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Occurrence of Antibodies Reactive with More than One Variant of the Putative Envelope Glycoprotein (gp70) Hypervariable Region 1 in Viremic Hepatitis C Virus-Infected Patients

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The hepatitis C virus (HCV) is a frequent cause of chronic liver disease. A mechanism proposed as being responsible for virus persistence is evasion of the host immune response through a high mutation rate in crucial regions of the viral genome. We have sequenced the hypervariable region 1 (HVR1) of the virus isolated from three serum samples, collected during 18 months of follow-up, from an asymptomatic HCV-infected patient. A synthetic peptide of 27 amino acids, corresponding to the HVR1 sequence found to be predominant in both the second and third samples, was used as the antigen for detection of antibodies by enzyme-linked immunosorbent assay (ELISA). We observed reactivity against this HVR1 sequence in the first serum sample before the appearance of the viral isolate in the bloodstream; the reactivity increased in the second and third samples while the cognate viral sequence became predominant. Moreover, our results show that antibodies from all three samples recognize a region mapping at the carboxyl-terminal part of the HVR1 and are cross-reactive with the HVR1 sequence previously found in the same patient. The presence of anti-HVR1 antibodies was investigated in a further 142 HCV patients: 121 viremic and 21 nonviremic. Two synthetic peptides were used, the first corresponding to the sequence derived from the patient described above and the second one synthesized according to the sequence of the HCV BK strain. A high frequency of positive reactions against both HVR1 variants was detected in the samples from the viremic individuals. Finally, antibodies cross-reactive with both variants were shown to be present by competitive ELISA in 6 of 10 viremic patients. The potential negative implications of this observation for the host are discussed.

Hepatitis C virus (HCV) is the major etiologic agent of posttransfusional and sporadic non-A non-B hepatitis (1). Most HCV-infected individuals will develop chronic hepatitis with an attendant risk of cirrhosis, liver failure, and hepatocellular carcinoma (4).

The HCV genome is a positive-strand RNA molecule of 9.5 kb. The genome organization has been found to be very similar to those of human flavivirus (dengue and yellow fever viruses) and animal pestivirus (hog cholera virus and bovine viral diarrhea virus) (23). The genome encodes for a large precursor polyprotein, whose cleavage results in mature structural and nonstructural viral proteins. The 5′ end of the genome encodes for a basic polypeptide (core) and a first envelope glycoprotein (E1), followed by a second envelope glycoprotein (E2) in pestivirus and a secreted nonstructural protein (NS1) in flavivirus. HCV seems to be more closely related to pestivirus, and the second glycoprotein (E2 [gp70]) most probably represents a virion envelope protein (23, 39).

Both pestivirus E2 and flavivirus NS1 have been shown to induce a protective immune response in vaccinated hosts (33, 45). Recently, it was also shown that immunization of chimpanzees with HCV E1-E2 recombinant protein complexes protects animals from viral challenge with the same viral isolate (3). Humoral immune response against recombinant E1 and E2 in humans has been investigated, and preliminary studies have shown the presence of antibodies only in a limited number of patients (12, 13, 25). Recently, the use of purified proteins expressed in baculovirus (28) and in vaccinia virus has led to the detection of anti-E1 and anti-E2 antibodies in most of the patients studied (2). The immune response against envelope proteins was found in chronic patients carrying the virus but rarely in acute or chronic nonviremic patients (12, 13, 25).

Previous studies have identified a hypervariable region (HVR1) at the amino terminus of the HCV E2 protein (11, 15, 16, 43) that undergoes a gradual diversification of its sequence during the course of infection (10, 19, 21, 26, 31, 34). Antibodies restricted to a viral isolate have also been described (17, 39, 44). The occurrence of new antigenic variants may allow escape of virus when neutralizing antibodies are produced against the original sequence. For other RNA viruses, like human immunodeficiency virus (14) and foot-and-mouth disease virus (7), epitopes able to induce neutralizing antibodies have been located in regions showing a high degree of variability.

We report here the follow-up of HVR1 viral sequences and anti-HVR1 antibodies in a viremic HCV-infected patient. We investigated the reactivity of serum samples against a predominant HVR1 sequence found in the patient. We show that antibodies recognizing a single carboxyl-terminal region within the HVR1 sequence precede the occurrence of the viral variant they react to and then coexist for a long period in the bloodstream with the viral isolate. These antibodies are also

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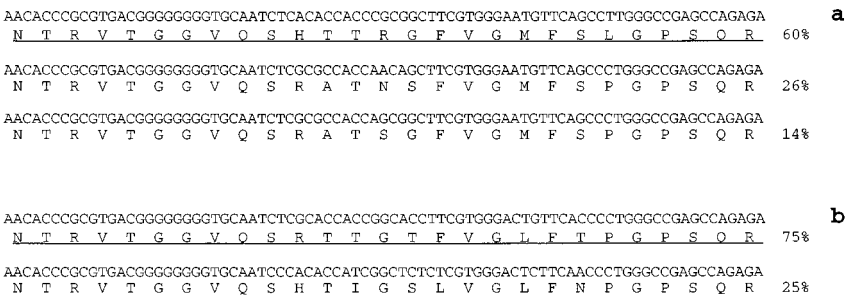

FIG. 1. Nucleotide and deduced amino acid sequences of HVR1 obtained from HCV infecting an asymptomatic patient. Amino acids are indicated by the single-letter code. Predominant virus variants found at the time of the diagnosis (a) and 11 months after the diagnosis (b) are underlined. The frequencies of representation of all sequences are indicated.

shown to be cross-reactive with the viral variant previously present in the blood of the patient.

To further investigate this phenomenon we studied the humoral anti-HVR1 response in 142 HCV-infected individuals. We found that 25% of the viremic patients were reactive with two HVR1 sequences randomly chosen and only distantly related (50% of homology). Furthermore, in 6 of 10 patients tested by competitive enzyme-linked immunosorbent assay (ELISA), cross-reactive antibodies were found.

MATERIALS AND METHODS

Patients. We followed the HVR1 viral sequence modifications and the production of specific antibodies in one patient (patient c8) for 18 months. The patient was asymptomatic and was found to be positive for anti-HCV antibodies (second-generation ELISA; Ortho Diagnostics, Raritan, N.J.) in November 1992 during blood donation. Serum samples were collected 11 and 18 months after diagnosis.

Serum samples from 142 HCV-seropositive (second-generation ELISA; Ortho Diagnostics) subjects were tested for anti-HVR1 antibodies. Of the 142 subjects, 19 were intravenous drug abusers, 46 had chronic liver disease without known epidemiological risk factors, and 77 were found to be anti-HCV positive upon screening for blood donation.

Detection of HCV RNA and genotyping. HCV RNA was detected by nested reverse transcription-PCR with conserved primers localized in the 5′ noncoding region of the viral genome (8). HCV genotyping was performed by PCR amplification of core region sequences with universal and type-specific primers to generate DNA fragments of different sizes, specific for the five most common HCV genotypes (29, 30). The genotype 2a specific primer was modified because of several nucleotide differences between Italian and Japanese isolates of the genotype 2a sequence (37).

HVR1 sequencing. First-strand cDNA (35) was produced from viral RNA extracted from the patient's serum. Amplification was performed in a volume of 100 μl with *Taq* polymerase, buffer (Boehringer), and the oligonucleotide primers already described (6), 5′ ATAACGGGTCACCGATGGCATGGGATAT 3′ and 5′ CACCACCACGGGGCTGGGAGTGAAGCAAT 3′. PCR was performed for 20 cycles (94°C for 1 min, 53°C for 2 min, and 72°C for 3 min) in a Techne PHC2 thermocycler. An aliquot of this reaction was subjected to an additional 20 cycles of amplification with the same set of primers. The PCR products were purified from 1% agarose gel with Gene-clean II and directly sequenced by the dideoxynucleotide chain termination method (36). To determine the frequency of each sequence variant in the samples, the PCR products were blunt-end ligated in Bluescript KS+ vector, previously digested with *Eco*RV. Plasmids were transfected into *Escherichia coli* DH5α cells. Colonies carrying the insert were identified by α-complementation (35), and 20 independent clones were sequenced.

Peptide synthesis. The peptides were synthesized by standard solid-phase methods, using Fmoc protection and kieselguhr-polyamide resin. They were all purified to >95% homogeneity by reverse-phase high-performance liquid chromatography (Ultrasphere C-18 column [250 by 4.6 mm], 80 Å; linear gradients of acetonitrile in water containing 0.1% trifluoroacetic acid). The peptides were solubilized in dimethyl sulfoxide at a concentration of 2 mg/ml. A multiple-chain peptide, containing eight identical peptides covalently attached to a polylysine core, was synthesized according to the protocol previously described (32, 40).

Immunization of mice. BALB/c mice (7 weeks old) were immunized intraperitoneally with 50 μg of the multiple-chain peptide emulsified in complete Freund adjuvant. The mice were boosted intraperitoneally twice, at 3 and 5 weeks, with the antigen emulsified in incomplete Freund adjuvant.

ELISA. ELISA plates (96 wells, MaxiSorp; Nunc) were coated with each peptide at a concentration of 10 μg/ml diluted in 100 mM $NaHCO_3$ buffer, pH 8.6, overnight at 4°C. Saturation buffer (phosphate-buffered saline [PBS], 10% newborn calf serum, 0.1% Tween 20) was used for blocking the plates for 1 h at room temperature. The serum samples were diluted 1:10 (unless noted otherwise in the text) in the saturation buffer and incubated overnight at 4°C. The second antibody, alkaline phosphatase-conjugated anti-human immunoglobulin G (Sigma), at a dilution of 1/5,000, was incubated for 1 h at room temperature. The plates were washed 10 times with PBS–0.05% Tween 20 at each step. Development of the reaction was performed by adding the substrate (4-nitrophenylphosphate) in diethanolamine buffer, and optical density values were measured at 405 nm. All samples were run in duplicate. Cutoff values were calculated as the mean of 20 negative controls (negative for HCV by second-generation ELISA, Ortho Diagnostics) plus 5 times the value of the standard deviation.

Competitive ELISA. For the epitope mapping, ELISA plates were coated with the peptide corresponding to the sequence of HVR1/c8b (see Fig. 1 and 2), and the five overlapping peptides shown in Fig. 2 (b1 to b5) were used as competitors. The serum samples were diluted 1/20 in saturation buffer containing serial dilutions of competitor peptides, immediately added to the ELISA plates, and incubated overnight at 4°C. The incubation with the second antibody and the development were performed as described above. In order to clarify the ability of antibodies to discriminate between HVR1 variants, the ELISA reactivity against HVR1/BK was inhibited by the addition of the alternative variant HVR1/c8b (see Fig. 2).

Nucleotide sequence accession number. The sequences of the HVR1 clones have been submitted to the EMBL data library with the following accession numbers: X79669, X79670, X79671, X79672, and X79673.

RESULTS

Sequence analysis of the HVR1 variants during the follow-up of patient c8. The first 27 amino acids of the HCV E2 protein show a high degree of variability in different HCV isolates, and new mutations accumulate rapidly during the course of infection. We have amplified by PCR the DNA encoding the HVR1 of the HCV E2 protein in one asymptomatic patient (patient c8). Direct sequencing of the PCR product obtained at the time of diagnosis showed that more than one variant for HVR1 was present in the sample. The sequence was homogeneous in the regions flanking HVR1 (data not shown) and belonged to the genotype described as type 1b according to the classification system of Simmonds et al. (38). To determine the frequency of each variant, the PCR product was cloned and 20 independent clones were sequenced. The predominant sequence at time zero of our study, HVR1/c8a, accounted for 60% of the viral population; the other two sequences shown in Fig. 1a represented, respectively, 26 and 14% of the viral population. The second blood sample was collected after 11 months, and two novel sequences were found: HVR1/c8b, present in 75% of the viral population, and another sequence, which was present in the remaining 25% (Fig. 1b); we were not able to detect any of the variants present at time zero of our study in this blood sample. Furthermore, the PCR product obtained after 18 months was homogeneous, as judged by direct sequencing, showing undoubtedly that the predominant sequence was still HVR1/c8b, the same as the specimen col-

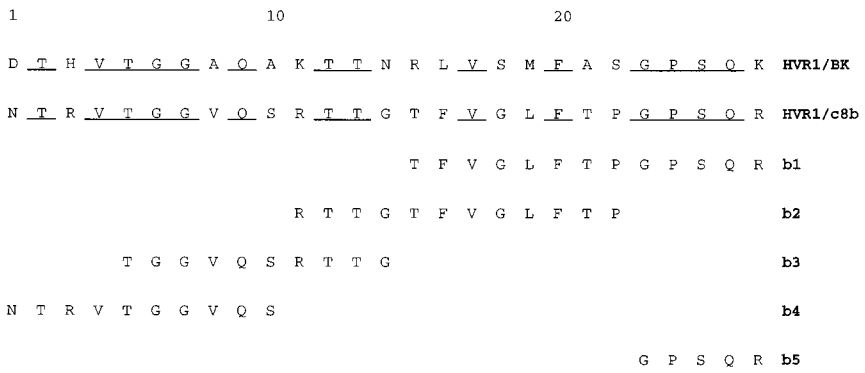

FIG. 2. Peptide sequences used for immunological analysis. Shown are the full-length HVR1 sequence of the BK strain (HVR1/BK) and the predominant isolate (HVR1/c8b) found in patient c8 at 11 and 18 months after the diagnosis. Amino acids conserved between the two are underlined. The five small overlapping peptides (b1 to b5), used for epitope mapping, are also shown.

lected 7 months before. Cloning of this specimen was thus considered unnecessary.

Follow-up of the antibody titer against one HVR1 variant in patient c8. We investigated the humoral anti-HVR1/c8b response in patient c8 over time. The three serum samples collected during the follow-up were tested against a synthetic peptide corresponding to the HVR1/c8b sequence with ELISA (Fig. 2). Antibody titer was determined by the end point titer approach. Samples were serially diluted in the assay down to negative values. We took as the titer the last dilution which gives a reaction above that of the negative controls run over the same dilution series. The first sample, taken prior to the occurrence of the viral variant in the blood, was positive at a 1/24 dilution; the titer increased in the two subsequent samples, in which the HVR1/c8b sequence was predominant (Fig. 3).

Epitope mapping and determination of cross-reactivity in patient c8. In order to map the epitope recognized within the HVR1/c8b sequence, the reactivity in ELISA to this sequence was subjected to competition with five smaller overlapping peptides (Fig. 2). The data for the blood sample collected at 18 months are shown in Fig. 4. The peptide containing the last 13 carboxyl-terminal amino acids (aa), b1 (Fig. 2), was able to compete for the reaction with the same strength as that of the full-length peptide; the peptide b2, which overlaps with b1 by 8 amino acids, was also able to compete for the reaction but to a much lesser extent, indicating that some residues in the last 5 amino acids are crucial for a good binding. We can exclude the possibility that the last 5 conserved residues were active by themselves, since the small peptide b5 (Fig. 2), containing only the last 5 carboxyl-terminal residues, could not compete for the reaction. No competition at all was achieved with the other small peptides, not even at a high concentration for the competitors (6×10^{-4} M). Comparable results were obtained with the samples collected at time zero and after 11 months (data not shown).

We investigated by competitive ELISA the cross-reactivity of anti-HVR1/c8b antibodies with the previous variant HVR1/c8a (Fig. 1). Four amino acid substitutions between HVR1/c8b and HVR1/c8a occurred in the region involved in the binding of antibodies. ELISA reactivity against the HVR1/c8b sequence was clearly competed for by the alternative variant, HVR1/c8a, in all the samples collected, as shown in Fig. 4 for the sample collected at 18 months.

Humoral immune response to HVR1 in 142 HCV-seropositive individuals. Immune reactivity to HVR1 variants in a collection of serum samples positive for HCV antibodies was investigated. These serum samples were analyzed by PCR for the presence of the virus, and 121 of them were found to be positive. Most of the viremic individuals (113) have been characterized for the viral genotype: 16 were infected by genotype 1a, 50 were infected by genotype 1b, 37 were infected by

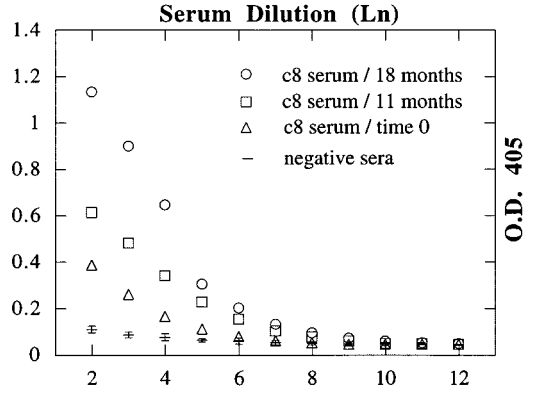

FIG. 3. Titration of antibodies specific for the HVR1/c8b sequence in patient c8 during follow-up. Error bars indicate the standard deviation from the mean of the negative controls. OD 405, optical density at 405 nm.

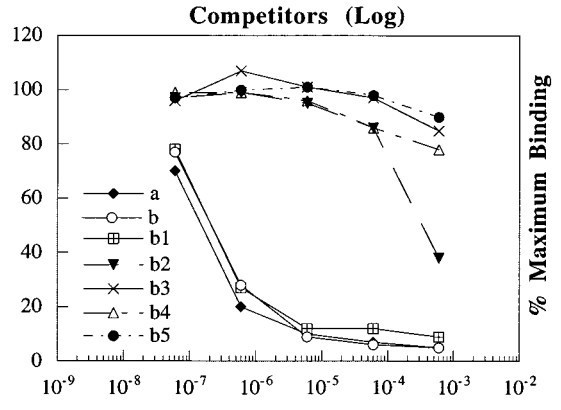

FIG. 4. Competition for binding to HVR1/c8b peptide in c8 serum sample collected at 11-month follow-up. Competition was performed by adding equivalent molar concentrations of the HVR1/c8a peptide (a), of the HVR1/c8b peptide (b), and of the five smaller overlapping peptides encompassing the HVR1/c8b sequence (b1 to b5).

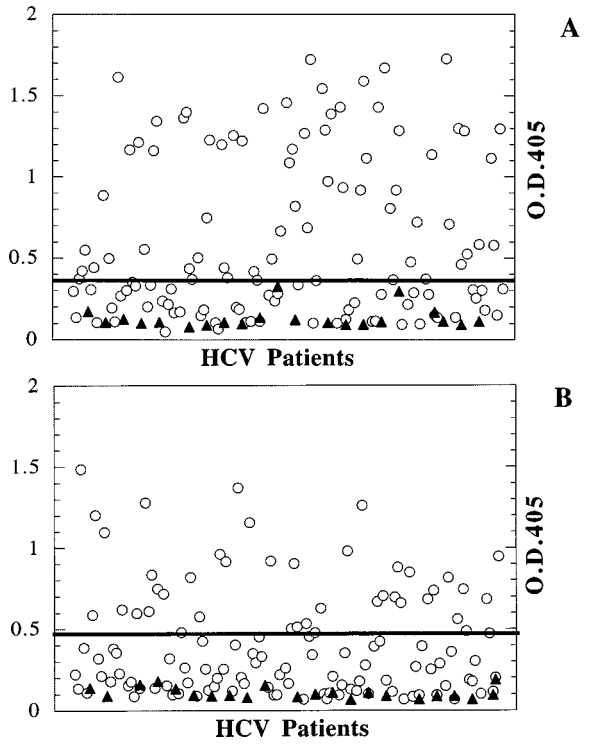

FIG. 5. Immune reactivity in ELISA against HVR1/c8b (A) and HVR1/BK (B) in HCV-infected patients. Symbols: ○, viremic patients; ▲, nonviremic patients. The cutoff value is represented by a line. OD 405, optical density at 405 nm.

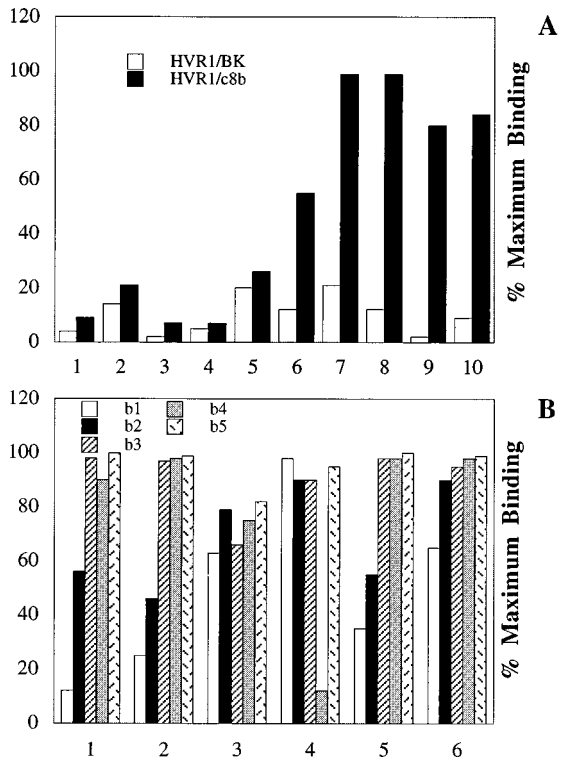

FIG. 6. (A) Competition for binding to the HVR1/BK peptide in serum samples from 10 HCV-infected patients. HVR1/BK and HVR1/c8b were used as competitors at a concentration of 6×10^{-4} M. (B) Competition for binding to HVR1/BK in serum samples, from six patients, which exhibited cross-reactive antibodies. The five small overlapping peptides encompassing the HVR1/c8b sequence (b1 to b5) were used as competitors at a concentration of 6×10^{-4} M.

genotype 2a, and 10 were infected by genotype 3a (38). This collection of serum samples was tested by ELISA against the HVR1 variant derived from the patient described above (HVR1/c8b) and a second peptide corresponding to the sequence of the prototype BK. The sequences of the two 27-amino-acid-long peptides (Fig. 2) differ by 13 amino acids, and they are both derived from viruses of genotype 1b. More than half (58%) of the viremic individuals tested were positive for antibodies to the HVR1/c8b sequence (Fig. 5A), and 32% of them were positive for antibodies to the BK variant (Fig. 5B). Interestingly, 25% were positive for antibodies to both. A higher frequency of positive results for antibodies against HVR1/BK occurred in individuals infected by genotype 1b than in individuals infected by other genotypes (data not shown). The relevance of this observation needs to be confirmed by further investigations.

Determination of cross-reactivity in 10 viremic HCV-infected patients. Competition experiments were performed in order to investigate if antibodies able to recognize both variants were present in patients' sera. Ten HCV-infected individuals were chosen on the basis of their immune reactivity against both HVR1 variants tested. The ELISA plates were coated with HVR1/BK, and the reactivity to this sequence was subjected to competition with the same variant that was used for coating and with the alternative variant HVR1/c8b (Fig. 6A). Cross-reactive antibodies were found in the serum samples of six patients. Epitope mapping with these samples was performed (Fig. 6B) by competition of the reactivity to HVR1/BK with the five overlapping peptides corresponding to the HVR1/c8b variant (b1 to b5; Fig. 2). Interestingly, for five of six patients, the major epitope was located between aa 16 and 22, as in the patient described above. In this region only valine at position 17 and phenylalanine at position 20 are conserved between HVR1/c8b and the BK variant and may thus be crucial for binding. For one patient the epitope was located in the amino-terminal part (from aa 1 to 10; Fig. 2), where HVR1 is much more conserved.

Determination of reactivity against HVR1 in immunized mice. Mice were immunized with a construct containing eight copies of the peptide HVR1/c8b linked to a polylysin core. These multimeric structures of peptides have been proven to be good immunogens and to elicit a high titer of antibodies (18, 40). Sera of immunized animals were tested by ELISA for their reactivity against the linear form of the peptide HVR1/c8b. Three of ten mice immunized have been found to be positive. The mouse with the strongest antibody titer was chosen for the characterization of the immune response by competition experiments (data not shown). The mouse serum, as well as most of the human sera studied, was reactive for the region between aa 16 and 22. Moreover, the mouse serum analyzed was cross-reactive with the variant HVR1/c8a but not with HVR1/BK. Further experiments are in progress, with the aim of characterizing the immunogenic properties of HVR1 in this animal model.

DISCUSSION

In many viral infections the host immune system produces antibodies able to bind and inactivate the virions, generally referred to as neutralizing antibodies. The production of neutralizing antibodies in many viral infections coincides with the onset of recovery from the disease. HCV causes chronic infec-

tions in most patients, and the mechanism proposed as responsible for persistence is the emergence of viral mutants that can evade the host immune response. In other diseases caused by RNA viruses, including foot-and-mouth virus disease (7) and human immunodeficiency virus (14), the targets of neutralizing antibodies are epitopes that show a high degree of sequence variation between different isolates and within one isolate over time. The amino terminus of the HCV E2 (gp70) protein, HVR1, shows similar properties. In fact, several variants have been isolated (10, 15, 16, 43), and sequence diversification during the course of infection has been described (10, 19, 21, 26, 31, 34). From a structural point of view, this region lacks conserved secondary structural motifs and changes may not interfere with the global conformation of the protein (41). Humoral immune response seems to play a major role in sequence diversification in infected chimpanzees (42). Moreover, an agammaglobulinemic patient has been shown to carry the same viral isolate for a long time (20).

We have investigated the immune response against HVR1 in a viremic HCV-infected patient over a period of 18 months. The follow-up of this patient showed that anti-HVR1 specific antibodies coexist in the bloodstream and even precede the viral sequence they recognize. The antibody titer increased in the samples taken subsequent to time zero, but the viral isolate survived in the bloodstream in the presence of increasing amounts of specific antibodies for 7 months. A single epitope, mapping at the carboxyl-terminal part of this hypervariable region (aa 16 to 22), was recognized by all three blood samples collected at different times. Cross-competition experiments provided an explanation for the occurrence of antibodies prior to the detection of the viral sequence in the bloodstream. In fact, these antibodies were demonstrated to be cross-reactive with the viral variant present earlier, differing by 4 amino acids in the epitope region. On the basis of these data, a simple correlation between presence of isolate-specific anti-HVR1 antibodies and clearance of the viral isolate cannot be accepted. Moreover, further studies are needed to clarify which characteristics are required by anti-HVR1 antibodies in order to acquire virus neutralization ability.

Until now, a few patients have been characterized for their immune response against HVR1 (22). We have tested the serum samples of 142 HCV-seropositive individuals for their reactivity against two HVR1 variants by ELISA. Antibodies against one of the two HVR1 variants have been found with high frequency (58%) in viremic individuals. None of the 21 nonviremic individuals tested were found to be positive for anti-HVR1 antibodies. These data are in agreement with those published concerning the immune response against full-length E2 recombinant protein, which also seems to be more frequently recognized by viremic individuals (2, 28). Recently, Mink and coworkers (24), searching for immunogenic epitopes within the conserved part of the E2 sequence from a yeast peptide library, identified only one antigenic domain recognized by 20% of the HCV-seropositive individuals. We thus believe that HVR1 itself may be an immunodominant epitope accounting for most of the reaction against E2, even though we cannot rule out the existence of other crucial epitopes.

The detection of anti-HVR1 antibodies prior to the appearance of the specific viral sequence in a viremic patient has already been described (17). Our findings confirm this observation, suggesting that cross-reactivity may be quite a common event for anti-HVR1 antibodies. We have found that 25% of the patients tested were positive for antibodies against two HVR1 variants which shared only 50% of their amino acid sequence. Cross-competition experiments were performed with samples from 10 patients who were positive for antibodies to both variants. We show that in six patients of ten the immune reaction against one HVR1 variant can be competed for by the variant itself and, to a lesser extent, by the alternative variant. One common epitope, which maps at the carboxyl-terminal part of HVR1 (aa 16 to 22), was recognized by five of the six serum samples containing cross-reactive antibodies. Moreover, preliminary results suggest that the same region is also immunogenic for mice in which we have induced cross-reactive antibodies by immunization with a single HVR1 variant.

Complex cross-reactivity has previously been shown for peptides derived from the gp120 V3 loop of human immunodeficiency virus (9, 27). The mechanism for viral escape described as the "original antigenic sin," observed first in influenza virus, infection and in togavirus, paramyxovirus, and enterovirus (5), has recently been suggested to be active also for human immunodeficiency virus infection (27). A strong immune response to an immunodominant and cross-reactive antigenic determinant, achieved after the first immunization, limits and fixes the B-cell repertoire that could be selected afterwards. In fact, a second infection with related strains, or the generation of such viruses during persistent infection, would not select new B cells but would give a secondary immune response to the original antigen, which is inefficient to neutralize the new variant. We propose that such an immunological mechanism is also active for HCV infection, at least in some patients. If further investigations confirm that HVR1 of the E2 (gp70) contains immunodominant and cross-reactive epitopes, this would obviously have to be taken into account when designing an envelope-based vaccine.

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