

## Humoral Immune Response to Hypervariable Region 1 of the Putative Envelope Glycoprotein (gp70) of Hepatitis C Virus

NOBUYUKI KATO,<sup>1</sup> HITOMI SEKIYA,<sup>1</sup> YUKO OOTSUYAMA,<sup>1</sup> TAKAHIDE NAKAZAWA,<sup>1</sup>  
MAKOTO HIJIKATA,<sup>1</sup> SHOWGO OHKOSHI,<sup>2</sup> AND KUNITADA SHIMOTOHNO<sup>1\*</sup>

*Virology Division, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104,<sup>1</sup>  
and Third Department of Internal Medicine, School of Medicine, Niigata University,  
1-757 Asahimachi, Niigata City 951,<sup>2</sup> Japan*

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We recently found that alterations of amino acids in hypervariable region 1 (HVR1) of the putative envelope glycoprotein (gp70) of hepatitis C virus (HCV) occurred sequentially in the chronic phase of hepatitis at intervals of several months. This finding suggests that mutations in HVR1 are involved in the mechanism of persistent chronic HCV infection involving escape from the immunosurveillance system. To explore this possibility, we examined the humoral immune response to HVR1 with our assay system, in which immunoprecipitation was carried out with sera from patients by using an HVR1 (27-amino-acid) dihydrofolate reductase fusion protein synthesized by *in vitro* transcription and translation. Results showed that HVR1 contains a sequence-specific immunological epitope that induces the production of antibodies restricted to the specific viral isolate. Furthermore, analysis of the kinetics of the appearance of antibodies in two patients with chronic hepatitis, with whom successive alterations of amino acids of HVR1 have been observed, showed that the titers of anti-HVR1 antibodies usually reached maximal levels several months after the isolation of HCV having the specific sequence of HVR1. This observation suggests that anti-HVR1 antibodies are involved in the genetic drift of HVR1 (minor antigenic variation) by immunoselection. However, the coexistence of HVR1 as an antigen and its specific antibody was sometimes observed. The possibility that HVR1 acts as a neutralizing epitope is discussed.

Hepatitis C virus (HCV) is the major etiological agent of posttransfusional non-A, non-B hepatitis throughout the world (5, 27) and is also considered to be a causative agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma in Japan (33, 35, 42). However, neither the mechanism(s) by which HCV causes these hepatic diseases nor even the pathogenic effect of HCV is yet clear.

HCV has been proposed to belong to a new genus of the *Flaviviridae* distinct from flaviviruses and pestiviruses (16, 31). The HCV genome is a positive-stranded RNA molecule of about 9.5 kb and encodes a large polypeptide precursor of about 3,000 amino acids. Comprehensive structural analysis of HCV genomes revealed the existence of at least six different genotypes (HCV-I to HCV-VI) in the world (1-3, 7, 14, 20, 21, 26, 32, 47). The nucleotide sequences of the entire HCV genomes from HCV-I to HCV-IV have been determined (4, 6, 15, 19, 36-38, 44, 46). The genotypes show differences of about 20 to 35% in the nucleotide sequences of their virus genomes, and different isolates belonging to the same genotype showed 5 to 8% differences in nucleotide sequences and 4 to 5% differences in amino acid sequences (17, 19, 36, 46). These sequence diversities are distributed along the whole genome except for the conserved 5' non-coding region. In particular, the putative envelope regions encoding gp35 and gp70, which have been identified in an *in vitro* translation study (11), show especially marked sequence diversity (10, 46).

By comparison of the amino acid sequences of many HCV isolates (HCV-I and -II genotypes), we identified two hypervariable regions (HVR1 [27 amino acids] and HVR2 [7 amino acids]) in the N-terminal region of gp70 of the HCV-II

genotype (10, 23), which is the major genotype in Japan (21). HVR1 (21 amino acids) in the same region as in the HCV-II genotype is also present in the HCV-I genotype (23, 49). The amino acid sequence of HVR1 has been found to change during periods of 2 to 13 years (34, 36, 50). However, these findings probably do not reflect the actual rate of variability in HVRs, because we recently observed that the rate of variability (0.5 to 1.7 amino acids per month) in HVR1 was much higher than the values reported previously (0.06 to 0.32 amino acid per month) (34, 36, 50) during periods of several months in the chronic phases of patients with hepatitis (22). In addition, alterations of amino acids in HVR1 mostly occurred in a sequential fashion (22). These observations suggest that mutations in HVR1, which would result in escape from the immunosurveillance system, are involved in the mechanism of persistent chronic HCV infection. To examine this possibility, we developed a new system for detection of anti-HVR1 antibodies and analyzed HVR1s obtained from patients with chronic hepatitis. We report here that HVR1 contains an immunological epitope that is specific for the homologous virus isolate, and that, in some cases, an HCV with mutated HVR1 could escape recognition by preexisting anti-HVR1 antibodies.

### MATERIALS AND METHODS

**Materials.** Oligonucleotide primers for polymerase chain reaction (PCR) were synthesized in an Applied Biosystems model 380A apparatus. *Thermus aquaticus* DNA polymerase (*Taq* polymerase) was from Perkin-Elmer Cetus (Norwalk, Conn.). An *in vitro* RNA synthesis kit with T7 RNA polymerase was obtained from Nippon Gene Co. (Toyama, Japan). A rabbit reticulocyte lysate was from Promega Corporation (Madison, Wis.). The DNA ligation kit

\* Corresponding author.

and restriction enzymes were from Takara Shuzo (Kyoto, Japan). Protein G-Sepharose was from Zymed Laboratories (South San Francisco, Calif.). [<sup>35</sup>S]methionine was from Amersham (Amersham, United Kingdom).

**Patients.** Patient I was a 22-year-old woman diagnosed with acute non-A, non-B hepatitis without a history of blood transfusion on her first visit to the hospital of Niigata University (Niigata, Japan) in March 1991. The disease developed to the chronic stage, and serum samples were collected until 15 months after the onset of disease. Antibody against C100-3, a viral nonstructural protein, was detectable with an anti-HCV kit (Ortho Diagnostic Systems) in May 1991. Patient II was a 28-year-old man with chronic non-A, non-B hepatitis without a history of blood transfusion. He visited the hospital of Niigata University in March 1988 and received interferon therapy (total of 84 MU of beta interferon) for 2 months starting in April 1988. However, the disease was not cured and has remained in the chronic stage. Serum samples were collected until 48 months after the patient's first visit to the hospital. Antibody against C100-3 was detectable in March 1988.

**Construction of expression plasmids.** An expression plasmid, pTZ19RSVdhfr1, was constructed by insertion of the *Bam*HI-*Hind*III fragment of pTZSV2dhfr1, containing the dihydrofolate reductase (DHFR) gene derived from *Escherichia coli* (39), into the *Bam*HI-*Hind*III site of the pTZ19R

vector, which contains the T7 phage promoter just upstream of the multicloning site. DNA fragments containing only HVR1 were obtained by PCR with plasmid DNAs containing various HVR1s derived from patients I and II. Patient-derived HVR1 plasmids were obtained from the products of RT-nested PCR (cDNA synthesis and PCR amplification) with RNA from a patient's serum as described previously (22). The sequences of primers for PCR were selected from the nucleotide sequence data in our previous report (22). The sense primer contained a *Hind*III recognition site plus a GCCACCATGG sequence, a consensus sequence for translation (25), in addition to the nucleotide sequence (20-mer) of HVR1. The reverse primer also contained a *Bam*HI recognition site in addition to the nucleotide sequence (20-mer) of HVR1. The PCR was carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 25 cycles with 3 U of *Taq* polymerase, using about 10 ng of isolated plasmid DNA containing HVR1. The reaction cycle was as follows: primer annealing at 55°C for 45 s, primer extension at 72°C for 2 min, and denaturation at 94°C for 1 min. PCR products (expected size, 96 bp) were digested with *Hind*III and *Bam*HI and isolated as described previously (21). The isolated DNA fragment containing HVR1 was cloned into the *Hind*III-*Bcl*I site of the pTZ19RSVdhfr1 vector. The nucleotide sequence of HVR1 of a clone was confirmed by the dideoxy-nucleotide chain

		384	410	Months
I-1		Q T R T V G G Q V G H S V R G F T S L F S A G S A Q N		0
I-2		- - - - -		2
I-3		- - - - - L - - - - -		5.5
*I-4-1		- - - - - M - - G - - L - - - - -		
I-4-3		- - H - - - - M - - G - - L - - - - -		8
I-5		- - H - - - - H - - G - - L - N - - - - -		11
		384	410	Months
II-1		S T R V T G G Q Q G R A V H G I A S L F S L G A S Q K		0
II-2		- - - - - H - A - S L T - - - R - - - - - N		5
II-3-1		N - - - - - R - - - - A - S L T - - - P - - - - N		8
*II-3-5		- - H - - - A L - - - - A Y - - T - F L - H - P - - -		
II-4-3		- - Q - M - - - - - A Y S L - - - L - P - - - - -		14

FIG. 1. Sequence variabilities of HVR1 of HCV obtained from patients with chronic hepatitis. (A) Deduced amino acid sequences of HVR1 (positions 384 to 410) from patient I are shown. Amino acid sequences are indicated by the single-letter code. The amino acid sequences of the predominant species at each time point and a minor species (indicated by an asterisk) are shown. Capital letters indicate amino acids different from those in I-1. The number of months after diagnosis is shown. (B) Deduced amino acid sequences of HVR1 from patient II. As in panel A, the amino acid sequences are those of the predominant sequence at each time point and a minor species (II-3-5). Capital letters indicate amino acids different from those in the sequence of II-1. Detailed sequence data for patients I and II are in reference 22.

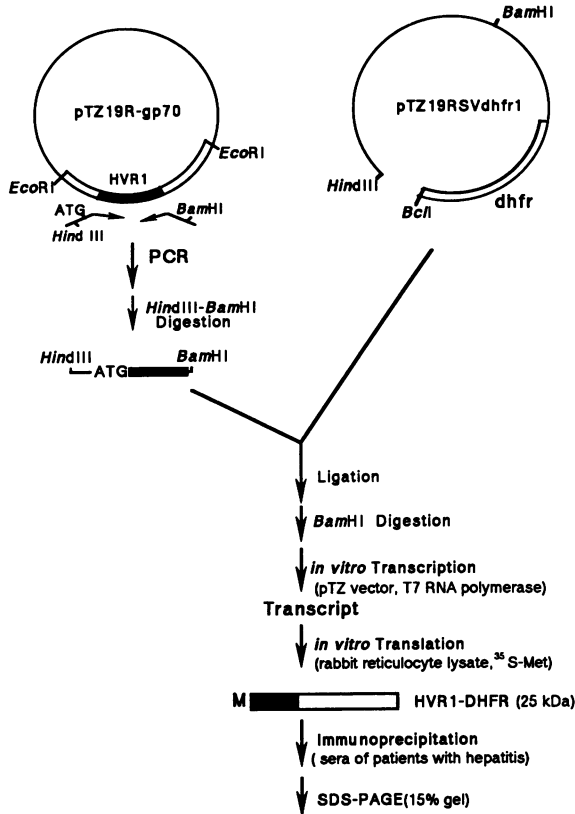


FIG. 2. Construction of expression plasmids and system for detection of specific antibodies against HVR1. Details of the construction of plasmids are described in Materials and Methods. After in vitro transcription and translation, immunoprecipitates with sera from patients containing anti-HVR1 antibodies were analyzed by SDS-PAGE.

termination method with an A.L.F. DNA sequencer (Pharmacia).

**In vitro transcription and translation.** All purified plasmids were linearized by cutting them at the unique *Bam*HI site downstream of the *dhfr* gene. They were then used as templates for in vitro transcription. RNA transcripts were synthesized in vitro with T7 RNA polymerase as described previously (18). The transcripts were translated with a rabbit reticulocyte lysate with [<sup>35</sup>S]methionine for radiolabeling.

**Immunoprecipitation.** Immunoprecipitation was carried out by a standard procedure (43) with slight modification. Briefly, a reticulocyte lysate containing an in vitro-translated HVR1-DHFR fusion protein was adjusted to a volume of 0.5 ml with NET-gel buffer (43) and mixed with 50 μl of recombinant protein G-Sepharose with gentle rocking for 4 h at 4°C. The mixture was then centrifuged for 1 min at 12,000 × g, and the supernatant was subjected to immunoprecipitation for 12 to 16 h at 4°C with sera from patients with hepatitis or serum from a healthy human volunteer at a 1:100 dilution. As a positive control, anti-DHFR rabbit antibody (39) was used at a 1:500 dilution. Protein G-Sepharose (30 μl) was added to the immune complex and incubated for 1 h at 4°C on a rocking platform. Washing of the immune complex and sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-PAGE) were carried out by standard procedures (43). Immunoprecipitates after SDS-PAGE were detected with a Bio-Imaging analyzer (BAS 2000; Fuji Photo Film Co.).

**RESULTS**

**Sequence variability of HVR1 of a putative envelope glycoprotein (gp70).** We recently analyzed the rates of sequence variability in regions including HVR1 and HVR2 of HCV isolated successively at intervals of a few months from two patients (patients I and II) with chronic C-type hepatitis by PCR. The nucleotide sequences of three to five independent cDNA clones from each time point were determined to obtain the predominant sequence of each specimen (22). We found that the amino acid sequence of HVR1 but not HVR2 from patient I showed marked sequential changes with time (22), while alterations of amino acid sequences of this region from patient II were partially sequential (22). Figure 1 shows the amino acid sequences of HVR1 of the predominant HCVs (frequency of more than 50%) obtained at each time point from patients I and II. I-4-1 and II-3-5 were, respectively, minor species first observed 8 months postdiagnosis (p.d.) of patients I and II. From these data for the two patients, we speculated that these genetic drifts in HVR1 reflect the results of pressure by immunological selection. This speculation led us to examine the humoral immune response to HVR1s in patients I and II.

**System for detection of specific antibodies against HVR1.** We established a system for detection of anti-HVR1 antibodies in sera from patients with hepatitis. This system is summarized in Fig. 2. Since HVR1 is located in the amino-terminal region of the gp70 protein, when it is fused into the amino-terminal portion of the DHFR protein, it may show

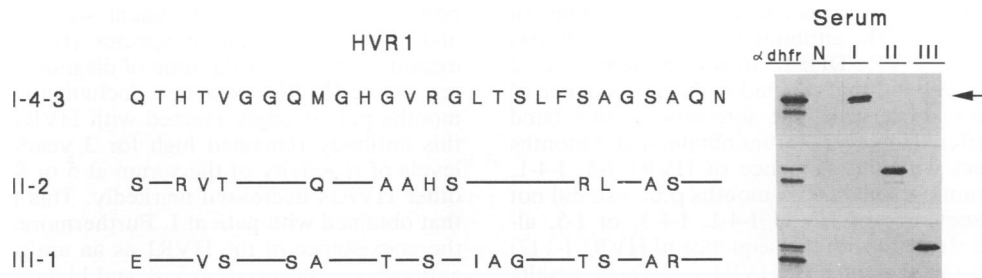


FIG. 3. HVR1 contains a sequence-specific immunological epitope which induces antibodies restricted to the homologous viral isolate. Three different HVR1 species (I-4-3, II-2, and III-1) were analyzed with sera obtained at least 6 months later from the patients from whom these HVR1s were isolated. N indicates serum from a healthy (normal) volunteer. The arrow denotes the position of the synthesized HVR1-DHFR fusion protein (25 kDa).

antigenicity similar to that in the native gp70 molecule. Details of the method are described in Materials and Methods.

**HVR1s in each virus isolate contain specific immunological epitopes.** We examined whether the immunological epitope is present on HVR1s (I-4-3, II-2, and III-1) obtained from patients I, II, and III, respectively. Patient III was a patient with acute C-type hepatitis due to HCV of the HCV-I genotype (9). As shown in Fig. 3, these HVR1s showed about 50% sequence diversity. We used sera obtained at least 6 months after the respective HVR1s were obtained. HVR1 I-4-3 was selectively precipitated as a 25-kDa protein with serum from patient I but not serum from patient II or III or a healthy volunteer. HVR1s II-2 and III-1 were also selectively precipitated only with sera from patients II and III, respectively (Fig. 3). The 25-kDa band of the immunoprecipitate was actually that of an HVR1-DHFR fusion protein, because we obtained a strong band of the same size with anti-DHFR antibody as a positive standard. The second band (20 kDa) is probably that of a product translated from an internal methionine codon of the DHFR coding region, because the sera of the patients did not react with this 20-kDa protein (Fig. 3). The above results show that HVR1 contains an immunological epitope that is specific for the homologous virus isolate.

**Titration of anti-HVR1 antibody.** The titers of HVR1-specific antibody were examined by analysis of serial twofold dilutions of serum for immunoprecipitation with the products of HVR1 II-1. As shown in Fig. 4A, the amount of immunoprecipitate decreased linearly with increases in the dilution of the serum used in this assay system and a specific immunoprecipitate could be detected even in 3,200-fold-diluted serum. The radioactivity of the immunoprecipitate was linearly proportional to the amount of serum used (Fig. 4B). Since the HVR1-DHFR fusion protein is present in excess in the reaction mixture, in this assay system the intensity of the signal depends on the titer of the antibody. Linear correlations were also found between the amounts of serum and the immunoprecipitates of the fused peptides of I-4-1 and I-5 (data not shown). Therefore, we were able to analyze the time courses of the appearance of humoral immune responses to various amino acid sequences of HVR1 obtained from patients I and II.

**Humoral immune responses to the amino acid sequences of HVR1 from patient I.** As shown in Fig. 1A, we observed sequential alterations in the amino acid sequence of HVR1 from patient I as the disease developed from acute to chronic hepatitis. Five different HVR1-DHFR fusion proteins were synthesized according to the sequence variations of HVR1 as shown in Fig. 1A, and the presence of specific antibodies against these sequences of HVR1 was examined at eight times, as shown in Fig. 5A. This figure shows the results of immunoprecipitations of the antibody in the serum from this patient with these HVR1-DHFR fusion proteins. At 2 months p.d., a faint band that reacted with the sequence of HVR1 I-1 (2) was observed. The intensity of this band increased thereafter. However, serum obtained at 2 months p.d. did not react with the sequence of HVR1 I-3, I-4-1, I-4-3, or I-5. Serum obtained at 5.5 months p.d. also did not react with the sequence of HVR1 I-4-1, I-4-3, or I-5, although it reacted strongly with the sequence of HVR1 I-1 (2) and weakly with the sequence of HVR1 I-3. These results indicate that there are a few antibodies in serum until 5.5 months p.d. that recognize the restricted amino acid sequences in HVR1 as an epitope(s). In contrast, sera obtained at 8 and 11 months p.d. reacted with all the sequences of

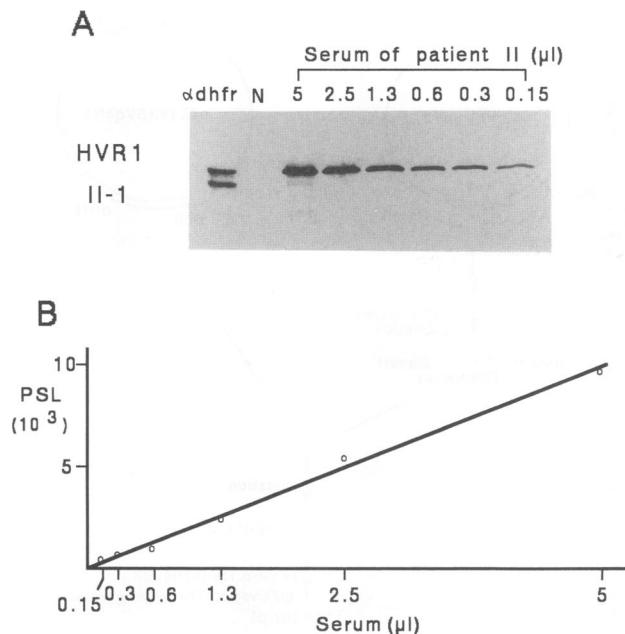
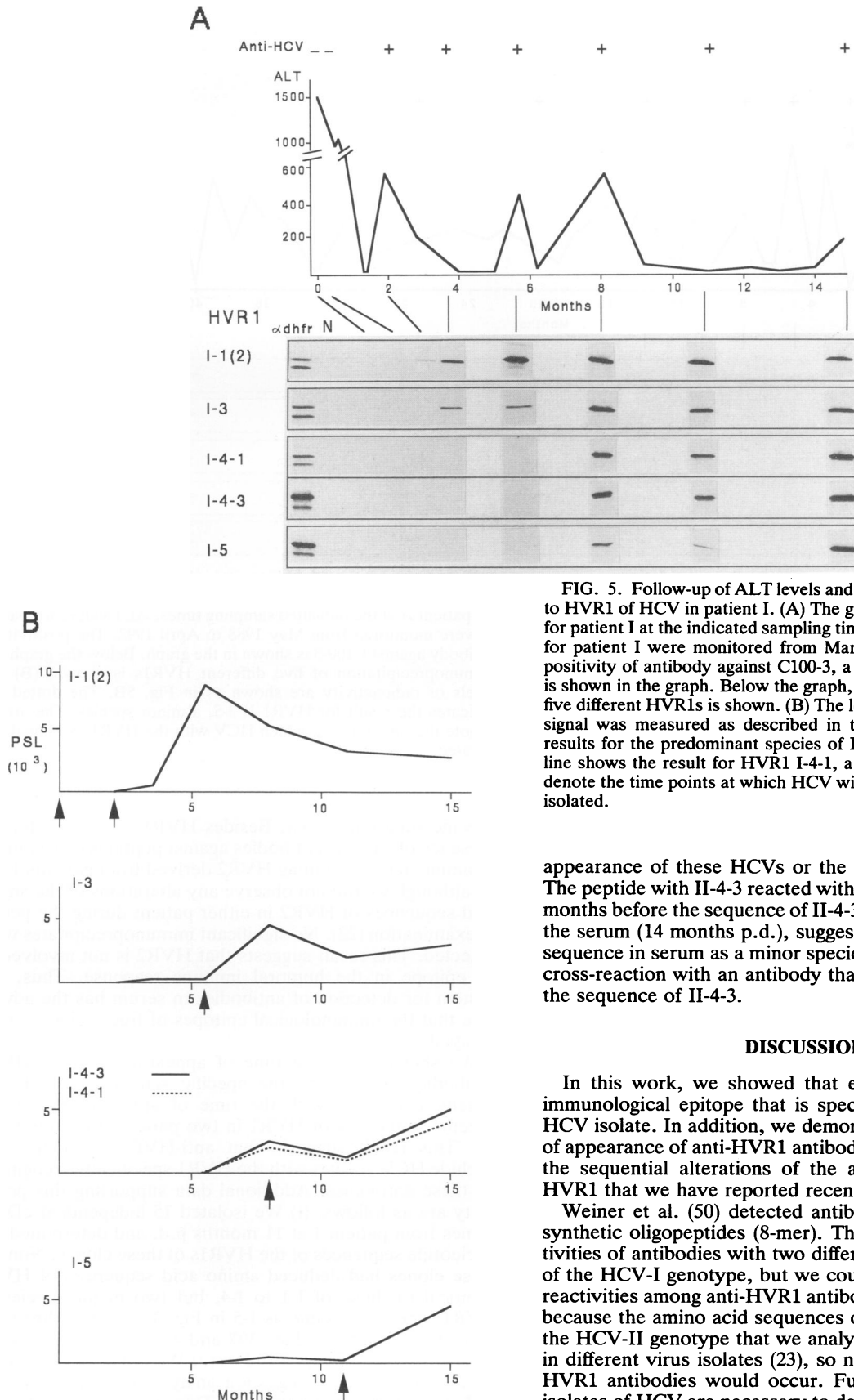


FIG. 4. Titration of anti-HVR1 antibody. (A) Twofold dilutions of serum were used for immunoprecipitation of HVR1 II-1. Serum obtained 2 years after HVR1 II-1 was obtained was used. (B) Levels of radioactivity of signals were measured with a Bio-Imaging analyzer (BAS 2000). PSL is the unit of radiation dose used in the BAS 2000.

HVR1s examined, although in a few cases their reactivities were relatively weak. These results indicate the coexistence of HCV having the specific amino acid sequence of HVR1 and antibody against its HVR1 in the serum of the patient. As the intensities of bands obtained by immunoprecipitation are thought to depend on the titers of antibodies, the radioactivities of all the bands seen in Fig. 5A were measured. Figure 5B shows the time courses of change in the relative titers of antibodies against the specific sequence of HVR1 obtained at each time point. The results clearly show that the titer of antibody against each sequence of HVR1 usually reached a maximum level several months after HCV with the specific sequence of HVR1 was isolated, although the levels of antibodies against HVR1s I-4-1 and I-4-3 were relatively high at 8 months p.d.

**Humoral immune responses to the amino acid sequences of HVR1s from patient II.** We next examined the kinetics of appearance of specific antibodies against HVR1s obtained from patient II with chronic hepatitis. The anti-HVR1 antibodies against four predominant species (II-1, II-2, II-3-1, and II-4-3) and a minor species (II-3-5) of HVR1 were examined. Serum at the time of diagnosis did not react with any of the HVR1s examined, including II-1, but serum at 2 months p.d. strongly reacted with HVR1 II-1. The titer of this antibody remained high for 3 years (Fig. 6), but the levels of reactivity of the serum at 5 or 8 months p.d. with other HVR1s decreased markedly. This result is similar to that obtained with patient I. Furthermore, as with patient I, the coexistence of the HVR1 as an antigen and its specific antibody was observed at 5, 8, and 14 months p.d. However, kinetic analysis of antibodies in the sera revealed that the titers of antibodies against specific HVR1 species increased markedly after the appearance of HCVs having these HVR1 species, although we did not determine the exact times of



**FIG. 5.** Follow-up of ALT levels and humoral immune responses to HVR1 of HCV in patient I. (A) The graph shows the ALT profile for patient I at the indicated sampling times. ALT values (units/liter) for patient I were monitored from March 1991 to June 1992. The positivity of antibody against C100-3, a viral nonstructural protein, is shown in the graph. Below the graph, the immunoprecipitation of five different HVR1s is shown. (B) The level of radioactivity of each signal was measured as described in the legend to Fig. 4B, and results for the predominant species of HVR1 are shown. A dotted line shows the result for HVR1 I-4-1, a minor species. The arrows denote the time points at which HCV with the HVR1 examined was isolated.

appearance of these HCVs or the exact amounts of virus. The peptide with II-4-3 reacted with serum obtained nearly 6 months before the sequence of II-4-3 became predominant in the serum (14 months p.d.), suggesting the presence of this sequence in serum as a minor species or the occurrence of a cross-reaction with an antibody that happened to recognize the sequence of II-4-3.

**DISCUSSION**

In this work, we showed that each HVR1 contains an immunological epitope that is specific for the homologous HCV isolate. In addition, we demonstrated that the kinetics of appearance of anti-HVR1 antibodies mainly conformed to the sequential alterations of the amino acid sequence of HVR1 that we have reported recently (22).

Weiner et al. (50) detected antibody against HVR1 with synthetic oligopeptides (8-mer). They observed cross-reactivities of antibodies with two different sequences of HVR1 of the HCV-I genotype, but we could not detect any cross-reactivities among anti-HVR1 antibodies. This was probably because the amino acid sequences of HVR1 in particular of the HCV-II genotype that we analyzed are highly divergent in different virus isolates (23), so no cross-reaction of anti-HVR1 antibodies would occur. Further analyses of many isolates of HCV are necessary to determine whether HVR1-

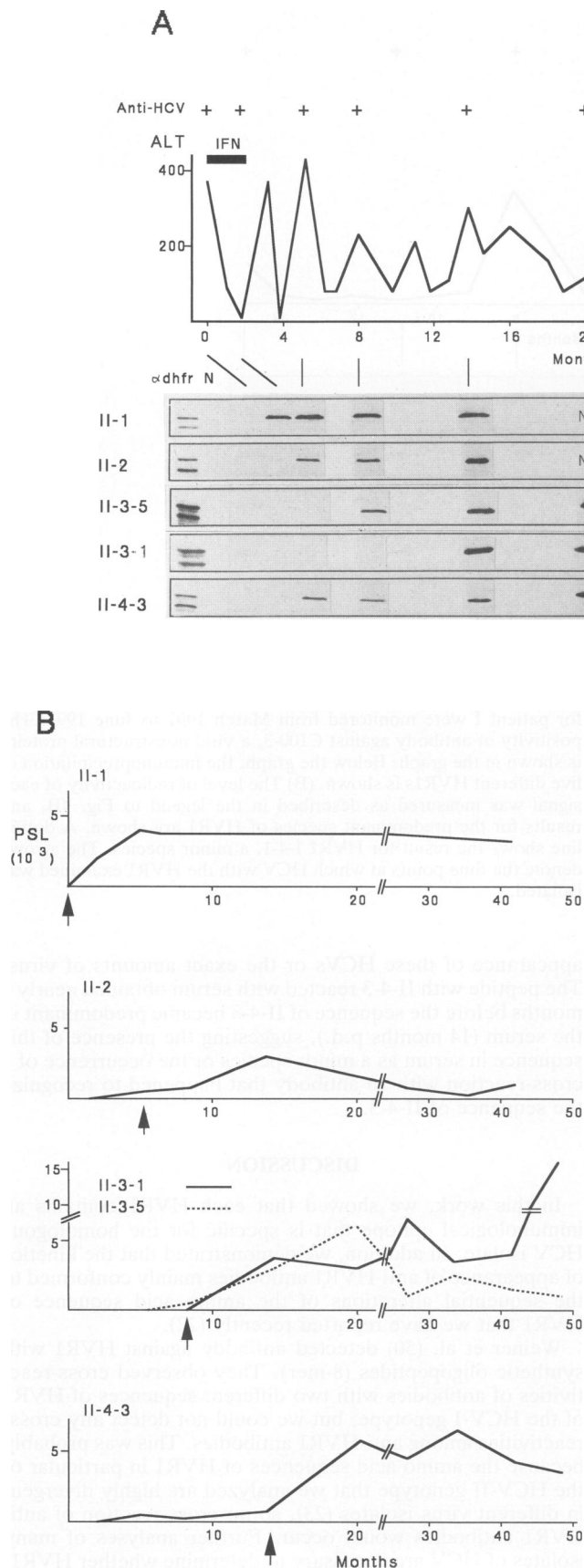


FIG. 6. Follow-up of ALT levels and humoral immune responses to HVR1 of HCV in patient II. (A) The graph shows the ALT profile for patient II at the indicated sampling times. ALT values for patient II were monitored from May 1988 to April 1992. The positivity of antibody against C100-3 is shown in the graph. Below the graph, the immunoprecipitation of five different HVR1s is shown. (B) The levels of radioactivity are shown as in Fig. 5B. The dotted line indicates the result for HVR1 II-3-5, a minor species. The arrows denote the time point at which HCV with the HVR1 examined was isolated.

specific subgroups exist. Besides HVR1, we tested for the presence of specific antibodies against peptides consisting of 17 amino acids, including HVR2 derived from patients I and II, although we did not observe any alterations of the amino acid sequences of HVR2 in either patient during the period of examination (22). No significant immunoprecipitates were detected. This result suggests that HVR2 is not involved as an epitope in the humoral immune response. Thus, our system for detection of antibodies in serum has the advantage that the immunological epitopes of free regions can be assayed.

We showed that the time of appearance of anti-HVR1 antibodies recognizing the specific sequences of HVR1s usually correlated with the time of appearance of these specific sequences of HVR1 in two patients (patients I and II). This result suggests that anti-HVR1 antibodies can exclude HCV species with the HVR1 specifically recognized by these antibodies. Additional data supporting this possibility are as follows. (i) We isolated 15 independent cDNA clones from patient I at 11 months p.d. and determined the nucleotide sequences of the HVR1s of these clones. None of these clones had deduced amino acid sequences of HVR1 identical to those of I-1 to I-4, but two major species of HVR1 (one is the same as I-5 in Fig. 1 and the other is its variant, in which residues 397 and 401 are both Ser) were obtained at this time point (24). (ii) We obtained affirmative results by restriction digestion analysis with the amplified cDNA products from patient II. This analysis also indicated

that HCV species with sequences of HVR1 different from those of previous dominant isolates became major species during the following few months (22). Therefore, we believe that sequential alteration of the amino acid sequence of HVR1 is due to the selective pressure of the immune system.

However, we cannot conclude from the present results that anti-HVR1 antibodies are general neutralizing antibodies against HCV infection, because at several times we detected the coexistence of HCV species with specific HVR1 amino acid sequences and their specific antibodies. With regard to this point, several problems must be overcome. One is how to determine the exact time of appearance of an HCV having a mutated HVR1 amino acid sequence. Another is how to monitor the amounts of individual HCV species having different HVR1 sequences. These problems are difficult because of limitations in the available samples and experimental techniques. A third problem is how to determine the exact site and number of epitopes in HVR1. We suspect, however, that the immunological epitope identified in HVR1 may be a neutralizing epitope against HCV infection for the following two reasons. First, HCV has been proposed to be a member of a new genus distinct from the pestiviruses (hog cholera virus and bovine viral diarrhea virus) and the flaviviruses (dengue virus, yellow fever virus, etc.) of the *Flaviviridae* (16, 31, 45). The hydrophathy profile of the structural region of HCV (16, 45) and the gene organization of HCV (11–13) indicate that HCV is more closely related to pestiviruses than to flaviviruses. Therefore, the two putative HCV envelope glycoproteins, gp35 and gp70, which have been identified in an *in vitro* translation study (11), probably correspond to the envelope glycoproteins gp25 and gp53, respectively, of bovine viral diarrhea virus, and gp53 of bovine viral diarrhea virus is known to induce neutralizing antibodies (48). Second, it is known that the third variable region (about 30 amino acids) of the external glycoprotein (V3 loop of gp120) of human immunodeficiency virus type 1 contains a binding site for human and experimentally induced antibodies that are able to neutralize human immunodeficiency virus type 1 infection (8, 28, 30, 40, 41) and that these antibodies against the V3 loop of gp120 show virus-specific neutralizing activities (8, 29). Since HVR1 of HCV is located at the amino terminus of the putative envelope protein gp70, shows marked sequence variation, and induces antibodies restricted to the homologous viral isolate, we think that HVR1 functions as a neutralizing epitope. To prove this idea, it will be important to develop an experimental system to examine the blocking activity of anti-HVR1 antibody in HCV infection.

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#### REFERENCES

- Bukh, J., R. H. Purcell, and R. H. Miller. 1992. Sequence analysis of the 5' noncoding region of hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **89**:4942–4946.
- Cha, T.-A., E. Beall, B. Irvine, J. Kolberg, D. Chien, G. Kuo, and M. S. Urdea. 1992. At least five related, but distinct, hepatitis C viral genotypes exist. *Proc. Natl. Acad. Sci. USA* **89**:7144–7148.
- Chan, S.-W., F. McOmish, E. C. Holmes, B. Dow, J. F. Peutherer, E. Follett, P. L. Yap, and P. Simmonds. 1992. Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. *J. Gen. Virol.* **73**:1131–1141.
- Chen, P.-J., M.-H. Lin, K.-F. Tai, P.-C. Liu, C.-J. Lin, and D.-S. Chen. 1992. The Taiwanese hepatitis C virus genome: sequence determination and mapping the 5' termini of viral genomic and antigenomic RNA. *Virology* **188**:102–113.
- Choo, Q.-L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**:359–362.
- Choo, Q.-L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, A. Medina-Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **88**:2451–2455.
- Enomoto, N., A. Takada, T. Nakao, and T. Date. 1990. There are two major types of hepatitis C virus in Japan. *Biochem. Biophys. Res. Commun.* **170**:1021–1025.
- Goudsmit, J., C. Debouck, R. H. Melen, L. Smit, M. Bakker, D. M. Asher, A. V. Wolff, C. J. Gibbs, Jr., and D. C. Gajdusek. 1988. Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. *Proc. Natl. Acad. Sci. USA* **85**:4478–4482.
- Gunji, T., N. Kato, S. Mori, Y. Ootsuyama, M. Hijikata, M. Imawari, and K. Shimotohno. 1992. Correlation between the amount of hepatitis C virus RNA in serum and the disease activities of acute and chronic hepatitis C. *Int. J. Cancer* **52**:726–730.
- Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, and K. Shimotohno. 1991. Hypervariable regions in the putative glycoprotein of hepatitis C virus. *Biochem. Biophys. Res. Commun.* **175**:220–228.
- Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, and K. Shimotohno. 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by *in vitro* processing analysis. *Proc. Natl. Acad. Sci. USA* **88**:5547–5551.
- Hijikata, M., H. Mizushima, T. Akagi, S. Mori, N. Kakiuchi, N. Kato, T. Tanaka, K. Kimura, and K. Shimotohno. Two distinct proteinase activities required in the processing of a putative nonstructural precursor protein of hepatitis C virus. Submitted for publication.
- Hijikata, M., H. Mizushima, Y. Komoda, Y. Tanji, Y. Hirowatari, T. Akagi, T. Sato, S. Mori, N. Kato, T. Tanaka, K. Kimura, and K. Shimotohno. Proteolytic processing of putative nonstructural polyproteins of hepatitis C virus. Submitted for publication.
- Houghton, M., A. Weiner, J. Han, G. Kuo, and Q.-L. Choo. 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. *Hepatology* **14**:381–388.
- Inchauspe, G., S. Zebedee, D.-H. Lee, M. Sugitani, M. Nasoff, and A. M. Prince. 1991. Genetic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. *Proc. Natl. Acad. Sci. USA* **88**:10292–10296.
- Kato, N., M. Hijikata, M. Nakagawa, Y. Ootsuyama, K. Muraishi, S. Ohkoshi, and K. Shimotohno. 1991. Molecular structure of the Japanese hepatitis C viral genome. *FEBS Lett.* **280**:325–328.
- Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, and K. Shimotohno. 1990. Sequence diversity of hepatitis C viral genomes. *Mol. Biol. Med.* **7**:495–501.
- Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, and K. Shimotohno. 1990. A structural protein encoded by the 5' region of the hepatitis C virus genome efficiently detects viral infection. *Jpn. J. Cancer Res.* **81**:1092–1094.
- Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, and K. Shimotohno. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc. Natl. Acad. Sci. USA* **87**:9524–9528.
- Kato, N., S. Ohkoshi, and K. Shimotohno. 1989. Japanese isolates of the non-A, non-B hepatitis viral genome show



- sequence variations from the original isolate in the U.S.A. Proc. Jpn. Acad. 65B:219-223.
21. Kato, N., Y. Ootsuyama, S. Ohkoshi, T. Nakazawa, S. Mori, M. Hijikata, and K. Shimotohno. 1991. Distribution of plural HCV types in Japan. Biochem. Biophys. Res. Commun. 181:279-285.
  22. Kato, N., Y. Ootsuyama, S. Ohkoshi, T. Nakazawa, H. Sekiya, M. Hijikata, and K. Shimotohno. 1992. Characterization of hypervariable regions in the putative envelope protein of hepatitis C virus. Biochem. Biophys. Res. Commun. 189:119-127.
  23. Kato, N., Y. Ootsuyama, T. Tanaka, M. Nakagawa, T. Nakazawa, K. Muraiso, S. Ohkoshi, M. Hijikata, and K. Shimotohno. 1992. Marked sequence diversity in the putative envelope protein of hepatitis C viruses. Virus Res. 22:107-123.
  24. Kato, N., et al. Unpublished data.
  25. Kozak, M. 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. 266:19867-19870.
  26. Kubo, Y., K. Takeuchi, S. Boonmar, T. Katayama, Q.-L. Choo, G. Kuo, A. J. Weiner, D. W. Bradley, M. Houghton, I. Saito, and T. Miyamura. 1989. A cDNA fragment of hepatitis C virus isolated from an implicated donor of post-transfusion non-A, non-B hepatitis in Japan. Nucleic Acids Res. 17:10367-10372.
  27. Kuo, G., Q.-L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, G. E. Tegtmeier, F. Bonino, W.-S. Colombo, W.-S. Lee, C. Kuo, K. Berger, J. R. Shuster, L. R. Overby, D. W. Bradley, and M. Houghton. 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362-364.
  28. LaRosa, G. J., J. P. Davide, K. Weinhold, J. A. Waterbury, A. T. Profy, J. A. Lewis, A. J. Langlois, G. R. Dreesman, R. N. Boswell, P. Shaddock, L. H. Holley, M. Karplus, D. P. Bolognesi, T. J. Matthews, E. A. Emimi, and S. D. Putney. 1990. Conserved sequence and structural element in the HIV-1 principal neutralizing determinant. Science 249:932-935.
  29. Looney, D. J., A. G. Fisher, S. D. Putney, J. R. Rusche, R. R. Redfield, D. S. Burke, R. C. Gallo, and F. Wong-Staal. 1988. Type-restricted neutralization of molecular clones of human immunodeficiency virus. Science 241:357-359.
  30. Matsushita, S., M. Robert-Guroff, J. Rusche, A. Koito, T. Hattori, H. Hoshino, K. Javaherian, K. Takatsuki, and S. Putney. 1988. Characterization of a human immunodeficiency virus neutralizing monoclonal antibody and mapping of the neutralizing epitope. J. Virol. 62:2107-2114.
  31. Miller, R. H., and R. H. Purcell. 1990. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. Proc. Natl. Acad. Sci. USA 87:2057-2061.
  32. Mori, S., N. Kato, A. Yagyu, T. Tanaka, Y. Ikeda, B. Petchclai, P. Chiewsilp, T. Kurimura, and K. Shimotohno. 1992. A new type of hepatitis C virus in patients in Thailand. Biochem. Biophys. Res. Commun. 183:334-342.
  33. Muraiso, K., M. Hijikata, S. Ohkoshi, M.-J. Cho, M. Kikuchi, N. Kato, and K. Shimotohno. 1990. A structural protein of hepatitis C virus expressed in *E. coli* facilitates accurate detection of hepatitis C virus. Biochem. Biophys. Res. Commun. 172:511-516.
  34. Ogata, N., H. J. Alter, R. H. Miller, and R. H. Purcell. 1991. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proc. Natl. Acad. Sci. USA 88:3392-3396.
  35. Ohkoshi, S., H. Kojima, H. Tawaraya, T. Miyajima, T. Kamimura, H. Asakura, A. Satoh, S. Hirose, M. Hijikata, N. Kato, and K. Shimotohno. 1990. Prevalence of antibody against non-A, non-B hepatitis virus in Japanese patients with hepatocellular carcinoma. Jpn. J. Cancer Res. 81:550-553.
  36. Okamoto, H., M. Kojima, S.-I. Okada, H. Yoshizawa, H. Iizuka, T. Tanaka, E. E. Muchmore, D. A. Peterson, Y. Ito, and S. Mishiro. 1992. Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. Virology 190:894-899.
  37. Okamoto, H., K. Kurai, S. Okada, K. Yamamoto, H. Iizuka, T. Tanaka, S. Fukuda, F. Tsuda, and S. Mishiro. 1992. Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology 188:331-341.
  38. Okamoto, H., S. Okada, Y. Sugiyama, K. Kurai, H. Iizuka, A. Machida, Y. Miyakawa, and M. Mayumi. 1991. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. J. Gen. Virol. 72:2697-2704.
  39. Osumi, T., T. Tsukamoto, S. Hata, S. Yokota, S. Miura, Y. Fujiki, M. Hijikata, S. Miyazawa, and T. Hashimoto. 1991. Amino-terminal presequence of the precursor of peroxisomal 3-ketoacyl-CoA thiolase is a cleavable signal peptide for peroxisomal targeting. Biochem. Biophys. Res. Commun. 181:947-954.
  40. Palker, T. J., M. E. Clark, A. J. Langlois, T. J. Matthews, K. J. Weinhold, R. R. Randall, D. P. Bolognesi, and B. F. Haynes. 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. Proc. Natl. Acad. Sci. USA 85:1932-1936.
  41. Rusche, J. R., K. Javaherian, C. McDanal, J. Petro, D. L. Lynn, R. Grimaila, A. Langlois, R. C. Gallo, L. O. Arthur, P. J. Fischinger, D. P. Bolognesi, S. D. Putney, and T. J. Matthews. 1988. Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. Proc. Natl. Acad. Sci. USA 85:3198-3202.
  42. Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, Q.-L. Choo, M. Houghton, and G. Kuo. 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA 87:6547-6549.
  43. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
  44. Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama. 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. J. Virol. 65:1105-1113.
  45. Takeuchi, K., Y. Kubo, S. Boonmar, Y. Watanabe, T. Katayama, Q.-L. Choo, G. Kuo, M. Houghton, I. Saito, and T. Miyamura. 1990. The putative nucleocapsid and envelope protein genes of hepatitis C virus determined by comparison of the nucleotide sequences of two isolates derived from an experimentally infected chimpanzee and healthy human carriers. J. Gen. Virol. 71:3027-3033.
  46. Tanaka, T., N. Kato, M. Nakagawa, Y. Ootsuyama, M.-J. Cho, T. Nakazawa, M. Hijikata, Y. Ishimura, and K. Shimotohno. 1992. Molecular cloning of hepatitis C virus genome from a single Japanese carrier: sequence variation within the same individual and among infected individuals. Virus Res. 23:39-53.
  47. Tsukiyama-Kohara, K., M. Kohara, K. Yamaguchi, N. Maki, A. Toyoshima, K. Miki, S. Tanaka, N. Hattori, and A. Nomoto. 1991. A second group of hepatitis C viruses. Virus Genes 5:243-254.
  48. Weiland, E., R. Stark, B. Haas, T. Rumenapf, G. Meyers, and H.-J. Thiel. 1990. Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. J. Virol. 64:3563-3569.
  49. Weiner, A. J., M. J. Brauer, J. Rosenblatt, K. H. Richman, J. Tung, K. Crawford, F. Bonino, G. Saracco, Q.-L. Choo, M. Houghton, and J. H. Han. 1991. Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. Virology 180:842-848.
  50. Weiner, A. J., H. M. Geysen, C. Christopherson, J. E. Hall, T. J. Mason, G. Saracco, F. Bonino, K. Crawford, C. D. Marion, K. A. Crawford, M. Brunetto, P. J. Barr, T. Miyamura, J. McHutchinson, and M. Houghton. 1992. Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infection. Proc. Natl. Acad. Sci. USA 89:3468-3472.