Multiple Sequence-Reactive Antibodies Induced by a Single Peptide Immunization with Hypervariable Region 1 of Hepatitis C Virus

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Hypervariable region 1 (HVR1) of hepatitis C virus (HCV) is known to contain neutralizing epitopes. We previously found that murine antibodies against HVR1-#6 captured a different isolate, HCV-#7, and cross-reacted with the HVR1 peptide of HCV-#7. We investigated the inducibility and generality of cross-reaction of animal anti-HVR1 antibody responses in this study. Anti-HVR1-#7 antibodies, which were induced in mice and a chimpanzee by immunization, were found to be cross-reactive to HVR1-#6 peptide. Antibody responses against HVR1-#6-1 and HVR1-#7 peptides were detected in 11/165 (6.7%) and 26/165 (15.8%) HCV-infected individuals, respectively. Nine HVR1 sequences from six individuals, who were strongly positive for anti-HVR1-#7 antibodies, were only 50–64.5% identical to that of HVR1-#7. All nine of these HVR1 peptides were reactive to sera from the six patients and/or to antisera against HVR1-#6 and HVR1-#7 produced in mice and chimpanzees. Cross-inhibition tests of chimpanzee antisera indicated that a given species of anti-HVR1 antibodies was reactive to multiple HVR1 sequences. Fine epitope mapping of polyclonal and monoclonal anti-HVR1 antibodies showed that conserved subregions in HVR1 sequences determined the observed immunological cross-reactivity. Our data demonstrate that cross-reacting anti-HVR1 antibodies are inducible by a single peptide immunization.

INTRODUCTION

Hepatitis C virus (HCV) infection has been identified as the major cause of posttransfusional and community-acquired non-A, non-B hepatitis (Choo et al., 1989; Kuo et al., 1989; Alter et al., 1992). More than half of the infections become chronic, and a high number of patients has associated cirrhosis and hepatocellular carcinoma (Saito et al., 1990). Diagnostic procedures have been established following the molecular cloning and expression of the HCV genome (Choo et al., 1989; Kuo et al., 1989; Brown et al., 1992). Prevention of HCV infection, however, remains an urgent challenge due to the high mutation rate of HCV.

Hypervariable region 1 (HVR1), which is located in the amino-terminus of E2 of HCV, may contain epitopes for neutralizing antibodies (Weiner et al., 1992; Kato et al., 1993; Taniguchi et al., 1993; Kojima et al., 1994). Antibodies to HVR1 in human sera have been shown to block viral attachment (Shimizu et al., 1994; Zibert et al., 1995) and to protect chimpanzees from HCV infection (Farci et al., 1994). Animal antibodies raised to this region have prevented viral infection in cell cultures (Shimizu et al., 1996) and have provided effective prophylaxis in chimpanzee challenge experiments (Farci et al., 1996; M. Esumi et al., 1999). HCV isolates, however, show markedly different sequences in HVR1 among patients, and there are often multiple closely related variants or quasi-species in an individual during the course of the infection (Weiner et al., 1992; Martell et al., 1992; Kato et al., 1994; van Doorn et al., 1995). The high HVR1 mutation rate is hypothesized to be caused by immune selection, which may generate escape mutants and lead to persistent infection even after development of antibodies against HVR1 in the sera of patients (Weiner et al., 1992; Kato et al., 1993; Taniguchi et al., 1993). Hence, it remains doubtful whether HVR1 may be used to develop a prophylactic vaccine because of this high HVR1 sequence heterogeneity among diverse virus isolates. The development of an effective and readily applicable vaccine for HCV infection may therefore require a broadening of the reactivity of anti-HVR1 antibodies.

We recently demonstrated that HCV can be captured by murine anti-HVR1 antibodies against different HCV isolates in vitro and that the HVR1 sequence from the captured HCV is cross-reactive with anti-HVR1 antibodies in ELISA (Esumi et al., 1998). To investigate the applicability of an anti-HVR1 antibody response in the development of a broadly reactive pattern, we examined the cross-reactivity of anti-HVR1 antibody response not only in HCV-infected patients but also in experimental animal models immunized with synthetic HVR1 peptides.
Furthermore, we tried to determine the basis of this cross-reactivity by fine epitope mapping of chimpanzee anti-HVR1 antibodies and mouse monoclonal antibodies (mAbs) cross-reactive to different HVR1 sequences.

RESULTS

Reactivity of anti-HVR1-#7 antibodies to HVR1-#6-1 peptide

We recently found that murine anti-HVR1 antibodies against HCV-#6 were able to capture a different HCV isolate, i.e., HCV-#7, in vitro and that the antibodies were cross-reactive to HVR1-#7 (Esumi et al., 1998). Chimpanzee antiserum obtained by hyperimmunization with HVR1-#6 peptides was also reactive to HVR1-#7 (data not shown). The amino acid sequences of these two HVR1 are shown in Fig. 1a. To further characterize this cross-reactivity, we examined mutual reactivity, that is, anti-HVR1-#7 antibody cross-reactivity to HVR1-#6-1 peptide. We obtained anti-HVR1-#7 antibodies by immunizing mice and a chimpanzee with synthetic HVR1-#7 peptide. The humoral immune response to these peptides was similar in six mice, three each of the BDF1 and C57BL/6J strains (data not shown). The antibodies against HVR1-#7 in all three BDF1 mice and two out of three C57BL/6J mice were reactive with HVR1-#6-1 peptide. Figure 1b shows the representative titration of anti-HVR1-#7 antibodies to HVR1-#6-1 peptide from a BDF1 mouse. Similarly, as shown in Fig. 1b, antibodies against HVR1-#7 produced in the chimpanzee were also reactive to HVR1-#6-1. Therefore anti-HVR1-#7 antibodies induced in mice and a chimpanzee had the capacity to recognize a different HVR1 sequence, HVR1-#6-1. Taken together with the finding of anti-HVR1-#6 antibodies reactive to the HVR1-#7 sequence (Esumi et al., 1998), the antibodies against HVR1-#6 and HVR1-#7 sequences are presumably mutually cross-reactive to each of these two HVR1 sequences.

HVR1 sequences in HCV-infected patients sera positive for anti-HVR1-#6-1 and/or HVR1-#7 antibodies

To examine whether animal antisera against HVR1-#6-1 and HVR1-#7 react with other HCV HVR1 sequences, we first identified these HCV HVR1 candidates by screening HCV-infected individuals who were positive for anti-HVR1-#6-1 and/or anti-HVR1-#7 antibodies. The HVR1 sequences in these individuals were presumed to be regions potentially reactive to the two anti-HVR1 antibodies. This was based on the observation that most HCV-infected patients develop antibodies against HVR1 (Weiner et al., 1992; Kato et al., 1993; Kojima et al., 1994). Table 1 shows the anti-HVR1 antibody positive rates in a total of 165 HCV-infected individuals. As previously reported (Scarselli et al., 1995; Jackson et al., 1997), multi-

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Note. The antibody response was tested by peptide ELISA. Values of absorbance at 450 nm more than 0.25 were considered positive. Positive rates of respective anti-HVR1 antibodies are shown as percentages in parentheses.
ple HCV-infected patient sera were reactive to an unrelated patient’s HCV HVR1 sequence. Of the 29 patients positive for either of these two anti-HVR1 antibodies, 27 were positive for HCV RNA with three genotypes, 1b, 2a, and 2b. Interestingly, eight samples were simultaneously positive for antibodies to both #6-1 and #7 sequences.

We selected six samples strongly reactive to HVR1-#6-1 and/or HVR1-#7 peptides, S1422, S6669, P33-1, P33-2, S3636-1, and S3636-2. The first three sera were positive for both antibodies, whereas the last three were positive only for anti-HVR1-#7 antibodies. The deduced amino acid sequences of HVR1 in these individuals and the sequences of HVR1-#6-1 and #7 differed substantially. The identity of these HVR1 sequences was only 50–64.5%.

To determine the evolutionary relationship between these HVR1 sequences, a 101-bp fragment from the beginning of E2 region covering the HVR1 sequence was used to construct a phylogenetic tree (Fig. 2b). NT-1–NT-5 constituted 101-bp sequences of HVR1 at five consecutive time points from an HCV-infected patient (Kurosaki et al., 1994) and were used as a reference group.

FIG. 2. Alignment of deduced amino acid sequences of HVR1 (amino acid 384-414) from HCV-infected individuals (a) and phylogenetic tree of the nucleotide sequences (b). (a) Six patients, S1422–S8350, were positive for antibodies against HVR1-#6-1 and/or HVR1-#7 peptides. Patient P32, negative for both anti-HVR1-#6-1 and anti-HVR1-#7, served as a negative control. The subclone analysis shown on the right indicates quasispecies in S33, S3636 and S1. (b) NT-1–NT-5 were reference related HVR1 sequences. The scale shows the genetic distance. NC, negative control sequence of patient P32. Bootstrap analysis was performed 1000 times and the values are shown at each branch.
The reference sequences, NT-1–NT-5, were closely related, whereas other HVR1 clones were not closely related to each other, most notably not to either #6-1 or #7. Bootstrap values also confirmed the unrelatedness of these sequences except for quasispecies of HCV within the individual patients, P33, S1, and S3636.

Antibody responses to HVR1 sequences of HCV-infected patients and immunized animals

To determine whether the HCV-infected individual sera selected by the presence of an antibody response to HVR1-#6-1 and/or HVR1-#7 were also reactive to each other's HVR1 sequences, each respective synthetic peptide of these nine HVR1 sequences (Fig. 2a) was tested for the reactivity to each serum of these patients by ELISA. P32 served as a negative control peptide. All positive reactivity between HVR1 peptides and patient sera was confirmed by >50% inhibition on the inhibition test (data not shown). Figure 3a shows that HVR1 peptides of S1422, S3636-1, S3636-2, S1-1, S1-2, and S8350 reacted with antibodies in four to all of these six patients, and peptide P33-2 weakly reacted with one patient serum S1.

Sera from HCV-infected individuals contain all antibodies generated in response to the antigenic drift of HCV or multiple infection, that is, not only from the response to the present HCV infection but also from that to past infection. It is difficult to accurately determine whether different antibodies to different HVR1 or cross-reactive antibodies react to the unrelated HVR1 sequences. We examined the cross-reactivity of animal antisera against HVR1-#6-1 and HVR1-#7 to the respective synthetic peptides. Figure 3b shows that, of a total of nine HVR1 peptides, eight reacted with both chimpanzee anti-HVR1 antibodies against HVR1-#6-1 and HVR1-#7. Similarly, seven and six HVR1 peptides were capable of reacting with mouse antibodies against HVR1-#6-1 and HVR1-#7, respectively. Only one peptide, S1422, did not react with anti-HVR1 antibodies produced by either the chimpanzees or mice, but this peptide reacted with the sera from five HCV-infected individuals including patient S1422 (Fig. 3a). On the other hand, the S6669 and P33-1 peptides showed no reactivity in HCV-infected individuals (Fig. 3a) but reacted with chimpanzee anti-HVR1 antibodies and/or mouse anti-HVR1 antibodies (Fig. 3b). The negative control sera showed no reactivity to any peptide of these HVR1 sequences.

Inhibition of cross-reactivity with HVR1 peptides

To determine whether or not antibodies cross-reactive with various HVR1 sequences are the same species, we performed cross-inhibition tests with various HVR1 pep-
tides in each ELISA of chimpanzee antisera. Figure 4 shows one-to-one cross-reaction of 10 HVR1 peptides, which were cross-reactive to anti-HVR1-#6 and anti-HVR1-#7 antibodies. In most peptides, the cross-reaction was mutually inhibited by various soluble competing peptides except for negative control peptide P32. The reactivity of anti-HVR1-#6 to peptide S6669 was partially inhibited by several HVR1 peptides, but peptide S6669 inhibited all peptide reactions (Fig. 4a). Similarly, the reactivity of anti-HVR1-#7 antibodies to S6669 or P33-1 was also partially inhibited by most peptides, whereas peptide P33-1 completely inhibited all peptide reactions, as did peptide S6669 except in the case of P33-1 (Fig. 4b). Our findings suggest that these chimpanzee antisera essentially contain a given species of the antibody cross-reactive to multiple HVR1 sequences.

Fine epitope mapping of cross-reacting antibodies

To determine the molecular basis of the immunological cross-reactivity to HVR1, epitope mapping was performed using these two mutually cross-reacting antibodies, anti-HVR1-#6 and -#7 antibodies, and a serial of synthetic peptides as shown in Fig. 1a. We first identified that the cross-reacting epitopes were located within amino acid 395-414 region. Figure 5a shows that anti-HVR1-#6 antibodies were reactive to peptides 1-31 and 12-31 but not to peptide 1-20 of HVR1-#7 and that anti-HVR1-#7 antibodies were reactive to peptides 1-31 and 12-31 but not to peptide 1-20 of HVR1-#6. It is notable that neither anti-HVR1-#6 nor anti-HVR1-#7 reacted to peptide 11-30, but both antibodies cross-reacted to peptide 12-31. This suggests that the single amino acid at the
C terminus in this study is essential for the cross-reactivity. We then performed cross-inhibition ELISA using various length peptides as competing antigens to define the shortest sequence for the cross-reactivity. Because the cross-reacting epitopes were located within amino acid 395-414 as shown above, ELISA plates were coated by corresponding peptide 12-31. Figure 5b shows that the reactivity of anti-HVR1-#6 antibodies was significantly inhibited by peptides 12-31, 21-31, 22-31, and 24-31 derived from HVR1-#7. Similarly, peptides from same regions derived from HVR1-#6-1 significantly inhibited the reactivity of anti-HVR1-#7 antibodies (Fig. 5c). Peptide 26-31 showed no significant inhibition. Interestingly, the cross-inhibition also was abolished by a single amino acid deletion from the C terminus (Figs. 5b and c). Therefore the region of amino acid 407-414 in both HVR1-#6-1 and HVR1-#7 was considered the cross-reactive epitope of chimpanzee antibodies. A sequence comparison of this region in HVR1-#6-1 and HVR1-#7 (Fig. 1a) suggests that a conserved sequence in the C-terminal region of HVR1 gives rise to the cross-reactivity.

To further clarify whether the conserved sequence is responsible for the cross-reactivity to other HVR1 sequences after the antibodies were preincubated with corresponding peptides 21-31 and 24-31. The findings show that the cross-reactivity to other eight HVR1 sequences was significantly inhibited by both peptides 21-31 and 24-31. Figure 6 shows the representative results that the cross-reactivity to one of the other HVR1 sequences (S8350) was inhibited by peptides 21-31 and 24-31, respectively. The negative control peptide 1-20 showed no inhibition. The sequence at aa 24-31 of HVR1-S8350 is PAQKIQLV, of which six and five residues are identical to those of HVR1-#6 and HVR1-#7, respectively. These data indicate that the conserved sequence in the C-terminal region is indeed responsible for the cross-reactivity.

Monoclonal antibodies with cross-reactivity

To elucidate the mechanism of the cross-reactivity of HVR1 sequences furthermore, we developed two mAbs, each specific to HVR1-#6-1 and HVR1-#7, and examined their reactivity to other HVR1 sequences. The mAb against HVR1-#6-1, designated 30F3, was directed to amino acid 394-403 region, and the mAb against HVR1-#7, designated 5A9, to amino acid 384-393 region (data not shown). Figure 7 shows that both mAbs were cross-
reactive to another HVR1 sequence, respectively, among seven reactive peptides shown in Fig. 3b. mAb 30F3 was cross-reactive to HVR1-S1-1 (Fig. 7a), and mAb 5A9 was cross-reactive to HVR1-P33-1 (Fig. 7b). Neither of these two mAbs showed reactivity to other HVR1 sequences (data not shown). The epitope of each mAb for the cross-reactivity was determined by inhibition ELISA. The binding reactivity of mAb 30F3 to HVR1-#6-1 was inhibited not only by peptide #6-1(11-20) but also peptide S1-1(11-20) (Fig. 7c). Similarly, peptide 1-10 derived from both #7 and P33-1 inhibited the reactivity of mAb 5A9 to HVR1-#7 (Fig. 7d). The other 10-amino acid peptides in Fig. 1a showed no inhibition (data not shown). Amino acid comparison shows that 8 of 10 residues in amino acid 394-403 of #6-1 and S1-1 are identical (Fig. 7c) and that 7 of 10 in amino acid 384-393 of #7 are consistent with those of P33-1 (Fig. 7d). These data provide the further evidence that conserved subregions of HVR1 sequences indeed play an important role in the cross-reactivity.

**DISCUSSION**

We herein directly demonstrated by experimental immunization that immunological epitopes in HVR1 of HCV were reactive not only to the homologous HCV isolate but also to different HCV isolates. Such immunological cross-reactivity could be interpreted by the conserved subregions in different HVR1 sequences.

Scarselli et al., (1995) and Jackson et al., (1997) previously showed that anti-HVR1 antibodies in HCV-infected individuals could react with more than one variant of HVR1, as we also demonstrated in our present study. A single infected patient, however, had multiple quasispecies that could change progressively either by continuous mutation or by selective over-expression of a particular single variant (Weiner et al., 1992; Kato et al., 1993; Kurosaki et al., 1994). A wide range of anti-HVR1 antibodies might therefore be produced and accumulate in a patient over time, resulting in a broadening of the reactivity of anti-HVR1 antibodies. To exactly determine the immunological cross-reactivity of HVR1, we immunized experimental animals, chimpanzees and mice, for cross-reactive assessment (Fig. 3b). There were no accumulated antibodies against different HVR1 sequences in the sera of these experimental animals, but the anti-HVR1 antibodies reacted with multiple HVR1 sequences. Hence, our data provide direct evidence that HVR1 sequences can induce anti-HVR1 antibodies capable of reacting with many HVR1 sequences in vitro other than the original challenge sequence. This is in agreement with a recent observation on the cross-reacting mimotopes of HVR1 (Puntoriero et al., 1998). Additionally, the anti-HVR1 antibodies induced in the chimpanzees recognized more diverse sequences than those induced in mice (Fig. 3b). This probably means that the immune response to immunized HVR1 peptides in primates is more evolved than that in nonprimates.

The mechanism of HVR1 sequence cross-reactivity is not yet known. The cross-inhibition assay (Fig. 4) suggests that, at a minimum, a given antibody species reactive to the multiple HVR1 peptides is induced in immunized chimpanzees. The HVR1 sequences in our patients were remarkably divergent except several amino acids at the C termini (Fig. 2a). These relatively conserved C termini probably play roles in the cross-reactivity of anti-HVR1 antibodies. Actually, the epitope mapping of cross-reacting antibodies (Fig. 5) and the cross-

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**FIG. 6.** Inhibition of binding of anti-HVR1 antisera to solid phase HVR1-S8350 by soluble competing peptides. Chimpanzee anti-HVR1-#6 (a) and anti-HVR1-#7 (b) diluted adequately were preincubated with corresponding HVR1 peptides. The residual reactivity of the antisera was tested on ELISA plates coated by HVR1-S8350 peptide. The aa sequence of HVR1-S8350 is compared with those of HVR1-#6-1 (a) and HVR1-#7 (b) indicated at the top. Results are represented as a percentage of antibody binding measured under same conditions where competing peptides were replaced by PBS.
inhibition test to other HVR1 sequences (Fig. 6) demonstrate that antibody species against the C-terminal conserved sequences at amino acid 407-414 of HVR1 is responsible for the cross-reactivity to multiple HVR1 sequences. A recent report on the fine mapping of HVR1 using HCV-infected patient sera also suggests that the reactivity of isolate-independent anti-HVR1 antibodies is related partly to the C terminus of HVR1 (Zibert et al., 1997). Furthermore, fine mapping of the cross-reacting mAbs prepared from the mice immunized with HVR1-#6-1 or HVR1-#7 reveals that mostly identical amino acid sequences are responsible for the cross-reactivity (Figs. 7c and d). Therefore the cross-reactivity of the HVR1 sequences is mediated at least partially by the conserved fragments in HVR1.

Of interest is the observation that the cross-reactivity of antibodies against HVR1-#6 and HVR1-#7 was abolished completely after deletion of a single amino acid at the C terminus (Fig. 5a). Similarly, such a deletion also resulted in a complete loss of cross-reactivity as a cross-competing peptide (Figs. 5c and d). Indeed, we found that even a substitution of the residue at 414 blocked the significant inhibition (data not shown). This suggests that the residue at the C terminus of the peptide is an essential determinant for the cross-reactivity in this work. The mechanism responsible for this phenomenon remains unclear. It is likely to be related to the fact that immunization with the C-linked peptide results in antibodies reactive with a site in the peptide near the C-terminus (Dyrberg and Oldstone, 1986).

HVR1 has the potential to provide a viral antigen for vaccine development, and the cross-reactivity of HVR1 sequences is an essential consideration in the development of a broadly reactive vaccine to prevent HCV infection. We found that anti-HVR1 antibodies elicited by even a single peptide immunization in mice and chimpanzees
recognized many other HVR1 sequences in vitro. Our results highlight the applicability of HVR1 in the development of a broadly reactive antibody response. We compared 163 unrelated (exclusion of quasispecies) HVR1 sequences corresponding to amino acid 407-414 from the HCV database (National Institute of Genetics and Second Department of Medicine, Nagoya City University Medical School, Japan). The frequency of the amino acid identical to our cross-reacting epitope of chimpanzee antibodies was 20.2% (data not shown). It indicates that antibodies induced by a single HVR1 peptide immunization can react with 20% of HVR1 variants. Recently, a new strategy that can induce cross-reactive antibodies has also been developed using mimotopes of HVR1 (Puntoriero et al., 1998). However, it remains necessary to confirm whether the cross-reactivity of HVR1 sequences in vitro means cross-protection against HCV challenge in vivo. If so, it may eventually be possible to develop a polyvalent vaccine using a mixture of several HVR1 sequences that cover the reactivity of most HCV isolates.

MATERIALS AND METHODS

Human sera

Samples of HCV-infected sera were obtained from a panel of 56 blood donors who were positive for HCV RNA. Another 109 serum samples were taken from chronic HCV-infected patients. HCV infection was confirmed by the presence of anti-HCV antibodies and/or HCV RNA. The genotypes of HCV were determined as described previously (Okamoto et al., 1992). The sera were aliquoted and stored at −80°C.

Reverse transcription and PCR

Total RNA was extracted from 100 μl of serum after the addition of 1 ml of TRIzol Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. The extracted RNA was reversely transcribed into cDNA using 100 U Moloney murine leukemia virus reverse transcriptase (Life Technologies) and 100 pmol hexa random primer (Takara, Kyoto, Japan) in 20 μl of mixture. Half of the cDNA was subjected to nested polymerase chain reaction (PCR) by using 0.5 U Taq-DNA polymerase (Life Technologies) and 15 pmol of primers in a volume of 50 μl. One-tenth of the first PCR products was used in the second round of PCR. The outer primers were 5′-GCGGACGCTTGCCCTTACTATCC-3′ (nt 1410–1429) and 5′-GGGACACGGAGAGGTGGA-3′ (nt 1699–1680); and the inner primers, 5′-CATGGCGGGGAACTGGGCTA-3′ (nt 1430–1453) and 5′-CAGGGCAGTGCTTGTGTG-3′ (nt 1622–1599). The two rounds of PCR were both run with 35 cycles of 30 s at 94°C, 45 s at 55°C, and 1 min at 72°C, followed by extension for 10 min at 72°C.

HVR1 sequence analysis

Amplified PCR products of HVR1 were purified with a Microcon 100 (Amicon, Beverly, MA) and directly sequenced using a dye primer cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems, Foster City, CA) on a 310 automated DNA sequencer (Perkin-Elmer Applied Biosystems) according to the manufacturer's protocol. To detect HVR1 sequence variants in each sample, the purified PCR products were cloned into T-tailed pGEM4Z vectors by a T–A overhang cloning method (Marchuk et al., 1991). The plasmids were transformed into Escherichia coli JM109 competent cells (Toyobo, Osaka, Japan). At least five independent clones were analyzed for each sample with same primers as reported before (Esumi et al., 1998). The nucleotide sequences were translated by the DNASIS program version 3.6 (Hitachi Software Engineering, Yokohama, Japan). Phylogenetic analysis of 101-bp sequences covering the HVR1 was carried out with the neighbor-joining method (Saitou and Nei, 1987) using Clustal W 1.5 computer software (Thompson et al., 1994).

Peptide synthesis

The peptides were synthesized in an automated multipeptide synthesizer (PSSM-8, Shimadzu, Japan) according to the manufacturer's instructions. After being cleaved and lyophilized, each peptide was analyzed by reversed-phase high-performance liquid chromatography (HPLC) to check peptide purity. If two or more main HPLC peaks appeared, the peptides were subsequently purified by reverse HPLC with a SynProPep column (type RPC 18, 20 × 150 mm, Shimadzu). The authenticity of expected peptides was confirmed by amino acid sequence analysis using a PSQ10 amino acid sequencer (Shimadzu). The peptides used in the immunization of animals were coupled with an equal amount of maleimide-activated keyhole limpet hemocyanin (KLH) (PIERCE, Rockford, IL) by adding a cysteine at the C-terminus to confer an immune response to peptides in the animals.

Animal antisera

Immunogens and immunization procedures were basically the same as described previously (Esumi et al., 1998). HVR1-#6-1 and HVR1-#7 sequences, derived from HCV-#6 and HCV-#7, respectively, are shown in Fig. 1a. Three mice, which were of two different strains, BDF1 and C57BL/6J, were injected intraperitoneally and separately with 50 μg of HVR1-#6-1 and HVR1-#7 peptides conjugated with KLH, which were emulsified in an equal volume of complete Freund's adjuvant (CPA) (DIFCO, Detroit, MI), as the primary immunization for each of the mice. Next, three booster immunizations were carried out weekly in the same way except that the adjuvant was incomplete Freund's adjuvant (IFCA) (DIFCO). Five days
after the fourth booster immunization and 1 month after the third booster, antisera were collected (by sacrificing the mice) and aliquotted and stored at -20°C. The sera from mice that were immunized with KLH only, using the same method, served as a negative control.

Chimpanzee 260 was immunized subcutaneously using 200 µg of HVR1-#7 peptide coupled with KLH, at intervals of 4 weeks, which had first been emulsified with CFA and then with IFCA. The antiserum was collected 1 week after the fourth booster immunization. Anti-HVR1-#6 serum was obtained from chimpanzee 228 during a vaccination study of synthetic HVR1 peptides, #6-1, #6-2, and #6-3, quasispecies of HCV-#6 (Esumi et al., 1999). The animal was immunized with 500 µg each of synthetic peptides four times. The antiserum was collected 1 week after the final booster immunization.

Monoclonal antibodies

Three 8-week-old mice BDF1 were immunized intra-peritoneally by HVR1-#6-1 and -#7 peptides, respectively, as above. Spleen cells obtained 5 days after the last booster immunization were fused with myeloma cells P3-X63-Ag8-U1 (P3U1) from the BALB/c strain using polyethylene glycol 1500. Hybridoma cells were cloned by a limited dilution method in the presence of 10^6 peritoneal macrophages per well and were screened against both HVR1-#6-1 and HVR1-#7 peptides. Ascitic fluid was prepared in BALB/c mice, and the mAbs, 30F3 against HVR1-#6-1 and HVR1-#7 peptides. Ascitic fluid was pre-coated with each of the corresponding HVR1 peptides. Subsequent steps were the same as those of ELISA. The remaining binding reactivity of anti-HVR1 antibodies was expressed as the percentage of their binding reactivity measured under identical conditions except that the soluble competing HVR1 peptides were replaced by PBS.

ELISA and inhibition test

ELISA was performed on 96-well microtiter plates (Nunc, Roskilde, Denmark) as previously described (Ahmed et al., 1996). Serum samples were variously diluted with PBS containing 5% skim milk and 0.05% Tween 20, and 100-µl portions were added to the plate to determine the reactivity to the coated antigens. The negative control human sera were derived from 10 blood donors who were all negative for anti-HCV antibodies and HCV RNA, and had ALT levels <20 IU/L. The negative controls of chimpanzee sera and mouse sera were obtained from preimmunized chimpanzees and KLH-immunized mice, respectively. The cutoff was established as twice the average + 3 SD of negative control samples.

The inhibition ELISA test was performed to confirm the specificity of the positive binding reaction, to determine whether the same antibody species reactive to different HVR1 sequences, and to identify epitopes of cross-reactive anti-HVR1 antibodies. Adequately diluted antisera or mAbs were preincubated with various amounts of soluble competing HVR1 peptides in PBS (pH 7.4) for 1 h at 37°C. Mixtures then were transferred into ELISA wells precoated with each of the corresponding HVR1 peptides. Subsequent steps were the same as those of ELISA. The remaining binding reactivity of anti-HVR1 antibodies was expressed as the percentage of their binding reactivity measured under identical conditions except that the soluble competing HVR1 peptides were replaced by PBS.

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