Genetic Drift in Hypervariable Region 1 of the Viral Genome in Persistent Hepatitis C Virus Infection†

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The hypervariable region 1 (HVR1) of the putative second envelope glycoprotein (gp70) of hepatitis C virus (HCV) contains a sequence-specific immunological B-cell epitope that induces the production of antibodies restricted to the specific viral isolate, and anti-HVR1 antibodies are involved in the genetic drift of HVR1 driven by immunoselection (N. Kato, H. Sekiya, Y. Ootsuyama, T. Nakazawa, M. Hijikata, S. Ohkoshi, and K. Shimotoho, J. Virol. 67:3923-3930, 1993). We further investigated the sequence variability of the HCV genomic region that entirely encodes the envelope proteins (gp35 and gp70); these sequences were derived from virus isolated during the acute and chronic phases of hepatitis in one patient, and we found that HVR1 was a major site for genetic mutations in HCV after the onset of hepatitis. We carried out epitope-mapping experiments using the HVR1 sequence derived from the acute phase of hepatitis and identified two overlapping epitopes which are each composed of 11 amino acids (positions 394 to 404 and 397 to 407). The presence of two epitopes within HVR1 suggested that epitope shift happened during the course of hepatitis. Four of six amino acid substitutions detected in HVR1 were located within the two epitopes. We further examined the reactivities of anti-HVR1 antibodies to the substituted amino acid sequences within the two epitopes. HVR1 variants in both epitopes within the HVR1 escaped from anti-HVR1 antibodies that were preexisting in the patient's serum.

Most hepatitis C virus (HCV) infections cause chronic hepatitis; this persistent viral infection frequently develops into liver cirrhosis and hepatocellular carcinoma (3, 22, 26, 30, 34). Genetic analysis of HCV has been accelerated rapidly (4, 11, 12, 32, 37, 39) since the structure of the entire HCV genome was first determined in 1990 (16). A diagnostic system for HCV infection is now generally established (22, 26). Despite these advances, the mechanisms of viral replication and persistence are still not clear.

The HCV genome is a positive-stranded RNA molecule of about 9.5 kb and encodes at least 10 viral proteins (8–10, 14, 16). The viral proteins are produced from a large polyprotein precursor that is about 3,000 amino acids long. Their cleavage is mediated by a host cell signal peptidase and by two viral proteases. The most characteristic feature of the HCV genome is the remarkable sequence diversity exhibited by different viral isolates (1, 2, 5, 15, 18, 25, 36, 41). The quasispecies nature of HCV genome distribution in a single patient was also reported (24). Comparison of the amino acid sequences of many HCV isolates identified two hypervariable regions (HVR1 and HVR2) in the N-terminal region of a putative second envelope glycoprotein (gp70) (7, 20, 42). HVR1, particularly, shows marked sequence variability and a quasispecies nature (6, 19, 23, 27–29, 31), and it induces anti-HVR1 antibody (21, 40, 43).

Amino acid alterations in HVR1 occur sequentially during the chronic state of hepatitis at a rate of 0.5 to 1.7 amino acids per month. (19). HVR1 contains a B-cell epitope that is specific for the homologous virus isolate (21). We obtained the data suggesting that an HCV with an amino acid-substituted HVR1 could escape recognition by preexisting anti-HVR1 antibodies (21). However, it is still not clear whether HVR1 is the major mutation site in the HCV genome during the progression from acute to chronic hepatitis, nor is the relationship between the hypervariable sites and the site of the B-cell epitope understood. To clarify these points, we analyzed the sequence variability of the envelope protein (gp35 and gp70) coding region in HCV genomes derived from a patient whose sporadic acute hepatitis developed into chronic hepatitis, and we mapped HVR1 epitopes from acute-phase isolates. We report here that HVR1 is the major site affecting HCV genetic drift. Two overlapping B-cell epitopes were located within the HVR1, amino acid substitutions in each HVR1 epitope led to escape from recognition by preexisting anti-HVR1 antibodies, and qualitative changes in antibody accompanied HVR1 epitope shifts during the clinical course of hepatitis.

MATERIALS AND METHODS

Materials. Oligonucleotide primers for PCR were synthesized in an Applied Biosystems model 394 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 and restriction enzymes were from Takara Shuzo (Kyoto, Japan). Protein G-Sepharose was from Zymed Laboratories (South San Francisco, Calif.). [35S]methionine was from Amersham (Amersham, United Kingdom).

Patient. Patient 1 is a 22-year-old woman diagnosed with acute non-A, non-B hepatitis without a history of blood
transfusion. The detailed clinical course of this patient was described previously (21).

cDNA synthesis and PCR amplification (RT-nested PCR). RNAs from serum samples of patient I were prepared as described previously (17). RNA from 10 μl of serum was used for reverse transcriptase (RT)-nested PCR as described previously (20). The sequences of primers in the first PCRs for regions A, B, C, and D (see Fig. 1A) were 224, 5'-CATGTTG CTCCGGTCTTCTGGA-3'; 144, 5'-CTACTCCGGATCCCA CAAGC-3'; 185R, 5'-CAGACCTTATGGTCTGCA-3'; and 254, 5'-AAGGTTAGATGTAGTGCG-3', respectively, as sense primers (corresponding to positions 786 to 805, 1338 to 1358, 1773 to 1792, and 2211 to 2230 of the HCV-J [16], respectively). The sequences of primers in the second PCRs for regions A, B, C, and D were 225, 5'-GTGAATATCGCAACAGGGGA-3'; 146, 5'-ATTCCATGTGTTGGAAGCTG-3'; 184B, 5'-GGTC CAGTGTATGGTTCA-3'; and 256, 5'-AGCACAAGGGGTA GTGCTGCA-3', respectively, as sense primers (corresponding to positions 813 to 832, 1347 to 1366, 1871 to 1890, and 2256 to 2275 of the HCV-J [16], respectively). The sequences of primers in the first PCRs for regions A, B, C, and D were 225, 5'-GTGAATATCGCAACAGGGGA-3'; 146, 5'-ATTCCATGTGTTGGAAGCTG-3'; 184B, 5'-GGTC CAGTGTATGGTTCA-3'; and 256, 5'-AGCACAAGGGGTA GTGCTGCA-3', respectively, as sense primers (corresponding to positions 813 to 832, 1347 to 1366, 1871 to 1890, and 2256 to 2275 of the HCV-J [16], respectively).

cDNA cloning and sequencing. PCR products were cloned into the pTZ19R plasmid vector as previously described (15, 17). Nucleotide sequences were determined by the dideoxy nucleotide chain termination method, using an A.L.F. DNA sequencer (Pharmacia). System for detection of specific antibodies against mutated regions. Our assay system for anti-HVR1 antibodies (21) was used to detect antibodies against amino acid-substituted regions by replacing HVR1 with the amino acid sequences containing the substituted amino acid position. An expression plasmid, pTZ19RSVdhfr1 (21), was used to express a fusion protein with peptides (14 or 19 amino acids) containing the substituted amino acid position and dihydrofolate reductase (DHFR) derived from Escherichia coli by in vitro transcription and translation. Immunoprecipitates of fusion proteins with serum samples from patient I were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Preparation of deleted HVR1 mutants. The HVR1 I-1 sequence came from virus isolated from patient I during the acute phase of hepatitis (21). Plasmid DNA containing HVR1 I-1 sequence was used as a template for PCR with primers which were designed to obtain deletions or mutations. PCR was carried out under conditions described previously (19). After digestion with HindIII and BamHI, the PCR product was cloned into the HindIII-BclI site of the pTZ19RSVdhfr1 vector. The nucleotide sequences of HVR1 of the mutants were confirmed by the dideoxy nucleotide chain termination method, using an A.L.F. DNA sequencer (Pharmacia).

Nucleotide sequence accession number. Nucleotide sequence data from this study have been deposited with the DDBJ, EMBL, and GenBank data libraries under accession numbers D26394 to D26438.

RESULTS

Sequence variability of whole envelope proteins (gp35 and gp70). In a previous study we showed that HVR1 undergoes sequential mutations at intervals of a few months during chronic C-type hepatitis that developed from sporadic acute hepatitis (19). This result suggests that HVR1 is the first region of the viral genome to mutate after the onset of hepatitis, though there is no direct evidence to support this idea. To evaluate this assumption, we analyzed the nucleotide sequences encoding the envelope proteins of HCV genomes isolated from patient I (the same patient we had studied previously) at 0, 2, 6, 8, and 11 months postdiagnosis (p.d.). Four regions (Fig. 1A, regions A to D) encoding all of gp35 and gp70 were amplified by RT-nested PCR with primers corresponding to conserved regions (see Materials and Methods). The amplified products were cloned into pTZ19R for nucleotide sequence analysis. We determined the nucleotide sequences of three different cDNA clones from each sample. At 6 months p.d. we detected several mutations (Fig. 1B). One mutation was an amino acid change from Phe to Leu at position 399 in the HVR1 of gp70, and another was a switch from Leu to Phe in the carboxy-terminal portion of gp70 at position 766. In addition, changes occurred at amino acid positions 266 (Val to Ile; one clone) and 268 (Ala to Thr; two clones) in gp35. Amino acid substitutions observed in regions A and D showed sequence heterogeneities during the progression of hepatitis (Fig. 1B). Significantly, only mutations in HVR1 within the region encoding envelope proteins of HCV genome accumulated with the development of chronic-phase hepatitis in patient I.

Humoral immune responses to the mutated regions. Our prior study showed that there are sequence-specific anti-HVR1 antibodies with high titers in the serum samples of patient I (21). These antibodies may exert enough immunological pressure against HCV to drive the selection of specific adaptive amino acid substitutions. This assumption led us to examine the humoral immune response to envelope protein amino acid sequences containing the substituted amino acid positions in non-HVR1 regions, as shown in Fig. 1B. We synthesized two peptides (amino acid positions 259 to 277 and 759 to 772) fused to DHFR proteins. However, we could not detect production of specific antibodies against these two peptides (data not shown). HVR1 apparently encoded the major antigen(s) recognized by anti-HCV envelope protein antibodies.

Genetic mutations of HVR1 in patient I. In patient I, the HVR1 sequence accumulated mutations during the course of disease (sampled at 0, 2, 6, 8, and 11 months p.d.) and encoded potent antigens. This led us to a more extensive examination of HVR1 sequence heterogeneity; from each sample, we analyzed more than 12 cDNA clones. The predominant HVR1 species (Fig. 2) in each specimen was the same as that from our previous study, in which we sampled only three cDNA clones derived from each specimen (19). However, in HVR1 derived from the 8-month-p.d. sample (Fig. 2), we observed sequence heterogeneity. This result is consistent with the quasispecies nature of HVR1 in patients with chronic-phase hepatitis (6, 19, 20, 27, 31) or in a healthy carrier with a persistent infection of HCV (28). Furthermore, we observed that the sequence of HVR1 had become more homologous again in the serum at 11 months p.d. In our subsequent experiments, we focused on the representative HVR1 species to examine the relation between amino acid substitution in HVR1 and humoral immune selection to HVR1 in patient I.

Mapping of the amino-terminal portion of the B-cell epitope. Although an HVR1 antigen sometimes coexists with
FIG. 1. Structural analysis of HCV genomes from patient I. (A) Schematic presentation of the amplified regions of the HCV genome. Regions A to D were amplified by RT-nested PCR as described in Materials and Methods. Amino acid positions at both ends of each region are shown. (B) Deduced amino acid sequences in the amplified regions. Amino acid positions 169 to 801 are shown (amino acid positions 361 to 368, 505 to 510, and 644 and 645 could not be deduced, because these were in the primer regions for amplification). Amino acid sequences are indicated by the single-letter code. Capital letters indicate different amino acids from the amino acid sequence of 1-1-1. 1-1, 1-2, 1-3, 1-4, and 1-5 indicate the HCV genomes isolated from patient I at 0, 2, 6, 8, and 11 months p.d., respectively. The nucleotide sequences of three different cDNA clones from each sample were determined. The shaded bars indicate the regions which were examined for the existence of specific antibody.
FIG. 2. Genetic alterations of HVR1 in patient I. Capital letters indicate amino acids different from those in the sequence of HVR1-I-1. The number of clones indicates the actual number of plasmid clones obtained at each time point. The heavy lines show the predominant population at each time point.

its specific antibody, anti-HVR1 antibodies appear to be involved in the genetic alterations of HVR1 from patient I (21). However, there is no direct evidence that the B-cell epitope in HVR1 actually contains the substituted amino acid positions of HVR1 detected in patient I. To clarify this point, we carried out epitope mapping with the sequence of HVR1-I-1 (Fig. 3, abc) derived from acute-phase hepatitis. We first divided the sequence of HVR1-I-1 into three domains: a, b, and c. As shown in Fig. 3, it appeared that domain b plus c (positions 392 to 410) contained an antibody-binding epitope. Domain b by itself did not react with any serum samples from patient I. Domain a plus b together must have contained an additional sequence-specific antibody-binding epitope (Fig. 3), because an HVR1-I-1 variant, which mutated to Arg from Gln at position 391 of HVR1-I-1, completely lost the ability to bind antibodies (data not shown). Antibody titers against domain b plus c were much higher than those against domain a plus b; therefore, we focused on the epitope within domain b plus c. From HVR1-I-1 we made bc1 to bc8, which each have one amino acid deleted in order from the amino-terminal portion of domain b, and then examined the reactivities of these deleted forms against serum samples obtained at several time points from patient I. Antibody-binding activities against the HVR1-I-1 truncated sequences bc1 to bc5 were detected, but no activity was detected against bc6 to bc8 (Fig. 3). This result indicated that Arg (position 397) of bc5 is the amino-terminal residue of a B-cell epitope. Between bc2 and bc3 there was a drastic difference in the antibody-binding activity of serum from 6 months p.d. The radioactivity of the band shown with bc3, using serum from 6 months p.d., was actually more than 10 times lower than that obtained with bc2. The electrophoretic band intensities of samples obtained by immunoprecipitation depended on the titers of antibodies in patient serum samples (21). Therefore, we supposed that bc2 contained the amino-terminal residue of another different B-cell epitope. These results suggested that at least two major B-cell epitopes are present in the HVR1-I-1 sequence.

Mapping of the carboxy-terminal portions of the two epitopes. To pinpoint the carboxy-terminal residues of the two B-cell epitopes that we identified in HVR1-I-1, we constructed several truncated forms of those sequences that had single sequential deletions from the carboxy-terminal portion. The first epitope variants, bc25 to bc210, were deleted starting from position 405, and the second epitope variants, bc51 to bc57, were deleted beginning at position 409 (Fig. 4A). The first epitope started from His (position 394) appeared to require 11 amino acids (positions 394 to 404) for specific antibody binding; its variants bc25 and bc26 showed strong antibody-binding activity, but bc27 to bc210 showed no activity. In the second epitope, we prepared seven truncated forms, bc51 to bc57, beginning from Arg (position 397). The reactivity of bc51 to serum samples from patient I was the same as that of the undeleted form of domain b plus c. The antibody-binding activities of the rest of the variant series gradually decreased until activity was nearly imperceptible in bc55 for sera from patient I. From this analysis, we initially estimated that Gly (position 406) was the carboxy-terminal residue of the second epitope. However, bc55 and the HVR1 portion of bc54 were the same Gly “terminus,” because the first amino acid of the DHFR portion of the fusion protein was Gly, as shown in Fig. 4A. This result suggests that the carboxy-terminal residue of

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**FIG. 3.** Mapping analysis of the amino-terminal portion of the epitope within HVR1-I-1 from patient I. Various HVR1-I-1 truncated forms were constructed as described in Materials and Methods. HVR1-I-1 truncated peptides were fused with the amino-terminal portions of DHFR proteins, synthesized by in vitro transcription and translation, and immunoprecipitated with serum collected at various times from patient I. N indicates serum from a healthy (normal) volunteer. As a positive control, anti-DHFR rabbit antibody (edhfr) (33) was used. Immunoprecipitates of fusion proteins with sera were analyzed by SDS-PAGE. The dotted lines indicate the predicted amino-terminal portions of the two epitopes.
the second epitope is Ser (position 407). We tried, unsuccessfully, to identify the carboxy-terminal residue of the second epitope by preparing three additional variants of bc53 to bc55, each of which was substituted from Gly to Trp at the first amino acid position of the DHFR protein. The antibody-binding activities of these three variants, though one-half to one-third those of bc53 to bc55, basically mirrored those of their parents (data not shown). As shown in Fig. 4B, the titer of antibody against the second epitope reached the maximum level at 8 months p.d., and the titer of antibody against the first epitope reached a maximum at 6 months p.d. Clearly, the epitope shift occurred between 6 and 8 months p.d. in this patient.

HVR1 variants can escape recognition by preexisting antibodies against two B-cell epitopes in HVR1 I-1. Figure 5 shows the relation between the positions of the two overlapping B-cell epitopes (epitopes I and II) identified in HVR1 I-1 and gives the amino acid sequences of the predominant HVR1 obtained from each time point. Epitopes I and II are both composed of 11 amino acids (positions 394 to 404 and 397 to 407, respectively). Four of the six substituted amino acid positions were located within the two epitopes, although substituted His (position 386) and Met (position 392) residues were located outside of epitopes I and II. We examined the reactivities of the antibodies to the substituted amino acid sequences of HVR1. For epitope I, we prepared bc25L (Phe to

FIG. 4. Two immunological epitopes present within HVR1 I-1 from patient I. (A) Mapping analysis of the carboxy-terminal portions of two epitopes. Various HVR1 I-1 truncated forms were constructed as described in Materials and Methods. Sera collected at various times from patient I were tested for relative binding activities to HVR1 I-1 truncated forms. The shaded and black boxes indicate the positions of epitopes I and II, respectively. G I S indicates the sequence of the amino-terminal portion of the DHFR protein. (B) The level of radioactivity of each signal was measured with a Bio-Imaging analyzer (BAS 2000). PSL is the unit of radiation dose used in the BAS 2000.
could escape recognition by preexisting antibodies. We analyzed the sequence variabilities of the HCV envelope proteins over the course of the disease and found three amino acid substitutions in different areas, including an amino acid substitution in HVR1 as early as 6 months p.d. HVR1 is probably the first site of genetic drift in HCV after the onset of hepatitis.

This study agreed well with our recent study which showed that HVR1 was one of the positions first mutated during relapse (8 months after onset) of another patient, patient M, diagnosed with acute C-type hepatitis contracted through a needle-stick injury (35). Since the mutated positions except HVR1 were different between patients I and M, HVR1 is probably the common mutated region of the HCV genome in the early phase of hepatitis.

Okamoto et al. (31) reported sequence variability of the HCV genome during an 8.2-year infection in a chimpanzee. They observed that eight amino acids in HVR1 changed during an 8.2-year infection. In our previous study (19) as well as this study, however, five amino acid substitutions in HVR1 were already observed by 11 months p.d., and significant sequence heterogeneity in HVR1 was noted at 8 months p.d. Kurosu et al. (23) also reported the sequence variabilities of HVR1s obtained at approximately 1-year intervals from nine patients with chronic hepatitis. They observed that the substitution rate of HVR1 differed among individuals, with 1 to 12 amino acids in HVR1 changing during 1 year. These observations point to a general occurrence of amino acid substitutions in HVR1 during the early stage of hepatitis and suggest that amino acid substitution rates might reflect the level of immune response to HCV infection.

We demonstrated in this study the existence of two distinct immunological epitopes in HVR1-I; however, we could not detect any specific antibodies against the other amino acid-substituted regions of the putative envelope proteins. A similar result was obtained in the analysis of patient M (35). The lack of antigenicity of the amino acid-substituted regions other than HVR1 suggests that there is little additional selective pressure in the humoral immune system against HCV infection. The possibility remains that other amino acid-substituted positions might be parts of a conformational epitope(s), because our system for specific antibodies detects only linear epitopes. Another possibility is that the non-HVR1 amino acid substitutions in this study might allow HCV to escape from cytotoxic T lymphocytes. Further analysis will be necessary to evaluate these possibilities.

By epitope mapping with our assay system, we detected two epitopes (I and II) within HVR1-I-I which both required at least 11 amino acids. Two other groups detected antibodies against HVR1 with synthetic oligopeptides, an 8-mer (43) or a 10-mer (40). In our study, any 10-mer derived from HVR1-I-I did not show antibody-binding activity with either epitope. Thus, the previously reported results may not reflect all of the humoral immune response to HVR1.

In the case of patient I, we observed an epitope shift of three amino acids within HVR1 between 6 and 8 months p.d. Probably, similar shifts of B-cell epitopes are frequently driven by immunological pressure during the clinical course of hepatitis. However, since the position of epitope II (positions 397 to 407) identified in patient I was identical to that identified in the other patient, patient M (35), and was very close to that (positions 396 to 407) observed in patient Q (43), positions 397 to 407 are more likely to represent an immunological epitope within the HVR1 that is shared by most strains of HCV despite the overall remarkable amino acid sequence diversity of the virus.

The two epitopes that we described contained four of the six

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FIG. 6. HVR1 mutants can escape from preexisting antibodies. (A) Reactivities of HVR1 I-1 mutants to serum samples from patient I. Several mutant forms of the two B-cell epitopes in HVR1 I-1 were constructed as described in Materials and Methods, and their reactivities to serum samples collected at various times from patient I were examined. The boxes of bc25 and bc51 indicate the positions of epitopes I and II, respectively. (B) The level of radioactivity of each signal was measured as described in the legend to Fig. 4B.

substituted amino acid positions, as shown in Fig. 5. The other two substituted amino acid positions, outside of both epitopes, may modify cell tropism during HCV propagation or may be parts of a conformational epitope(s) or mutations affecting escape from cytotoxic T lymphocytes, as described above. Modification of cell tropism by an amino acid substitution is also seen in the third variable region of human immunodeficiency virus type 1 (38). In contrast, the two epitopes that we identified contained four of six substituted amino acid positions in HVR1 I. This result suggests that, in this patient, amino acid substitutions in HVR1 resulted from the selection by immunological pressure.

The analysis of reactivities of amino acid-substituted HVR1 sequences to the preexisting anti-HVR1 antibodies revealed that most HCV having the amino acid-substituted HVR1 sequences could escape from the antibodies which recognize the presubstituted sequences in regard to both epitopes I and II identified in HVR1 I. In particular, an HCV species with Gly at position 395, Ser at position 397, and Leu at position 399 obtained from 11-month-p.d. serum was not recognized by the
antibodies produced in patient I. Antibodies against the HVR1 sequence of this HCV species were no longer detected even in the 19-month-p.d. serum (13). From these results, we propose that the frequent mutations in HVR1 are involved in HCV persistent infection. According to our proposal, during chronic hepatitis, clearance of HCV by anti-HVR1 antibodies is slower than the rate of escape from the antibodies by amino acid substitution. In the case (patient M) described above, the data are difficult to evaluate: the amino acid-substituted HVR1 sequence did not display an escape pattern from anti-HVR1 antibody (35); the anti-HVR1 antibody level was rather low and the time of antibody production was too late (detected at 8 months p.d.) to be compared directly with the results (detected at 2 months p.d.) of patient I presented here.

There is no direct evidence that anti-HVR1 antibody is a neutralizing antibody. An experimental system, including cell culture, needs to be developed to determine whether HVR1 contains a neutralizing epitope.

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