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Expression of Processed Envelope Protein of Hepatitis C Virus in Mammalian and Insect Cells

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The putative envelope protein of hepatitis C virus (HCV) was expressed in insect cells by using a baculovirus expression vector and in monkey COS cells under the control of exogenous promoters. The expressed envelope proteins, identified by immunoblot analysis using sera from patients with chronic HCV infection, were a series of glycoproteins of 35 to 24 kDa (gp35-24) in insect cells and a single species of glycoprotein of 35 kDa (gp35) in monkey cells. The size difference of these proteins was due to the different degrees of glycosylation. The envelope proteins expressed in these cells were produced by common specific cleavage from the precursor protein, and cleavage positions of the envelope protein were mapped at about amino acids 190 and 380. The gp35-24 proteins expressed in insect cells were used for detection of antibody against HCV envelope protein in patient sera. The results showed that (i) the antibody is detected in 2 to 17% of various patients with hepatitis C, (ii) three patients were apparently cured after acquiring the antienvelope antibody, and (iii) in sera of patients with more than a 20-year history of infection, the antibody sometimes coexisted with HCV. These results suggest that the antienvelope antibody is neutralizing only in limited number of patients with hepatitis C.

Hepatitis C virus (HCV) is a major cause of posttransfusion and sporadic non-A, non-B hepatitis (NANBH), affecting 1 million patients per year worldwide (1, 3, 12). HCV has a positive-strand RNA genome of 9.4 kb which encodes a large polyprotein of about 3,000 amino acids (aa) (4, 10, 20). Hydropathy analysis of the deduced amino acid sequences of the polyprotein suggested that HCV is distantly related to flaviviruses or pestiviruses (15, 24), although the putative HCV structural region is significantly shorter than those of these viruses. The 5′-terminal region of about nucleotides (nt) 330 to 1500 is proposed as the structural region of HCV which presumably encodes nucleocapsid (core) protein and envelope protein (17, 24). A 22-kDa protein (p22) was previously identified as the HCV core protein by transient expression in monkey COS cells (7) and in insect cells (2).

The most important features of HCV are that, unlike other RNA viruses, HCV causes persistent infection for over 30 years and eventually leads to development of hepatocellular carcinoma (HCC) (16, 18). Therefore, it is important to determine how HCV can escape from the host immune system, especially from neutralization of infectivity by antibody (Ab) against the viral envelope protein. As the first step in this approach, in this study we expressed and identified HCV envelope protein in insect cells and monkey COS cells. We found that an Ab against the expressed envelope protein was detected only in a small fraction of sera from patients with hepatitis C.

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MATERIALS AND METHODS

Cells and viruses. *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant viruses were grown and assayed in confluent monolayers of *Spodoptera frugiperda* cells in medium containing 10% fetal bovine serum as described previously (14). To express HCV envelope protein in monkey cells, COS1 cells (6) were plated on 50-mm dishes 24 h before transfection. They were transfected with 10 μg of plasmid DNA by the calcium phosphate precipitation method.

Construction of expression vectors. HCV cDNA clones used for expression were derived from healthy carriers of hepatitis C in Japan (23). Ac316 (2) is a recombinant baculovirus containing the HCV cDNA 316 spanning from nt 318 to 1670 (the numbering system is according to Kato et al. [10]) under the polyhedrin promoter. cDNA 316 denotes cDNA spanning roughly from nt 300 to 1600 (Fig. 1). The cDNA fragment was inserted into the pAcYM1 transfer vector (17), and the recombinant plasmids were cotransfected into *S. frugiperda* cells with infectious AcNPV DNA as described previously (14). To prepare recombinant baculoviruses Ac816 and Ac813, most of the core gene sequence (from nt 333, just after G of the initiator ATG at an *Hgi*AI site, to nt 789 at the *Dra*III site) was replaced by a 10-nt *Not*I linker, AGCGGCCGCT, to conserve the initiation codon and the open reading frame (cDNA 816). cDNA 813 spans from the same 5′ end of cDNA 816 to nt 1349 at the *Bam*HI site. These cDNAs were inserted into pAcYM1-derived charomid vectors (19) to yield Ac816 and Ac813. These expression charomid vectors will be described elsewhere.

pSR316x contains cDNA 316 under the SRα promoter (21). This plasmid was prepared from pSR316 (7) by adding

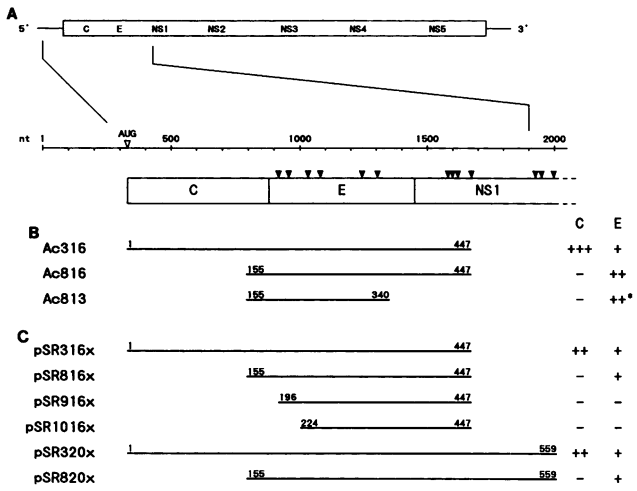

FIG. 1. Map of HCV cDNAs in expression vectors. (A) HCV genome structure. The coding frame of the polyprotein is represented by an open box, within which each protein region is shown. C, core; E, envelope; NS, nonstructural protein. (B) Map of the structural region. nt 1, 5′ end of the HCV genome; AUG, initiation codon of the polyprotein. Putative coding regions of core, envelope, and NS1 are shown as open boxes under the genome map. Filled triangle, possible N-glycosylation site. (C) Expression vectors. The name of the recombinant baculovirus or expression plasmid is shown at the left. Positions of amino acids at both ends of the cDNAs are shown. Note that the sequences around the initiation codon (318-AGACCGTGCATCATGAGC-335) are the same in all of the constructs and that an extra 5 aa derived from a *Not*I linker were expected at the amino terminus of the expressed protein except for Ac316, pSR316x, and pSR319x. The levels of expression of core (C) and envelope (E) proteins are summarized at the right. Asterisk, truncated envelope proteins were detected.

an *Xba*I linker at the 3′ end of the cDNA to terminate a read-through translation after an additional 6 aa. pSR816x contained cDNA 816. pSR916 and pSR1016 were identical in structure to pSR816 except that the *Not*I linker was ligated to nt 913 at the *Fsp*I site and to nt 997 at the *Sph*I site, respectively. To construct pSR320x and pSR820x, cDNA clone pS1-1520 (nt 1602 to 2008) was newly isolated from healthy carrier J1 (25). pSR320x and pSR820x are identical to pSR316x and pSR816x, respectively, except that the 3′ parts of their cDNAs (from nt 1620 at the *Hin*fI site to nt 2008) were derived from pS1-1520.

Protein and immunofluorescence analyses. *S. frugiperda* cells were infected with each recombinant virus or wild-type AcNPV at a multiplicity of 5 PFU per cell in 35-mm tissue culture dishes and incubated at 26.5°C for 24 h. Each dish was then labeled with 20 μCi of [^{35}S]methionine (ICN, Irvine, Calif.) for 4 h, and the cells were lysed and immunoprecipitated with patient serum KK. Western immunoblotting analysis was carried out as described previously (13). In some experiments, cell lysates obtained 48 h after transfection were analyzed by Western blot followed by biotin-avidin detection (7).

For immunofluorescence analyses, *S. frugiperda* cells were infected with recombinant and wild-type baculoviruses at a multiplicity of 5 PFU per cell and incubated for 36 h. Infected cells were stained with Abs with or without fixation (13).

Detection of HCV RNA. HCV RNA present in patient sera was detected by cDNA synthesis with reverse transcriptase followed by nested polymerase chain reaction (RT-PCR), using primers within the HCV core gene (22, 27).

RESULTS

Expression of the HCV envelope protein in insect cells. To express the HCV envelope protein in cultured cells, we first prepared recombinant baculovirus Ac816 (Fig. 1B), which contained HCV cDNA (aa 155 to 447) covering the entire region of the predicted envelope gene spanning from about aa 190 to 380 (24) under the control of the polyhedrin promoter. The protein expressed in infected *S. frugiperda* cells was then examined by Western blotting using more than 100 sera obtained from patients with NANBH. Typical examples were shown in Fig. 2A together with the results of another recombinant baculovirus, Ac316 (2), which contained both core and envelope genes and was previously shown to express HCV core protein p22. Most of the patient sera used (such as serum YY) did not detect any specific protein band in the Ac816-infected cells (Fig. 2A, lane 5), whereas they detected major proteins of 22 kDa (p22) and 52 to 40 kDa (uncleaved precursor) in the Ac316-infected cells (lane 4) (2). However, some sera, such as serum AF, did detect a series of bands of about 35 to 24 kDa in the Ac816-infected cells (lane 2).

These results showed that (i) proteins of 35 to 24 kDa are HCV envelope protein expressed by the baculovirus expression system and (ii) most of the patient sera contain antibody against HCV core protein p22 (anti-C Ab) but do not contain antibody against HCV envelope protein (anti-E Ab). The multiple bands of the expressed envelope protein can be explained by the incomplete glycosylation in this expression system (see below). Serum AF detected not only core-related protein bands of 22 kDa and 52 to 40 kDa but also faint bands of the 35 to 24 kDa in the Ac316-infected cell lysate (Fig. 2A, lane 1). The result suggests that Ac316 also expressed envelope proteins identical to those expressed by Ac816, although in lesser amount. The results also suggested that serum AF contained both anti-C Ab and anti-E Ab.

The putative envelope protein consists of about 190 aa (from aa 190 to 380) with six possible N-glycosylation sites (24). To confirm the predicted carboxy-terminal position of the envelope coding region, we prepared a third recombinant baculovirus, Ac813, which lacked a carboxy-terminal 40-aa portion of the envelope coding region (up to aa 340). With serum KK, which also contains both anti-E Ab and anti-C Ab, the Ac813-infected cell lysate yielded a series of bands, which appeared about 5 kDa shorter than the bands of 35 to 24 kDa detected in Ac816-infected cells (Fig. 2B, lanes 2 and 3). The results agreed with the predicted carboxy-terminal position of the envelope protein.

Expression of HCV envelope protein in mammalian cells. To examine whether the HCV envelope protein expressed in mammalian cells is different from that expressed in insect cells, we constructed two expression plasmids, pSR320x and pSR816x (Fig. 1). These plasmids were derivatives of pSR316 (7), in which HCV proteins were expressed under the control of the SRα promoter (21). Each plasmid was transfected to monkey COS cells, and the cell lysates were analyzed by Western blotting using serum KK (Fig. 3A). Unlike Ac816-infected insect cells, both pSR320x- and pSR816x-transfected monkey cells yielded a specific 35-kDa band corresponding to the envelope protein. These results strongly suggest that HCV envelope protein expressed in monkey cells is a single species of 35 kDa. These results also suggest that the envelope protein of 35 kDa was produced by

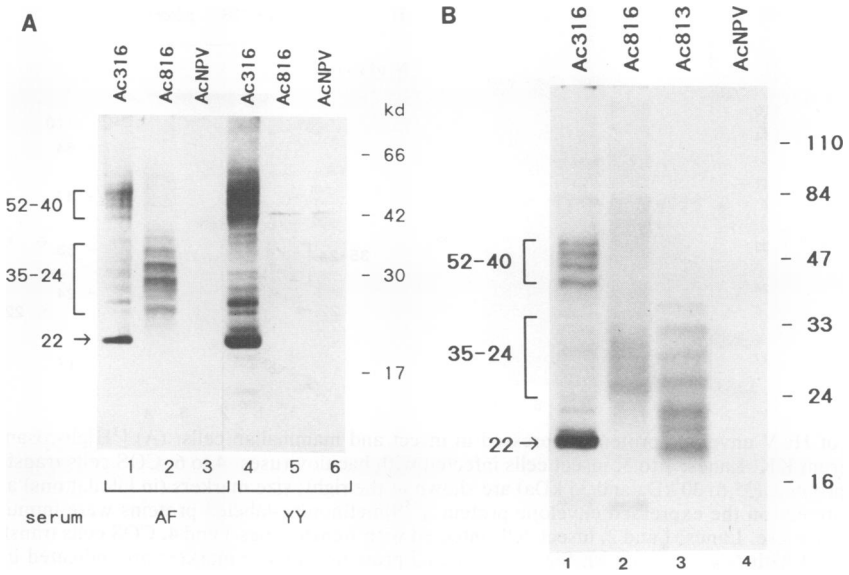

FIG. 2. Western blotting analysis of the HCV envelope proteins expressed in insect cells. At the left are shown sizes of the detected proteins (22 kDa, core protein [p22]; 35 to 24 kDa, envelope proteins; 52 to 40 kDa, uncleaved precursor proteins). Positions of size markers (in kilodaltons) are shown at the right. (A) Typical results obtained by using patient sera. HCV proteins were detected by the biotin-avidin method. (B) HCV proteins expressed by various recombinant baculoviruses. Serum KK (see Fig. 3A) was used. Proteins were detected by diaminobenzidine.

specific cleavages at both amino- and carboxy-terminal positions located within pSR816x, because apparently identical envelope protein was produced by using plasmid pSR320x, which juxtaposes an additional 500 or 400 nt at either end.

To confirm the predicted cleavage sites of HCV envelope protein, we prepared a series of expression plasmids (Fig. 1C). The results of the Western blotting analyses are shown in Fig. 3B. The 35-kDa protein was detected in cells transfected with all of the plasmids sharing the cDNA 816 region (lane 1 to 4). However, cells transfected with pSR916x or pSR1016x (starting from aa 196 and 224, respectively) did not yield the protein of 35 kDa (lanes 5 and 6). These results are consistent with the predicted map of the HCV envelope coding region (24).

HCV envelope proteins were glycosylated. To examine whether the expressed HCV envelope proteins were glycoproteins, we labeled proteins with [^{3}H]glucosamine, and the envelope protein was detected by immunoprecipitation with serum KK (Fig. 4A). The broad bands of 35 to 30 kDa were detected when Ac316 and Ac816 was expressed in insect cells (lanes 2 and 3, respectively). The results suggested that at least a higher-molecular-weight part of the expressed envelope proteins observed in Western blotting analysis (gp35-24) was glycosylated. A faint band of about 35 kDa was detected in COS cells transfected with either pSR316x

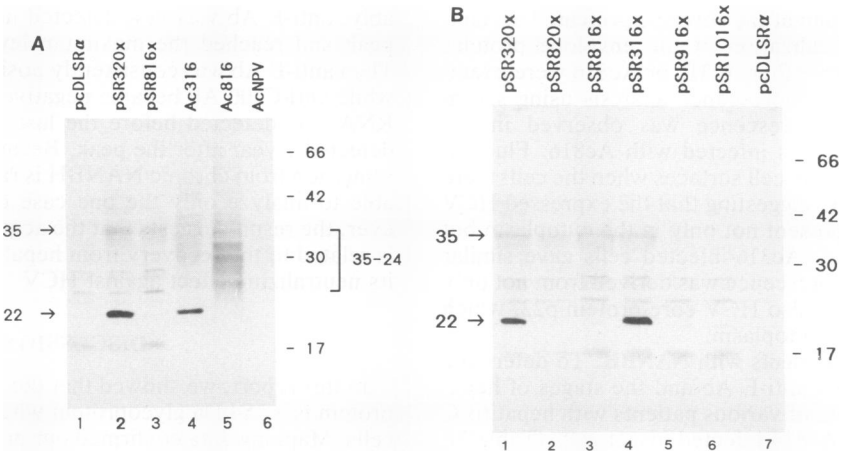

FIG. 3. Western blotting analysis of the HCV envelope protein expressed in mammalian cells. Proteins were detected by the biotin-avidin method using serum KK. (A) Expression of HCV proteins in mammalian cells (lanes 1 to 3) or in insect cells (lanes 4 to 6). Sizes of the detected proteins are shown (35 kDa and 35 to 24 kDa, envelope proteins; 22 kDa, core protein). Size markers (indicated in kilodaltons) are shown at the right. (B) HCV proteins expressed by a series of expression plasmids. Sizes of the detected specific proteins (left) and positions of size markers (right) are indicated in kilodaltons.

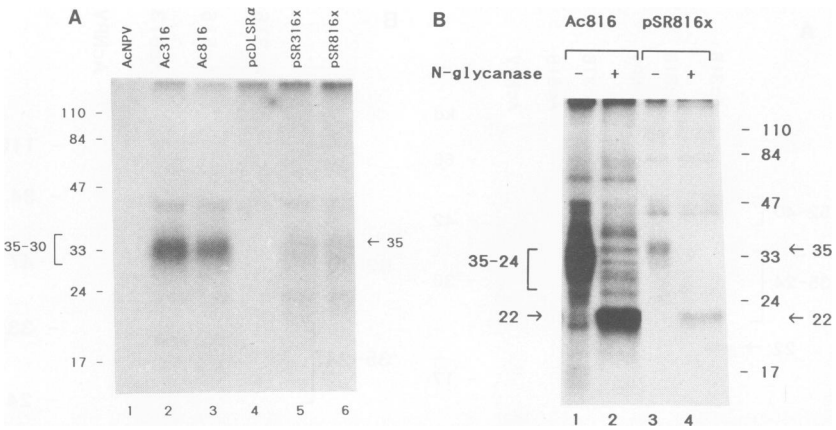

FIG. 4. Glycosylation of HCV envelope proteins expressed in insect and mammalian cells. (A) [^{3}H]glucosamine-labeled proteins were immunoprecipitated by serum KK. Lanes: 1 to 3, insect cells infected with baculoviruses, 4 to 6, COS cells transfected with plasmids. Sizes of the detected envelope proteins (35 to 30 kDa and 35 kDa) are shown at the right; size markers (in kilodaltons) are indicated at the left. (B) Effect of *N*-glycanase treatment on the expressed envelope protein. [^{35}S]methionine-labeled proteins were immunoprecipitated with serum KK and digested with *N*-glycanase. Lanes: 1 and 2, insect cells infected with Ac816; lanes 3 and 4, COS cells transfected with pSR816x. Cells were untreated (−) or treated with *N*-glycanase (+). Sizes of detected proteins and size markers are indicated in kilodaltons.

or pSR816x, suggesting that the envelope protein expressed in monkey cells was also glycosylated.

To confirm the results described above, we labeled proteins with [^{35}S]methionine, immunoprecipitated the HCV envelope protein with serum KK, and then digested the immunoprecipitated proteins with *N*-glycanase (Fig. 4B). The envelope proteins were detected as proteins of 35 to 24 kDa from Ac816 (lane 1) and of 35 kDa from pSR816 (lane 3), confirming the result of Western blotting presented above. After *N*-glycanase digestion, the sizes of these proteins apparently shifted to 22 kDa (lanes 2 and 4). The result show that the size of HCV envelope protein without glycosylation is 22 kDa and that the size difference of the envelope proteins expressed in insect and monkey cells was due to the different degrees of glycosylation. Because neither Ac816 nor pSR816x can express the HCV core protein p22, the 22-kDa protein detected here is different from the core protein. These results agreed with our previous prediction that both HCV core and HCV envelope proteins are about 190 aa long (24).

Intracellular distribution of the envelope protein. To examine the intracellular localization of the envelope protein, insect cells infected with either Ac316 or Ac816 were examined by indirect immunofluorescence analysis using serum KK (Fig. 5). Strong fluorescence was observed in the cytoplasm of the fixed cells infected with Ac816. Fluorescence was also observed on cell surfaces when the cells were stained without fixation, suggesting that the expressed HCV envelope protein was present not only in the cytoplasm but also on the cell surface. Ac316-infected cells gave similar results, although the fluorescence was derived from not only the envelope protein but also HCV core protein p22, which is located mainly in the cytoplasm.

Anti-E Ab in sera of patients with NANBH. To determine the relationship between anti-E Ab and the stages of hepatitis C, we examined sera of various patients with hepatitis C by Western blot using Ac816-infected insect cells (Table 1). The anti-E Ab was detected only in a limited number of patient sera (2 to 17%), though all of the patient sera shown in Table 1 were positive for the Ab against C100, a molecularly expressed protein of the junction region of NS3 and NS4 produced in yeast cells (12, 16), for the HCV anti-C Ab (2, 7), or for both. Interestingly, two patients who acquired the anti-E Ab at the acute phase of the disease belonged to apparently cured group; their alanine aminotransferase (ALT) levels became normal after 3 and 5 months, respectively, and HCV RNA became undetectable thereafter (Table 2). Another group of patients who developed the anti-E Ab included a small number of those with either chronic hepatitis (CH), liver cirrhosis (LC) or HCC, whose ALT levels were well controlled but still above the normal level (Table 1). We examined the presence of HCV by RT-PCR in the sera of two types of patients with LC. The percentage of sampled anti-E-positive patients in whom HCV RNA was detected was equal to that of the sampled anti-E-negative patients (Table 2), suggesting that in these patients the appearance of anti-E Ab is not associated with the elimination of HCV. To examine whether the appearance of anti-E Ab is associated with the curing of hepatitis C, we retrospectively analyzed a series of sera from a patient who had suffered active chronic hepatitis C for 13 months and then spontaneously recovered from the disease (Fig. 6). Remarkably, anti-E Ab was first detected just before the last ALT peak and reached the maximum level just after the peak. Then anti-E Ab was consistently positive for at least 2 years, while anti-C100 Ab became negative after 20 months. HCV RNA was detected before the last ALT peak but was not detected 1 year after the peak. Because such a spontaneous remission from chronic NANBH is rare, so far we have been able to analyze only the one case described above. However, the result suggests that the acquisition of the anti-E Ab is related to the recovery from hepatitis C, possibly through its neutralizing effect against HCV.

DISCUSSION

In this report, we showed that the putative HCV envelope protein is a 35-kDa glycoprotein when expressed in monkey cells. Mapping data confirmed our previous prediction based on sequencing analysis that the envelope protein is about 190 aa long with five or six possible N-glycosylation sites (24). However, the precise cleavage sites of the envelope protein have yet to be determined. Recently, Hijikata et al. (8) reported a similar 35-kDa glycoprotein by in vitro translation

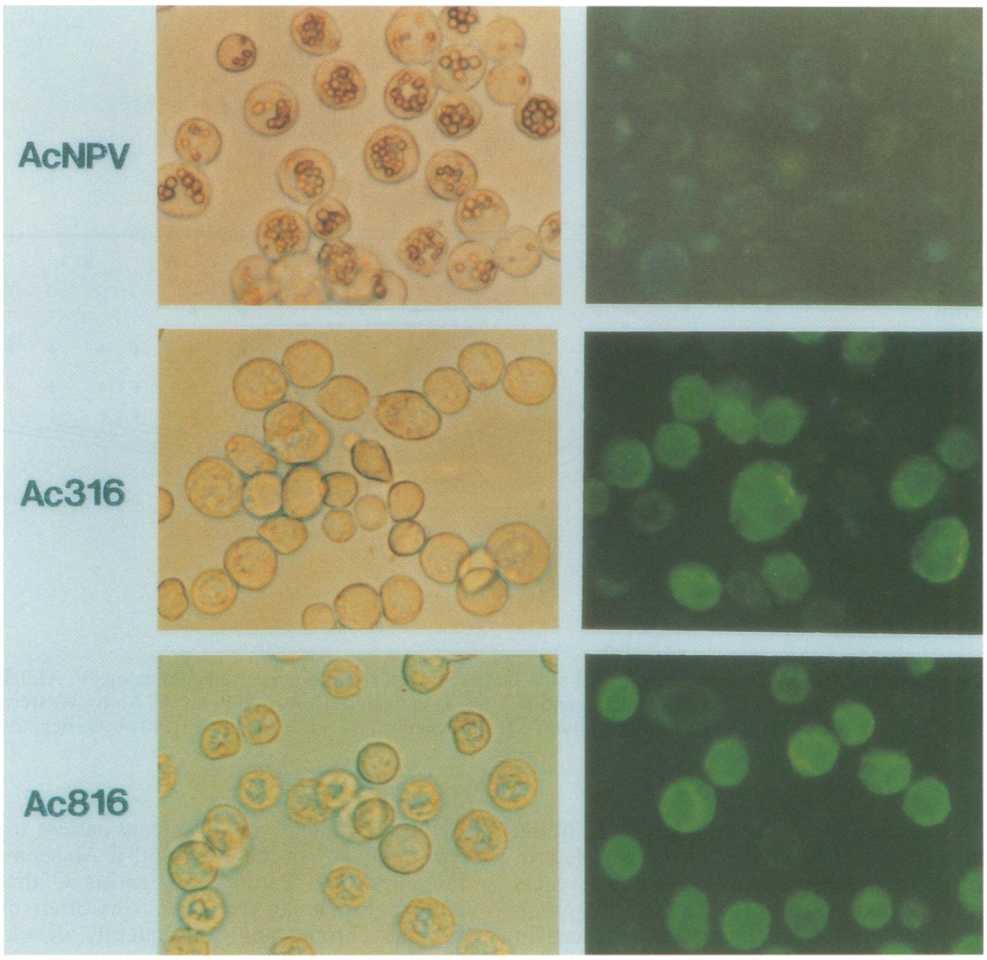

FIG. 5. Immunofluorescence analysis of infected cells. Insect cells infected with recombinant or wild-type AcNPV were examined by indirect immunofluorescence analysis using serum KK without fixation. Phase-contrast (left) and surface fluorescence (right) of the infected cells are shown.

studies, showing that the cleavage positions of the protein are consistent with those of our gp35 expressed in monkey cells.

Three cases were found in which the anti-E Ab appeared to work as a neutralizing antibody against HCV. Although only 2 sera among 14 collected from patients with acute NANBH were positive for the anti-E Ab, both were from patients completely recovered from hepatitis C after several months. In both cases, the anti-E Ab was detected when the first ALT elevation was observed, and it lasted even after the ALT level returned to normal (9). During the time course of one cured patient, the appearance of anti-E Ab preceded the recovery from active chronic hepatitis C. In each of these three cases, HCV RNA became undetectable by the RT-PCR method. While these observations are consistent with the notion that the anti-E Ab is a neutralizing Ab that can eliminate HCV, the anti-E Ab detected by the assay used in this present study is not necessarily essential for recovery; the Ab was detected in the sera of only some of the patients whose disease did not become chronic. One possible expla-

TABLE 1. HCV anti-E Ab in sera of patients with hepatitis C

Condition	No. of patients examined	No. (%) of cases detected
Acute hepatitis[a,b]	14	2 (14.2)
CH[a]	86	2 (2.3)
LC[a]	103	18 (17.5)
HCC[a]	107	9 (8.4)
Normal[c]	50	0 (0.0)

[a] Either anti-C100 Ab or anti-C Ab is positive.
[b] Typically monitored for 14 weeks, with five sera examined serially for anti-C100, anti-C, and anti-E Abs.
[c] Anti-C100 Ab-negative healthy adults.

TABLE 2. Detection of HCV RNA in NANBH patient sera

Patients	No. tested	No. (%) in whom HCV RNA was detected
Anti-E Ab-positive cured		
Active hepatitis stage	3[a]	3
Cured stage	3[a]	0
With LC		
Anti-E Ab positive	7	6 (86)
Anti-E Ab negative	7	6 (86)

[a] The same patients for whom data are shown in Table 1 (acute hepatitis [two cases]) and in Fig. 6 (one case).

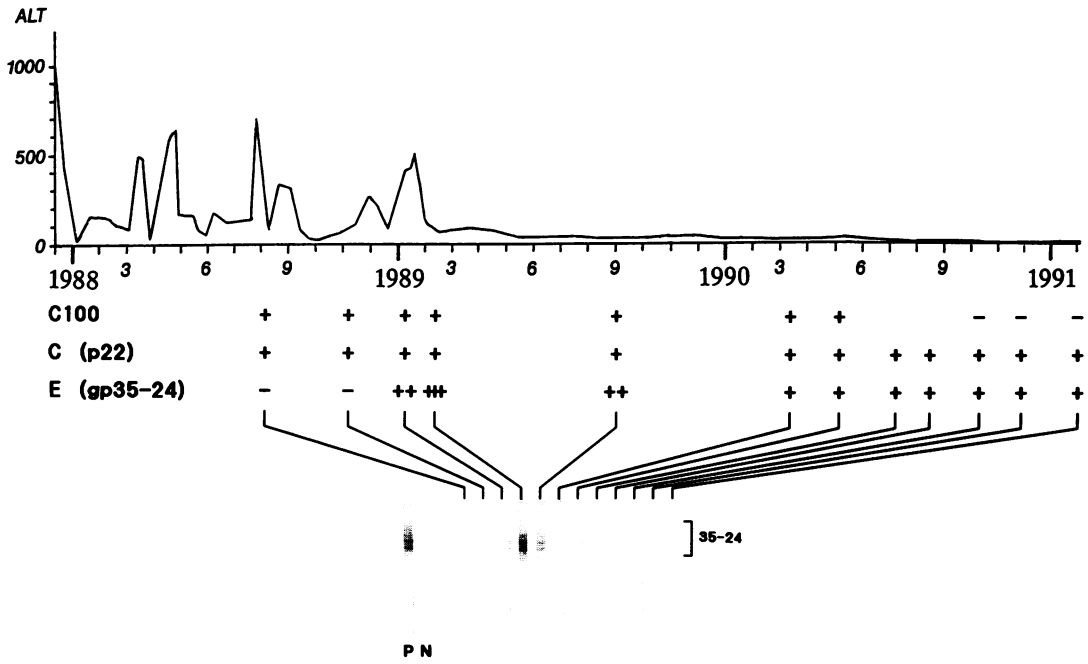

FIG. 6. Detection of anti-E Ab in a patient with NANBH who had recovered from the disease spontaneously. ALT levels are shown in Karmen units. Presence (+) or absence (−) of anti-C100 and anti-p22 Abs is indicated. Detection of anti-E Ab by Western blotting is shown, and relative intensities of reaction are expressed from negative (−) to maximum (+++). P, positive control; N, negative control.

nation is that in this study we examined the presence of the anti-E Ab by immunoblotting after denaturing gel electrophoresis. This procedure may not detect an Ab which reacts only to the native envelope protein. To examine this possibility, we are preparing an enzyme-linked immunosorbent assay system to detect the anti-E Ab reacting to a native form of envelope protein.

Anti-E Ab-positive patients whose ALT levels were well controlled but above normal were also found among those with CH, LC, and HCC. They seem to belong to a group distinct from the patients who recovered after the acute phase of the disease because (i) each of these patients had suffered from chronic hepatitis C for over 20 years and (ii) although their ALT levels seemed to be well controlled, their hepatitis states still continued and anti-E Ab coexisted with HCV in their sera. If the anti-E Ab is indeed a neutralizing antibody, one possible explanation is that the HCV present in these patients has already mutated and been selected to escape from the immune surveillance of the Ab, as in the case of human immunodeficiency virus.

We also showed in this study that the prevalence of the HCV anti-E Ab was apparently low, having been detected in only about 2 to 17% of patients with NANBH. In contrast, the anti-C Ab was detected far more often in sera of patients with hepatitis C (89% of patients with chronic NANBH [7] and 83% of patients with non-B HCC [26]). Two interpretations are possible. First, it may be much more difficult to elicit anti-E Ab in HCV infection than in infections with other viruses. The HCV envelope protein is mostly hydrophobic and much shorter than that of flaviviruses, suggesting that the immunogenicity of the HCV envelope protein is much weaker than that of the proteins of these viruses. Second, our expressed HCV envelope protein may fail to detect a significant fraction of anti-E Abs in patient sera, because amino acid sequences of the envelope protein differ by no less than about 10% from patient to patient (5). This low frequency of the HCV anti-E Ab seems to be related to the important feature of hepatitis C that, without cure, patients with acute hepatitis C very often (over 50% of cases) become chronic and subsequently develop fatal LC and HCC after over 30 years.

These results shown here were not sufficient to determine whether the anti-E Ab is a major neutralizing Ab against HCV. More extensive surveys in patients as well as protection experiments in chimpanzees are necessary. Recently, we showed that a recombinant vaccinia virus bearing the HCV envelope gene was able to raise an Ab against the envelope protein gp35-24 (11). Protection experiments of chimpanzees immunized by the recombinant vaccinia virus will be able to clarify this potentially important issue for the future development of HCV vaccines.

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