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		HEV RNA	HEV strain (genotype)
					IgM	IgG		
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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A型肝炎 ―我が国の最近の発生動向を中心に―

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1．はじめに

A型肝炎はピコルナウイルス科のA型肝炎ウイルス（HAV）によって引き起こされる急性疾患で，他のウイルス性肝炎（B型肝炎やC型肝炎）と違って，慢性化することはない．感染を仲立ちするベクターもなく，安全で有効なワクチンが開発され，人類が制圧可能な病気のひとつである．病原ウイルスは直径27nmの正二十面体でヒトエンテロウイルスと同じく腸管感染でヒトからヒトへ伝播する．ウイルスゲノムの塩基配列の相同性がエンテロウイルスのそれとかなり異なることなどから，ヘパトウイルス属として分類上明確に区別されるようになった．ヒトから分離されるHAVは4種，2亜型（IA, IB, II, IIIA, IIIB, VII）の遺伝子型であるが，抗原的には高度に保存されており，単一の血清型しか知られていない．我が国で分離される株の大部分は遺伝子型IAまたはIIIB型に属する（図１）[1]．他に3種の遺伝子型（IV, V, VI）のサル

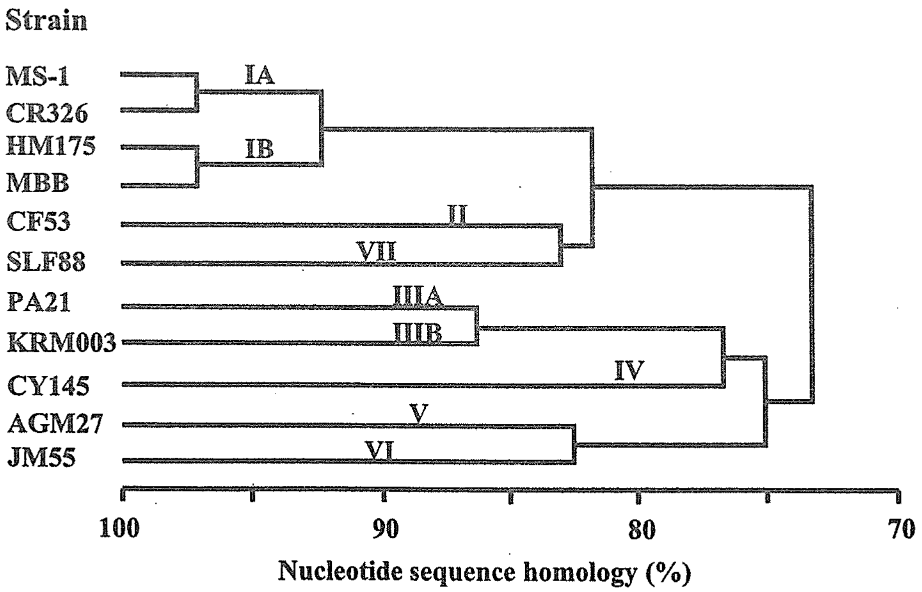

図１ HAV株の分子系統樹 VP1/2A領域，168塩基配列の比較

Hepatitis-A ―Review of hepatitis A virus infection in Japan―
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HAVが分離されているが，伝播はサルに限局されている．ウイルスの遺伝子解析は分子疫学必須の手法として実験室で広汎に使われるようになった．A型肝炎の場合は後述するように汚染食材を介して集団感染をひきおこすことが社会的な問題になっている．個々のウイルスRNAの塩基配列を調べて，感染源が明らかになれば，新たな発生に対処することも可能である．

2．感染の疫学的特徴

HAVは糞便中に排泄され，口から体内に入り感染する．糞口感染するウイルス病の特徴にたがわず，アジア，アフリカなどの途上国のように，人口過密，衛生環境の悪いところの乳幼児に蔓延している．こうした地域では成人する前に殆どすべてのひとがHAVに対する抗体を持つ．子供の頃感染すれば，不顕性の割合も多く，病気の症状も軽く非黄疸性のことが多い．日本や欧米のように衛生環境の整った地域では患者の年齢層もあがり，症状も重くなる．北欧やカナダでは散発症例の大部分は途上国から帰国する旅行者にみられる．人口10万人当たりの罹患率は1998年米国で8.6，オランダで6である．日本では0.33（2002年）[2]であるから，世界でも罹患率の最も低いグループに属する．図2に示すように日本の年齢別抗体保有率のパターンは，年々高年齢側に移動している．1994年の40歳未満の抗体保有率は1％以下であり，40歳以上では年齢に伴い上昇し，65歳以上では90％である[3]．このことは高齢になって急に感染が増加するのではなく，日本でも終戦直後までHAVが蔓延していたが，その後衛生環境が整備されるにつれて，蔓延状態は終息したことを物語っている．1960年以降生まれた人は殆ど抗体を持っていない．従って若年層は無論のこと，中高年層も感受性者が多くなり，国内での小流行の原因となっている．免疫のない人がHAVの常在している国々に旅行し，感染して帰国，発病することがしばしば見受けられる．

3．感染の経過

臨床経過から他のウイルス性肝炎と区別するのは難しい．診断にはウイルス学的あるいは血清学的な検査が必要である．定型的には発熱，筋肉痛，倦怠感，悪心，食欲不振，嘔吐といった前駆症状に続いて，肝障害が起こる．血清トランスアミナーゼ（ALT）などの肝臓由来の酵素やビリルビンの血中濃度が上昇し，黄疸は通常約4週間続く．これらの症状はウイルスによる直接の肝細胞障害ではなく，感染に対する宿主の免疫反応によるもので，一定の臨床経過を経て，ゆっくりではあるが完全治癒にむかう．感染後終生免疫が得られ，慢性のキャリアーも存在しない．感染年齢の高齢化に伴い，稀に劇症化して致死的になることが危惧されている．劇症化A型肝炎の報告は，日本では年に1～2

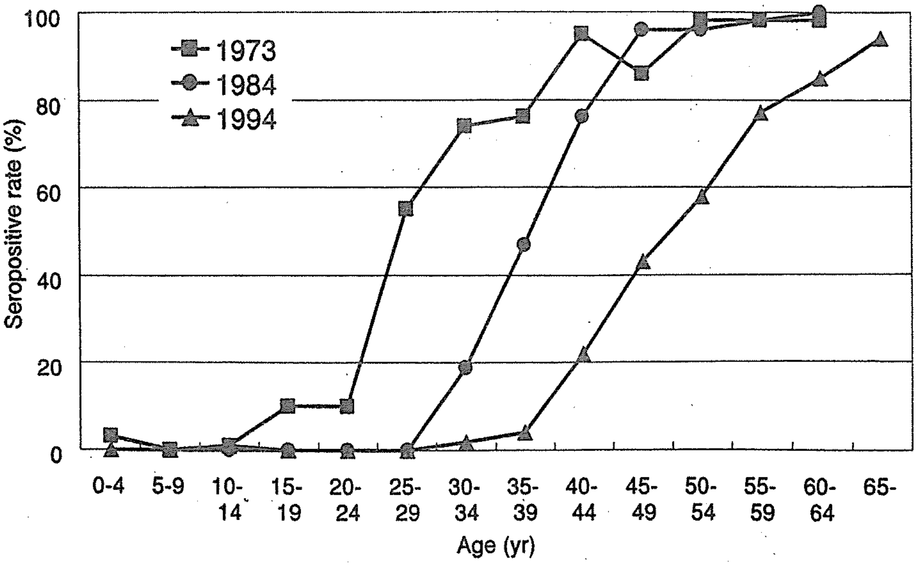

図2　日本人の抗HAV抗体保有状況の推移

例である．米国の統計ではA型肝炎の致死率は0.3%でその70%が50歳以上の高齢者である．妊婦が感染しても特に重い症状を呈することはなく，また胎児に感染して影響がでることも知られていない4)．

図3に典型的なA型肝炎の経過を示した．A型肝炎の潜伏期は約4週であるが，黄疸の出現する2～3週前の期間から，1週後まで感染性のウイルスは糞便中に検出される．血液中のウイルスは糞便中のそれよりはるかに少ない．最近のPCR法を使った研究では，HAV RNAは血液中でも3ヶ月にわたり検出し得る例が示されている．HAVのウイルス血症は考えられていた以上に長いようである5)．

発症とともに血清トランスアミナーゼが上昇し，HAV特異的IgM抗体とIgG抗体が血清中にほぼ同じ頃出現する．IgM抗体価は発症1ヶ月後がピークで，徐々に減衰し，3～6ヶ月後には検出限界以下となる．IgG抗体は終生持続する．

4．診　断

臨床的には他のウイルス性肝炎と区別しがたいので，疫学的にA型肝炎が疑われても最終診断は血清中のウイルス特異的IgM抗体か，糞便か血清中のHAV遺伝子の検出による．培養細胞によるウイルス分離には長期間を要するので診断目的には適さない．一般的な血清疫学調査などにはIgG抗体を測定する．

5．日本での発生動向

a）国内感染例

日本のA型急性肝炎の最近の特徴として罹患年齢の上昇化，春をピークとする季節性がある．年間500例前後の発生があり，国内の感染が主である．

2002年は全体で500例が報告された．感染地域の内訳は，国内感染例422例（85%），海外感染例69例（14%）であった（図4）．国

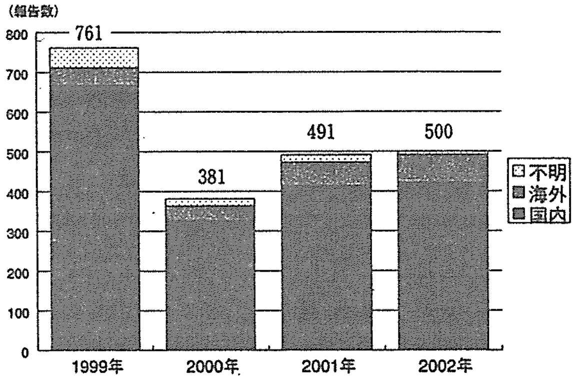

図4　年次別，地域別A型肝炎患者数

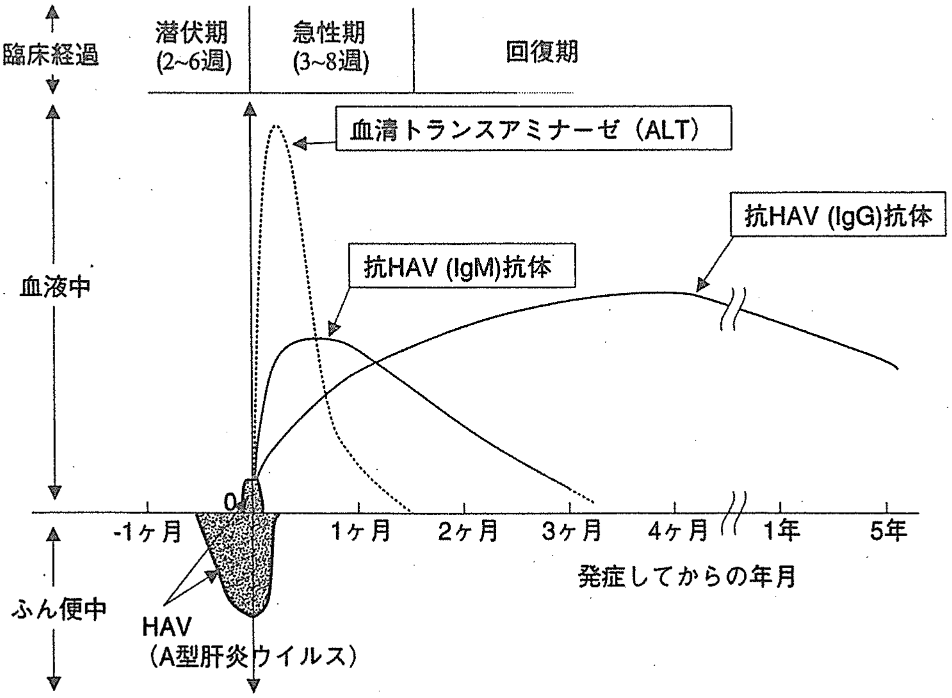

図3　A型肝炎ウイルス感染症の臨床ウイルス学的経過

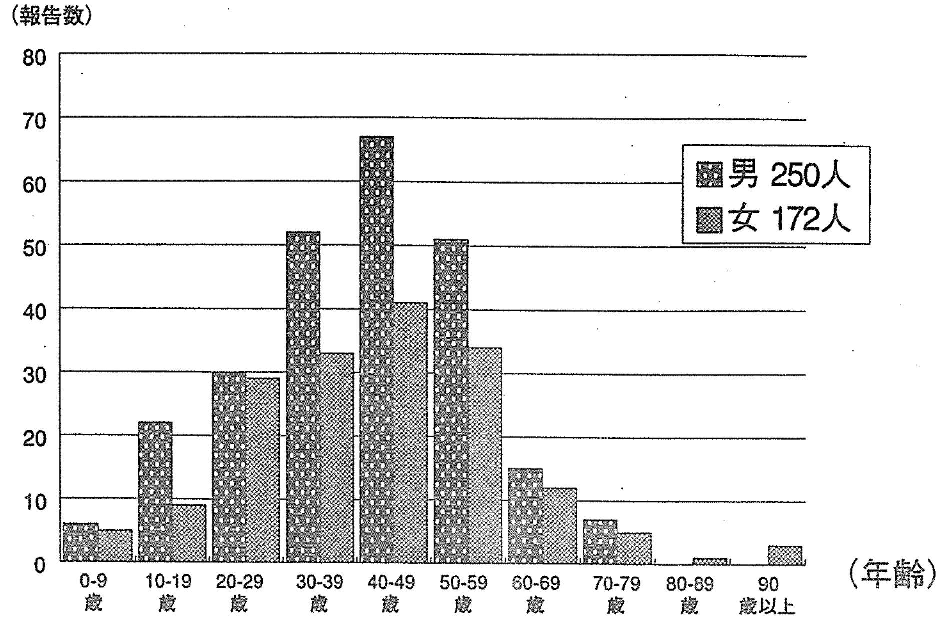

図５　2002年，国内で感染したＡ型肝炎患者の年齢分布

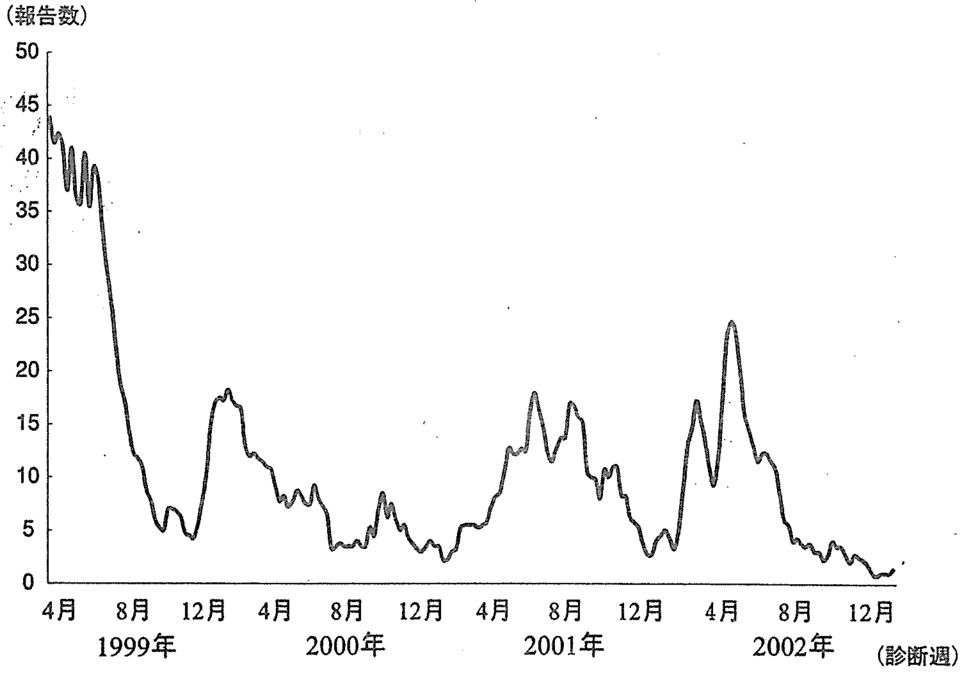

図６　Ａ型肝炎の流行パターン，1999～2002年

内感染者の年齢分布は，40歳代をピークにしてその前後の成人層に多く見られる（図５）．小児の報告は非常に少ない．20例を超えて報告された都道府県は，東京，千葉，神奈川，愛知の４県であった．人口10万人当りの罹患率では山形（1.05）と山口（1.8）の両県が1.0を越えた[2]．報告の多い地域の中でも，集団発生が確認されている地域と集団発生が確認されていない地域があった．1999年からの地域分布では，報告の多い地域は一定せず，毎年異なる．日本では上水道の改善によりHAVに汚染された環境から感染する機会が低くなったため，地域分布が一定していないと考えられる．

春をピークに冬から夏にかけて毎年流行が見られる（図６）．汚染された生牡蠣などの

喫食の影響が考えられている．オランダではひとの移動にともない，夏から秋に流行がみられ，秋に低い日本の流行様式とは異なる．季節性は米国にはみられない．

感染源を調べると，352人（83％）に飲食物が疑われ，牡蠣を含む海産物が原因と思われたものは121人（30％）になる．性行為で感染したと思われる報告例も１例あった（図７）．後述のように東南アジア，中国からの輸入海産物が感染の原因と考えられる報告もある．

家族内や，職場，学校などで周囲に感染が広がったのは120人（30％）で，272人（65％）の症例では周囲に同疾患の罹患者は認められなかった2)．福祉施設内感染は1998年以降報告されていないが，ひとたび汚染されれば，施設内では感染が広がりやすい状況に変わりはなく，従業員へのワクチン接種の徹底や衛生環境の整備にはより注意を払わなければならない．

b) 海外感染例

海外感染例は東南アジアの旅行者が多いとされている．2002年は中国での感染が疑われた例が多く例年とはやや異なる（図８）．2002年の中国での感染者は，長期出張中あるいは帰国後に発症し，７月に報告された例が多

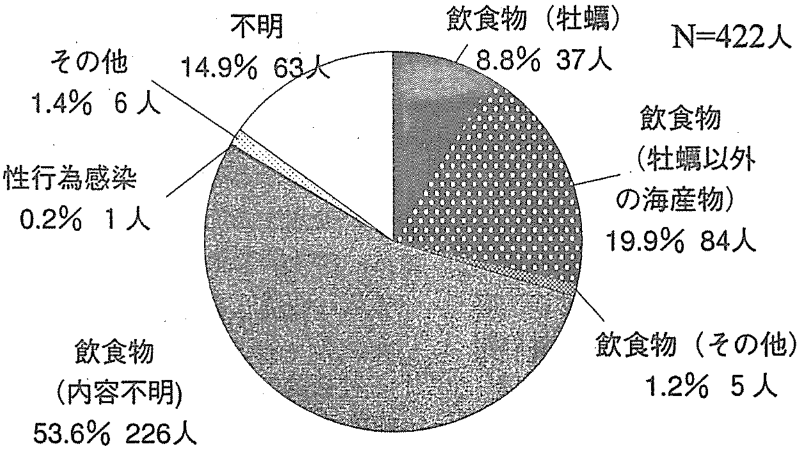

図７　2002年，国内で感染したＡ型肝炎の感染源，感染経路

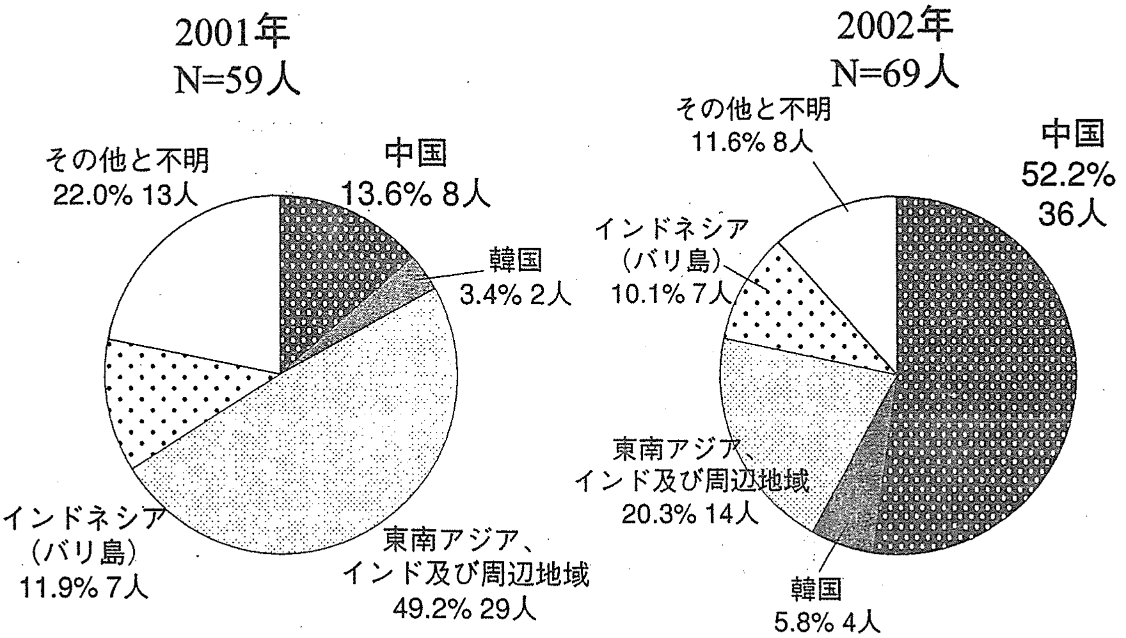

図８　2001～2002年，海外感染者の旅行先

かった．現地では飲食店が感染場所として疑われているが，明確な因果関係は確認されなかった．今後，海外進出している企業でもワクチン接種などのA型肝炎の感染対策が必要であり，同伴する家族にも病気の理解とワクチンの勧奨が重要となるであろう．海外で感染する主な患者層は，女性の患者は20歳代に，男性は30歳に患者数のピークがみられた（図9）．数は少ないが毎年同じような傾向が認められる．海外旅行に出かける年齢構成比と似ていて，国内で感染した患者の年齢分布（図5）と比べると興味深い．

6. 最近の国内外の流行例

汚染食材を介した集団発生は社会にあたえる影響も大きく，患者の糞便や血清，汚染が疑われた食材などから得られたHAV RNAの塩基配列を調べて，原因を究明する努力が関係者の間でなされている．A型肝炎の潜伏期は1ヶ月と長く，原因となった食材も保存されていることが稀なので，感染源や感染経路の特定は一般に困難である．今後の予防対策の一助に，感染源や感染経路が調査された最近の国内外の報告例をあげてみた．

(1) 2003年10月～11月，米国ペンシルベニア州にあるレストランAを利用した人の間で集団感染が報告された．調理従事者を含め患者数は555名，3名が死亡している．充分に加熱していない青ネギを含んだ食材が感染源とみられた．メキシコの農場から出荷され，ウイルスの遺伝子配列から同一の感染源と確認された[6]．

(2) 2002年3月～4月，東京都江東区の寿司店で調理従事者を含む喫食者24名がA型肝炎に感染した．調べたHAVの遺伝子型はIA型でかつ同一の塩基配列であった．調理従事者から寿司食材または調理器具を介した単一暴露が主原因とされた[7]．

(3) 2002年1月　浜松市や，2002年4月江戸川区の飲食店でウチムラサキ（通称，大アサリ）の喫食者にHAVとノロウイルス（NV）の2種のウイルスによる重感染と思われる食中毒が発生した．腹痛下痢，嘔吐等の食中毒症状を呈した後，A型肝炎を発病したものである．中国産のウチムラサキが原因と推定され，IA型のHAVが同定された．A型肝炎の患者数は4～5名であった[7]．

7. その他の感染経路

社会的に大きな問題となるのは血液製剤を介しての医療感染事故である．1990年代からイタリー，ドイツ，ベルギー，米国などで第8因子製剤による感染例が報告されたが，日本では確

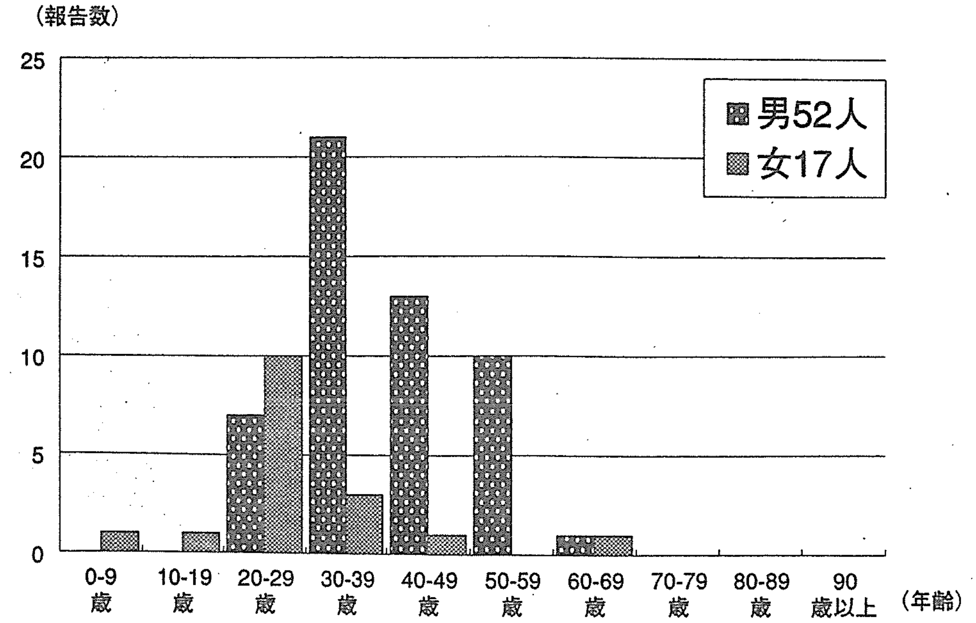

図9　2002年，海外感染者の年齢分布

認されていない[8]．HAVは非経口的に体内に侵入すると通常の経口感染よりはるかに感染効率が良いことがチンパンジーで確かめられている[9]．前述のようにウイルス検査の精度が上り，少数例とはいえ血清中にHAVが長期間存在する可能性が明らかになってきた．輸血，血液製剤，注射針等を介した非経口的感染にも注意を払わねばならない．血液製剤の材料となる血液の一層厳密な検査体制が求められている．

8. おわりに

日本でのA型肝炎は主に散発的な発生である．小規模な集団発生によるものが時々話題になるが，年間発生数は，減ることもなく，横ばい状態が続いている．抗体調査から推定されるように，日本ではHAV感受性者の割合が非常に多くなってきているが，近隣の東南アジア諸国は衛生環境の悪いHAV汚染地域である．東南アジア方面などのHAV汚染国への渡航者，医療関係者，調理従事者，福祉施設従業員等ハイリスク該当者にワクチンを徹底接種することが現時点での有効な予防策である．実際の患者数は報告された数の10倍になるという調査報告[10]もある．質の高いサーベイランスのデータを蓄積することや，血清疫学的，ウイルス学的調査研究がますます重要になってくるであろう．完全治癒するとはいえ，黄疸になれば，その症状は軽いものではなく，長期間の療養を要する．良いワクチンがあることと考えあわせ，B型肝炎ワクチンとともに全国的な規模での予防体制を導入する議論が必要であろう．

謝辞

図4から図9は感染症発生動向調査IDWR（厚生労働省・国立感染症研究所，感染症情報センター）2003年5月までの暫定集計によるものである．関係者に深謝いたします．

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Hepatitis E Virus Transmission from Wild Boar Meat

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We investigated a case of hepatitis E acquired after persons ate wild boar meat. Genotype 3 hepatitis E virus (HEV) RNA was detected in both patient serum and wild boar meat. These findings provided direct evidence of zoonotic foodborne transmission of HEV from a wild boar to a human.

Hepatitis E virus (HEV), a causative agent of human hepatitis E, is a single-stranded positive-sense RNA virus recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (1,2). HEV is transmitted primarily by the fecal-oral route through contaminated drinking water. However, recent studies have demonstrated that various animal species have serum antibodies to HEV, suggesting that hepatitis E is a zoonotic disease (3). In Japan, 4 hepatitis E cases have been linked directly to eating raw deer meat (4), and several cases of acute hepatitis E have been epidemiologically linked to eating undercooked pork liver or wild boar meat (5,6). These cases provide convincing evidence of zoonotic food-borne HEV transmission. We report direct evidence of HEV transmission from a wild boar to a human.

The Study

A 57-year-old woman came to Iizuka Hospital on March 12, 2005, with malaise and anorexia. Although she was a healthy hepatitis B virus carrier and negative for serologic markers of hepatitis A and C, testing upon admission showed elevated levels of liver enzymes (alanine aminotranferase 752 IU/L, aspartate aminotransferase 507 IU/L, and γ-glutamyl transpeptidase 225U/L). A serum sample collected on March 16 was positive for both immunoglobulin M (IgM) and IgG antibodies to HEV when tested by an antibody enzyme-linked immunosorbent assay using recombinant viruslike particles (7). This led to the diagnosis of hepatitis E. The hepatitis was typical, acute, and self-limiting, and the patient recovered by the end of March.

The patient's husband traditionally hunted boar for food 3 or 4 times a year, and she had eaten boar meat on 2 occasions. With her husband, she ate the meat as part of a hot pot on December 28, 2004, 11 weeks before her illness, and again, grilled, on January 19, 2005, along with 10 other people (including her husband) 8 weeks before her illness. Disease did not develop in the other 10 people. Except for this wild boar meat, the patient had not eaten meat or liver from other wild animals. Since she had not traveled abroad in the past 30 years, transmission must have occurred in Japan. Two portions of meat from the wild boar (meats 1 and 2) eaten on December 28, 2004, and 1 portion from the other wild boar (meat 3) eaten on January 19, 2005, remained and were frozen.

Juice was obtained from the sliced meat by centrifugation at 10,000 × *g* for 15 min. The supernatant was used for RNA extraction. A nested reverse transcription–polymerase chain reaction (RT-PCR) was conducted to amplify part of open reading frame 2 (ORF2), which corresponds to nucleotides (nt) 5939–6297 of the genotype 1 HEV genome (GenBank D10330), with external sense primer HEV-F1 (5′-TAYCGHAAYCAAGGHTGGCG-3′) and antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-3′). A nested PCR was conducted with internal sense primer HEV-F2 (5′-GGBGTBGCNGAGGAGGAGGC-3′) and internal antisense primer HEV-R1 (5′-CGACGAAATYAATTCTGTCG-3′). This procedure allows amplification of HEV 1, 3, and 4 genotypes. A PCR product of 359 bp including the primer sequences was obtained from meat 3 by nested PCR. However, meats 1 and 2 were negative. HEV RNA was not detected in the patient's serum by the same amplification method. This may have resulted from an extremely small amount of RNA.

New primers for the nested RT-PCR were designed for a region within the 359 base region based on the meat 3 sequences, which corresponded to nt 5983–6243. The first PCR was performed with external sense primer HEV-WB-F1 (5′-ACCTCTGGCCTGGTAATGCT-3′) and antisense primer HEV-WB-R2 (5′-GAGAAGCGTATCAGCAAGGT-3′). The nested PCR was performed with internal sense primer HEV-WB-F2 (5′-TATTCATGGCTCTCCTGTCA-3′) and internal antisense primer HEV-WB-R1 (5′-ACAGTGTCAGAGTAATGCCT-3′). These primers allowed amplification of 281 nt, including the primer sequences from the patient serum collected on March 16, 2005. In contrast, meats 1 and 2 were negative with these new primers.

To further analyze the RNA in the patient serum and meat 3, RNA genomes encoding an entire ORF2 were

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amplified as overlapping segments, nucleotide sequences were determined, and phylogenetic analysis was carried out with avian HEV as an outgroup. Avian HEV is a causative agent of chicken hepatitis-splenomegaly syndrome (8). Two sequences, 1 from the patient (DQ079629) and the other from meat 3 (DQ079630), were classified into genotype 3 (Figure). Only 1 nt difference was observed in the 1,980 nt of the entire ORF2; the nucleotide sequence identity was 99.95%. The difference was not accompanied by any amino acid changes. These data demonstrated that HEV infection was transmitted from the wild boar meat to the patient on January 19, 2005.

Conclusions

Currently, deer, pig, and wild boar are suspected sources of foodborne zoonotic transmission of HEV in Japan, and genotypes 3 and 4 of HEV are believed to be indigenous (4–6,9,10). Direct evidence for transmission of genotype 3 HEV from animals to humans was observed in acute hepatitis in 4 persons who had eaten uncooked deer meat that contained ≈10^7 copies of HEV RNA (4). However, the rare finding of HEV antibody-positive deer in Japan suggest that deer are not the major zoonotic reservoir of HEV in this country (11). In contrast, high antibody-positive rates in domestic pig and wild boar, including HEV genotypes 3 and 4, have been frequently detected, suggesting that persons who eat uncooked meat are at risk for infection with HEV (12,13). This report is the first to provide direct evidence of zoonotic foodborne genotype 3 HEV transmission from wild boar to a human.

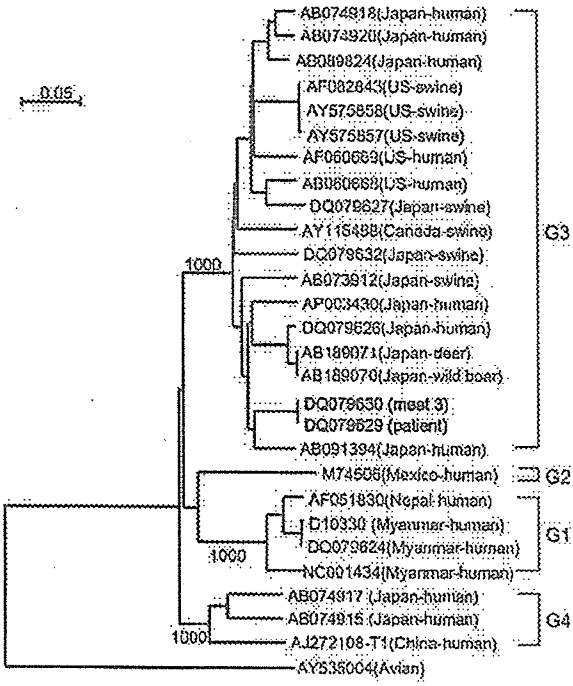

Figure. Phylogenetic tree of hepatitis E virus (HEV) reconstructed with avian HEV as an outgroup. Nucleotide sequences of the entire open reading frame 2 were analyzed by the neighbor-joining method. The bootstrap values correspond to 1,000 replications. The 2 nucleotide sequences characterized in this study are shown in **bold**. The horizontal scale bar at the top left indicates nucleotide substitutions per site.

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Dr Li is a senior researcher at National Institute of Infectious Diseases in Tokyo, Japan. His research focuses on epidemiology, expression of viral proteins, and the three-dimensional structure of hepatitis E virus.

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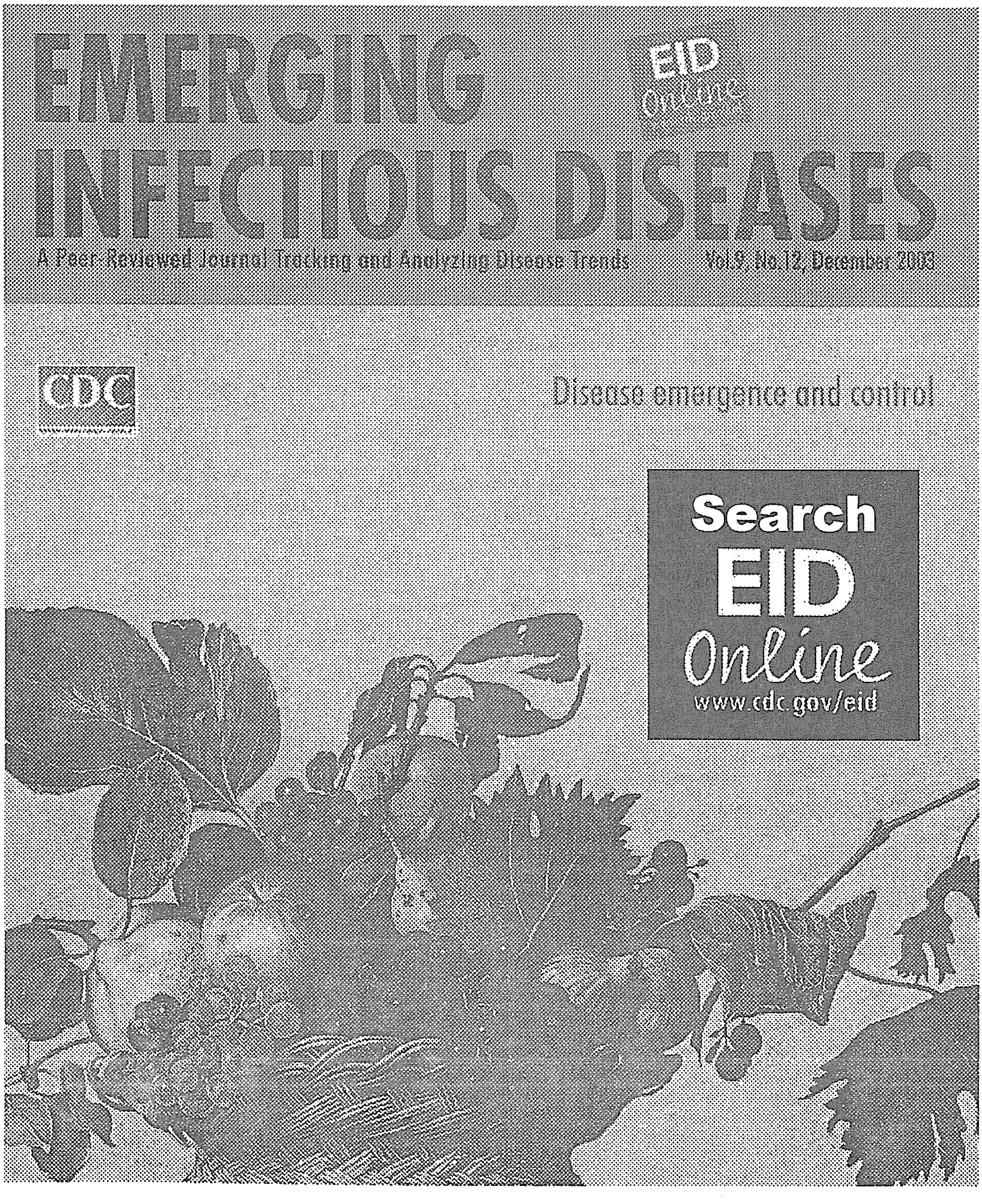

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Essential Elements of the Capsid Protein for Self-Assembly into Empty Virus-Like Particles of Hepatitis E Virus

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

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

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

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

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TABLE 1. Oligonucleotides used in the construction of baculovirus recombinants

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

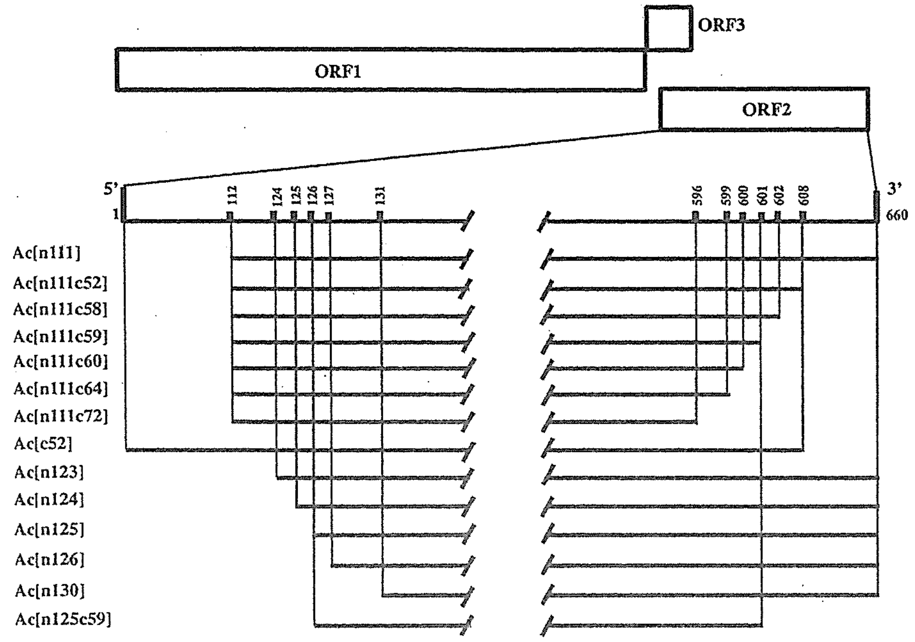

FIG. 1. Genome organization of HEV and schematic diagram of recombinant baculovirus vectors. DNA fragments encoding N- and C-terminal aa-truncated ORF2 were prepared by PCR with the primers listed in Table 1 and were used to construct 14 recombinant baculoviruses. Full-length pORF2 consisted of 660 aa.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

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

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

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

RESULTS

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

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

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

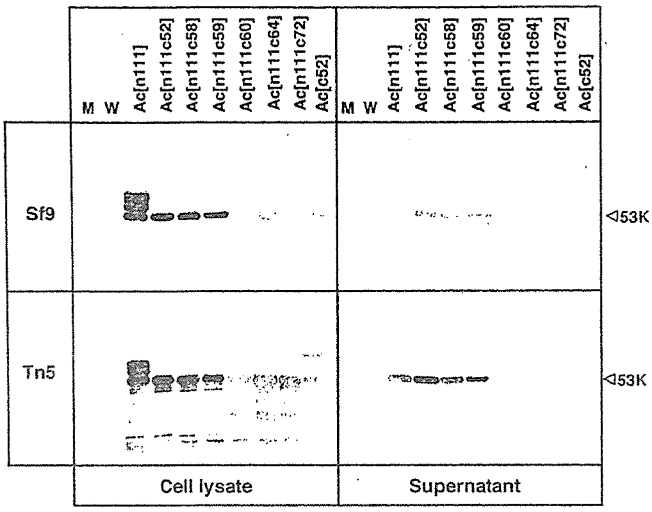

FIG. 2. Western blot assay of truncated pORF2 expressed in Sf9 and Tn5 cells. Eight recombinant baculoviruses, Ac[n111], Ac[n111c52], Ac[n111c58], Ac[n111c59], Ac[n111c60], Ac[n111c64], Ac[n111c72], and Ac[c52], were used to infect the insect cells. Ten microliters of the culture medium (right column)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

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

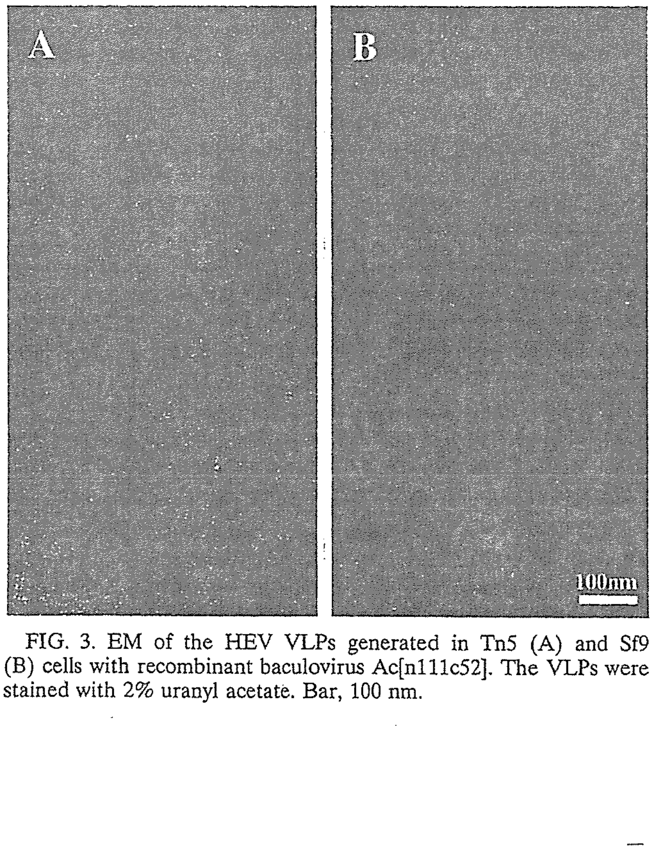

FIG. 3. EM of the HEV VLPs generated in Tn5 (A) and Sf9 (B) cells with recombinant baculovirus Ac[n111c52]. The VLPs were stained with 2% uranyl acetate. Bar, 100 nm.

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

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

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

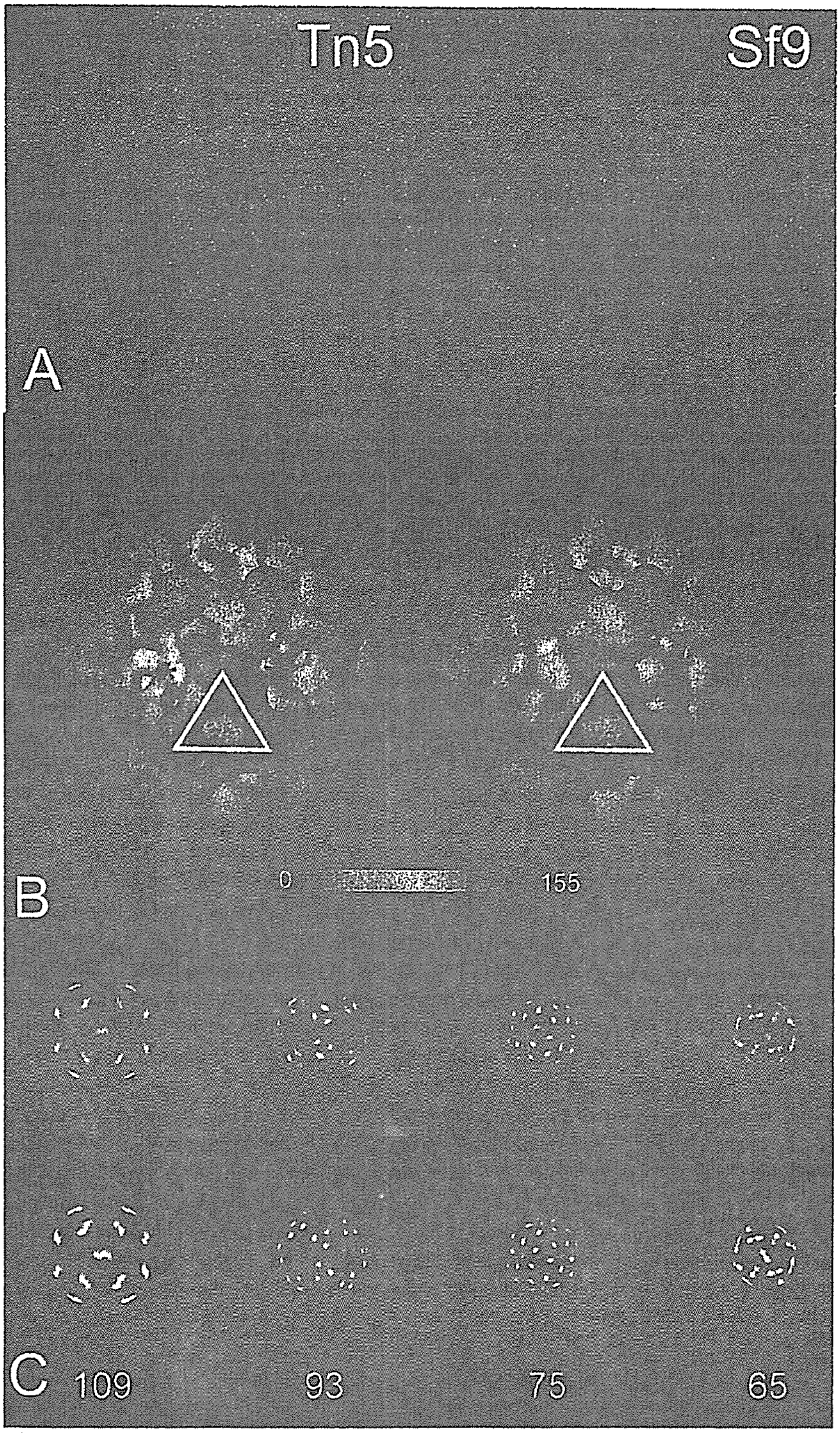

FIG. 4. HEV VLP structures determined by cryo-electron microscopy and image reconstruction. (A) Cryo-electron micrograph of ice-embedded HEV VLPs produced from Tn5 and Sf9 cells. The bar corresponds to 100 nm. (B) Surface-shaded representation of HEV VLP structures viewed along icosahedral twofold axes.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

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

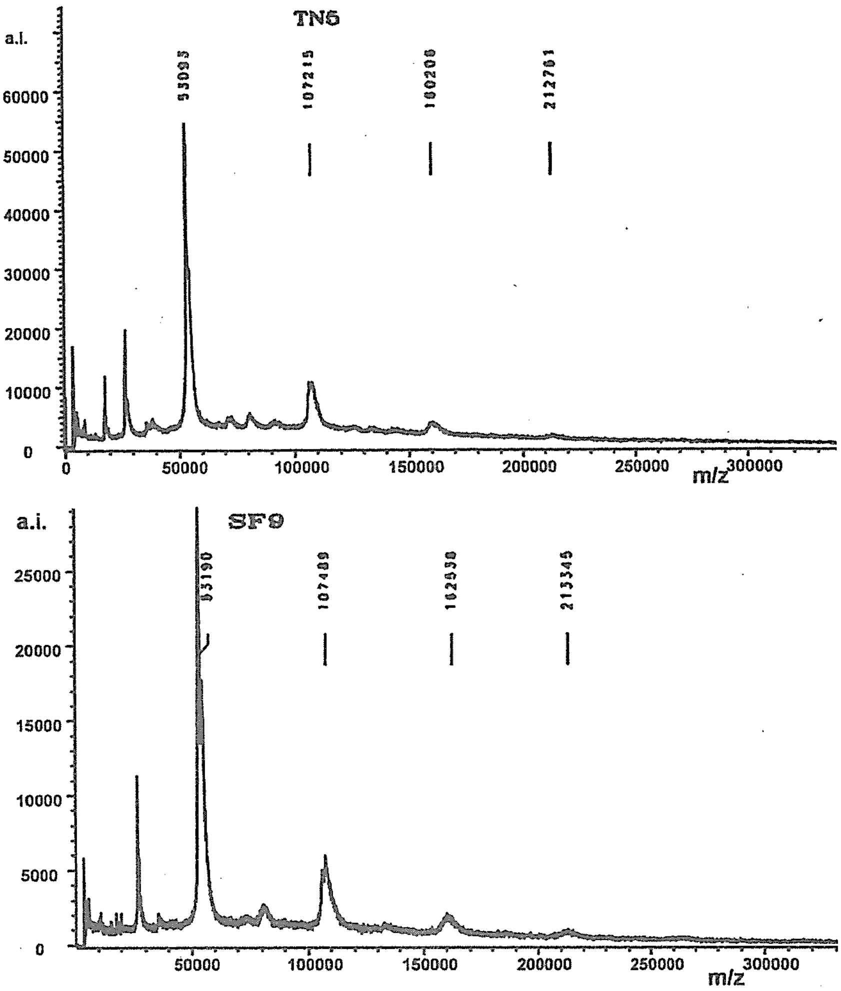

FIG. 5. Mass spectra from purified HEV VLPs displayed as the counts of isotope abundance (a.i.) versus mass/charge values (*m/z*). HEV VLPs produced from Tn5 (top panel) and Sf9 (bottom panel) cells with recombinant baculovirus Ac[n111c52] gave consistent mass spectra in which the abundant elements show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

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

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

DISCUSSION

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

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

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

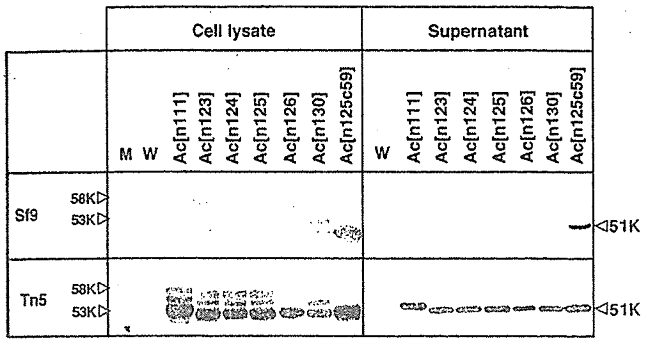

FIG. 6. Expression of N-terminally truncated pORF2 in Sf9 and Tn5 cells infected with Ac[n123], Ac[n124], Ac[n125], Ac[n126], Ac[n130], and Ac[n125c59]. A Western blot assay was carried out as described in the legend to Fig. 2. Ac[n111] was included for the expression of the 58K and 53K proteins. M,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

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

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

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

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

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

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