, and M. Carl.** 1995. Purification of a baculovirus-expressed hepatitis E virus structural protein and utility in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. **33:**3308–3311.
13. **He, J., A. W. Tam, P. O. Yarbough, G. R. Reyes, and M. Carl.** 1993. Expression and diagnostic utility of hepatitis E virus putative structural proteins expressed in insect cells. J. Clin. Microbiol. **31:**2167–2173.
14. **Huang, C.-C., D. Nguyen, J. Fernandez, K. Y. Yun, K. E. Fry, D. B. Bradley, A. W. Tam, and G. R. Reyes.** 1992. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology **191:**550–558.
15. **Jameel, S., M. Zafrullah, M. H. Ozdener, and S. K. Panda.** 1996. Expression in animal cells and characterization of the hepatitis E virus structural proteins. J. Virol. **70:**207–216.
16. **Jiang, X., D. O. Matson, G. M. Ruiz-Palacios, J. Hu, J. Treanor, and L. K. Pickering.** 1995. Expression, self-assembly, and antigenicity of a Snow Mountain agent-like calicivirus capsid protein. J. Clin. Microbiol. **33:**1452–1455.

17. **Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes.** 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J. Virol. **66:**6527–6532.

18. **Kajigaya, S., H. Fujii, A. Field, S. Anderson, S. Rosenfeld, L. J. Anderson, T. Shimada, and N. S. Young.** 1991. Self-assembled B19 parvovirus capsids, produced in a baculovirus system, are antigenically and immunogenically similar to native virions. Proc. Natl. Acad. Sci. USA **88:**4646–4650.

19. **Khuroo, M. S.** 1980. Study of an epidemic of non-A, non-B hepatitis: possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. Am. J. Med. **68:**818–824.

20. **Labbé, M., A. Charpilienne, S. E. Crawford, M. K. Estes, and J. Cohen.** 1991. Expression of rotavirus VP2 produces empty corelike particles. J. Virol. **65:**2946–2952.

21. **Li, F., H. Zhuang, S. Kolivas, S. A. Locarnini, and D. A. Anderson.** 1994. Persistent and transient antibody responses to hepatitis E virus detected by Western immunoblot using open reading frame 2 and 3 and glutathione *S*-transferase fusion proteins. J. Clin. Microbiol. **32:**2060–2066.

22. **Luo, L., Y. Li, and C. Y. Kang.** 1990. Expression of Gag precursor protein and secretion of virus-like Gag particles of HIV-2 from recombinant baculovirus-infected insect cells. Virology **179:**874–880.

23. **Meng, J., R. Guinet, and J. Pillot.** 1996. Infection of PLC/PRF-5 cells with the hepatitis E virus, p. 336–345. *In* Y. Buisson, P. Coursaget, and M. Kane (ed.), Enterically-transmitted hepatitis viruses. La Simarre, Tours, France.

24. **Panda, S. K., S. K. Nanda, M. Zafrullah, I. Ansari, M. H. Ozdenar, and S. Jameel.** 1995. An Indian strain of hepatitis E virus (HEV): cloning, sequence, and expression of structural region and antibody responses in sera from individuals from an area of high-level HEV endemicity. J. Clin. Microbiol. **33:**2653–2659.

25. **Purcell, R. H.** 1996. Hepatitis E virus, p. 2831–2843. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.

26. **Purdy, M. A., K. A. McCaustland, K. Krawczynski, A. Tam, M. J. Beach, N. C. Tassopoulos, G. R. Reyes, and D. W. Bradley.** 1992. Expression of a hepatitis E virus (HEV)-trpE fusion protein containing epitope recognized by antibodies in sera from human cases and experimentally infected primates. Arch. Virol. **123:**335–349.

27. **Stewart, L. M. D., and R. D. Possee.** 1993. Baculovirus expression vectors, p. 227–256. *In* A. J. Davison and R. M. Elliotts (ed.), Molecular virology: a practical approach. IRL Press, Oxford, England.

28. **Supanaranond, K., N. Takeda, and S. Yamazaki.** 1992. The complete nucleotide sequence of a variant of coxsackievirus A24. Virus Genes **6:**149–158.

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

30. **Tsarev, S. A., S. U. Emerson, G. R. Reues, T. S. Tsareva, L. J. Legters, I. A. Malik, M. Iqbal, and R. H. Purcell.** 1992. Characterization of a prototype strain of hepatitis E virus. Proc. Natl. Acad. Sci. USA **89:**559–563.

31. **Tsarev, S. A., T. S. Tsareva, S. U. Emerson, S. Govindarajan, M. Shapiro, J. L. Gerin, R. Robinson, A. E. Gorbalenya, and R. H. Purcell.** 1996. Prospects for prevention of hepatitis E, p. 373–383. *In* Y. Buisson, P. Coursaget, and M. Kane (ed.), Enterically-transmitted hepatitis viruses. La Simarre, Tours, France.

32. **Tsarev, S. A., T. S. Tsareva, S. U. Emerson, A. Z. Kapikian, J. Ticehurst, W. London, and R. H. Purcell.** 1993. ELISA for antibody to hepatitis E virus (HEV) based on complete open reading frame-2 protein expressed in insect cells: identification of HEV infection in primates. J. Infect. Dis. **168:**369–378.

33. **Uchida, T., K. Ishikawa, S. Mima, and T. Rikihisa.** 1996. The etiology of non-A, non-B, non-C, non-D hepatitis in Japan, p. 217–224. *In* Y. Buisson, P. Coursaget, and M. Kane (ed.), Enterically-transmitted hepatitis viruses. La Simarre, Tours, France.

34. **Uchida, T., K. M. Win, K. Suzuki, K. Komatsu, F. Iida, T. Shikata, K. Mizuno, S. Soe, H. Myint, K. M. Tin, and K. Nakane.** 1990. Serial transmission of a putative causative virus of enterically transmitted non-A, non-B hepatitis to *Macaca fascicularis* and *Macaca mulatta*. Jpn. J. Exp. Med. **40:**13–21.

35. **Wickham, T. J., and G. R. Nemerow.** 1993. Optimization of growth methods and recombinant protein production in BTI-Tn-5B1-4 insect cells using the baculovirus expression system. Biotechnol. Prog. **9:**25–30.

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

37. **Yarbough, P. O., E. Garza, A. W. Tam, Y. Zhang, P. McAtee, and T. R. Fuerst.** 1996. Assay development of diagnostic tests for IgM and IgG antibody to hepatitis E virus, p. 294–296. *In* Y. Buisson, P. Coursaget, and M. Kane (ed.), Enterically-transmitted hepatitis viruses. La Simarre, Tours, France.

38. **Yin, S., R. H. Purcell, and S. U. Emerson.** 1994. A new Chinese isolate of hepatitis E virus: comparison with strains recovered from different geographical regions. Virus Genes **9:**23–32.