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Monoclonal Antibodies to the Hypervariable Region 1 of Hepatitis C Virus Capture Virus and Inhibit Virus Adsorption to Susceptible Cells *in Vitro*

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To analyze the neutralizing-related activity of antibodies against the hypervariable region 1 (HVR1) of hepatitis C virus (HCV) in more detail, monoclonal antibodies (mAbs) against HVR1 were raised by immunizing various strains of mice with one of two synthetic HVR1 peptides that had been derived from two isolates of HCV. The epitope specificity of all six mAbs could be assigned by the use of a series of linear peptides in competitive ELISA. It seems that most subregions in the amino acid sequence of HVR1 can induce a humoral immune response in mice. All three mAbs specific to HVR1-6-1 had the ability to capture homologous HCV-6 and inhibit its absorption to susceptible cells *in vitro* despite the fact that the epitope of each mAb was at a different location in HVR1, whereas the other three mAbs specific to HVR1-7 could not capture HCV-6 nor inhibit the absorption of HCV-6 to susceptible cells. The data in this study suggest that mAbs against HVR1 can prevent the infectivity of HCV in an isolate-specific and epitope position-independent manner.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped virus with a positive-stranded RNA genome of nearly 9.5 kb encoding a polyprotein of about 3000 amino acids, which is post-translationally processed into functional proteins by cellular and viral proteinases. The structural proteins are located at the amino terminus, and include the core and two envelope glycoproteins, E1 and E2. The nonstructural proteins including NS2–NS5 antigens are encoded at the downstream end of the genome.

At the N-terminus of the E2 protein, there is a specific region of approximately 27–31 amino acids (aa) in length whose sequence shows considerable variability among different HCV isolates. This region is termed hypervariable region 1 (HVR1) (Hijikata *et al.,* 1991; Weiner *et al.,* 1991), and it has been postulated that the variability in the amino acid sequence of HVR1 among different isolates resulted from humoral immune pressure leading to the selection of escape mutants (Weiner *et al.,* 1992; Kato *et al.,* 1993). This interpretation has been further supported by the absence of such diversity in an agammaglobulinemic patient with HCV infection (Kumar *et al.,* 1994), and by the relatively less variability in the HVR1 sequence among HCV-infected hypogammaglobulinemic patients (Booth *et al.,* 1998) and among immunosuppressive patients (Ni *et al.,* 1999) than among normal HCV-infected patients. Indeed, antibodies to HVR1 in the human sera have the ability to block the absorption of HCV to cells *in vitro* (Shimizu *et al.,* 1994; Zibert *et al.,* 1995), and to protect chimpanzees from HCV infection (Farci *et al.,* 1994). Antibodies that were raised specifically against HVR1 in a rabbit inhibited viral infection in a cell culture (Shimizu *et al.,* 1996) and freed chimpanzees which were challenged by HCV from infection (Farci *et al.,* 1996). We also found that a chimpanzee in which higher titers of anti-HVR1 antibodies had been induced by active immunization with synthetic HVR1 peptides, could protect itself from infection upon being challenged by homologous HCV (Esumi *et al.,* 1999). Therefore, HVR1 may contain the neutralizing epitopes. However, it is questionable whether HVR1 may be applied as a candidate target for a vaccine because of the tremendous heterogeneity in this region among different HCV isolates. Although HVR1 shows great sequence variability, we found that animal polyclonal anti-HVR1 antibodies that had been raised against HCV-6, captured a different isolate of HCV-7, and that the anti-HVR1-6 antibodies cross-reacted with HVR1-7 (Esumi *et al.,* 1998). We further demonstrated that the cross-reactivity among HVR1 sequences was inducible by a single peptide immunization in mice and in chimpanzees, and that cross-reactivity is not a rare event (Zhou *et al.,* 1999). It appears that broadly cross-reactive anti-HVR1 antibodies can be induced with HVR1 peptides. Thus far, however, it remains unclear whether anti-HVR1 antibodies have neutralizing activity toward different HCV isolates. Therefore, it is of great interest to develop monoclonal antibodies (mAbs) specific to HVR1 to more specifically analyze the neutralizing phenomenon of anti-HVR1 antibodies.

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TABLE 1

Synthetic HVR1 Peptides Used for Immunization and Competitive ELISA

Designation	aa Position[a]	aa Sequence
6(1–31)	384–414 (1–31)	HTRVTGGVQGHVTSTLTSLFRPGASQKIQLV
7(1–31)	384–414 (1–31)	A-YT---A-S-TLRGV--F-S---------I
6(1–24)	384–403 (1–24)	HTRVTGGVQGHVTSTLTSLFRPGA
7(1–24)	384–403 (1–24)	A-YT---A-S-TLRGV--F-S---
6(1–10)	384–393 (1–10)	HTRVTGGVQG
6(7–16)	390–399 (7–16)	GVQGHVTSTL
6(11–20)	394–403 (11–20)	HVTSTLTSLF
6(17–26)	400–409 (17–26)	TSLFRPGASQ
6(22–31)	405–414 (22–31)	PGASQKIQLV
6(12–31)	395–414 (12–31)	VTSTLTSLFRPGASQKIQLV
6(12–30)	395–413 (12–30)	VTSTLTSLFRPGASQKIQL
6(17–31)	400–413 (17–31)	TSLFRPGASQKIQLV
7(1–10)	384–393 (1–10)	ATYTTGGAQS
7(7–16)	390–399 (7–16)	GAQSHTLRGV
7(11–20)	394–403 (11–20)	HTLRGVTSFF
7(17–26)	400–409 (17–26)	TSFFSPGASQ
7(22–31)	405–414 (22–31)	PGASQKIQLI
7(1–14)	384–397 (1–14)	ATYTTGGAQSHTLR
7(1–12)	384–395 (1–12)	ATYTTGGAQSHT
7(3–20)	386–403 (3–20)	YTTGGAQSHTLRGVTSFF
7(5–20)	388–403 (5–20)	TGGAQSHTLRGVTSFF

Note. Peptides 1–31 and 1–24 were used in the animal immunizations, and in ELISA as the antigens coated on the plate. A cysteine residue was added at the C-terminus of these peptides to couple with the carrier protein. The other peptides of various lengths were used as competing soluble antigens in competitive ELISA. Amino acid residues are represented by the single-letter code. Dashes indicate identical residues.

[a] Amino acid residues 384–414 at the N-terminus of the E2 protein are designated as aa residues 1–31 of hypervariable region 1 (HVR1) in the parentheses.

To date, cell culture systems in which efficient propagation of HCV occurs *in vitro*, and readily available animal models of HCV replication *in vivo*, have not been developed. In this study, we used alternative assays, i.e., the virus capture assay, which is based on the antibody–virus interaction (Hijikata *et al.,* 1993; Esumi *et al.,* 1998), and the assay of the inhibition of HCV absorption to susceptible cells (Shimizu *et al.,* 1996) to analyze the biological characteristics of six mouse mAbs specific to two HVR1 sequences, HVR1-6-1 and HVR1-7, which were mutually cross-reactive for the polyclonal antibodies (Esumi *et al.,* 1998; Zhou *et al.,* 1999). We attempted to search whether the mAbs have the neutralizing-related capacity to the infectivity of both homologous and heterologous HCV isolates *in vitro*.

RESULTS

Affinity and epitopes of anti-HVR1 mAbs

Three hybridomas that secrete HVR1-6-1-specific mAbs, 30F1, 30F3, and 30F4, and three hybridomas that secrete HVR1-7-specific mAbs, 5A2, 5A3, and 5A9, were produced. The 24-amino acid peptide and the 31-amino acid peptide of each of HVR1-6-1 and HVR1-7 that were used for immunizing mice of various strains, are shown in Table 1 (the first four peptides). Because we found that it was difficult to successfully produce anti-HVR1 mAbs with a strain of mice, we tried to immunize four strains of mice to develop the mAbs specific to HVR1-6-1 and HVR1-7. Table 2 summarizes the general characteristics of these mAbs. The affinity constant of each mAb to the respective 31-aa peptide was determined by surface plasmon resonance technology. As shown in Table 2, all six mAbs had a markedly low dissociation rate, and therefore each had a high affinity constant, ranging between 0.78 and 2.54 $\times$ 10^{-10} M. The titration curves of these mAbs against the respective HVR1 peptide in ELISA also indicated that these mAbs had comparable affinities (data not shown).

To define the linear epitopes responsible for mAb recognition, we performed competitive ELISA. The 24- or 31-aa peptide of HVR1-6-1 or HVR1-7 that had been injected in the mice, was immobilized on ELISA plates, and a series of synthetic HVR1 peptides covering different portions of the respective HVR1 sequence was used as the soluble competing antigens. The aa sequences of the HVR1 peptides coated on the plates and soluble competing antigens are shown in Table 1. Figure 1 shows the representative results. The epitopes of mAbs 30F1 and 30F3 were located in aa 1–10 and in aa 11–20 of HVR1-6-1, respectively (Fig. 1A), and the epitopes of mAbs 5A3 and 5A9 were defined in aa 7–16 and in aa

TABLE 2

Characteristics of Mouse mAbs against HVR1

mAb	Mouse	Immunogen[b]	Binding parameters[a] k_a ($s^{-1}M^{-1}$) ($\times 10^5$)	k_d (s^{-1}) ($\times 10^{-5}$)	K_D (M) ($\times 10^{-10}$)	Isotype	aa Position of epitope[c]
30F1	C57BL/6	6-1, 24 aa	3.71	7.71	2.08	IgG2b κ	1–10
30F3	BDF1	6-1, 31 aa	0.812	1.62	2.00	IgG2b κ	11–20
30F4	BALB/c	6-1, 31 aa	2.44	1.90	0.78	IgG1b κ	12–30
5A2	B6C3F1	7, 24 aa	2.57	6.52	2.54	IgG2b κ	3–14
5A3	B6C3F1	7, 24 aa	1.56	2.65	1.70	IgG2a κ	7–16
5A9	BDF1	7, 31 aa	1.70	2.41	1.42	IgG2b κ	1–10

[a] The binding activity of the mAb to the respective antigen was examined by BIACORE analysis. k_a, association rate constant; k_d, dissociation rate constant; K_D, affinity constant.

[b] Each mouse was immunized with either the full-length (31 aa) or the 24-aa peptide in which 7 aa at the C-terminus had been deleted, of HVR1-6-1 or HVR1-7.

[c] The epitopes were determined by competitive ELISA. The amino acid numbers of mAb recognition refer to Table 1.

1–10 of HVR1-7, respectively (Fig. 1B); each synthetic peptide with the sequence noted earlier exclusively and nearly completely inhibited the reactivity of the respective mAb to the corresponding coated immunogen. The epitope of mAb 30F4 was considered to be located in aa 12–30 of HVR1-6-1, since five 10-mer overlapping peptides did not inhibit the reactivity between the mAb and the immunogen, and only the peptides composed of aa

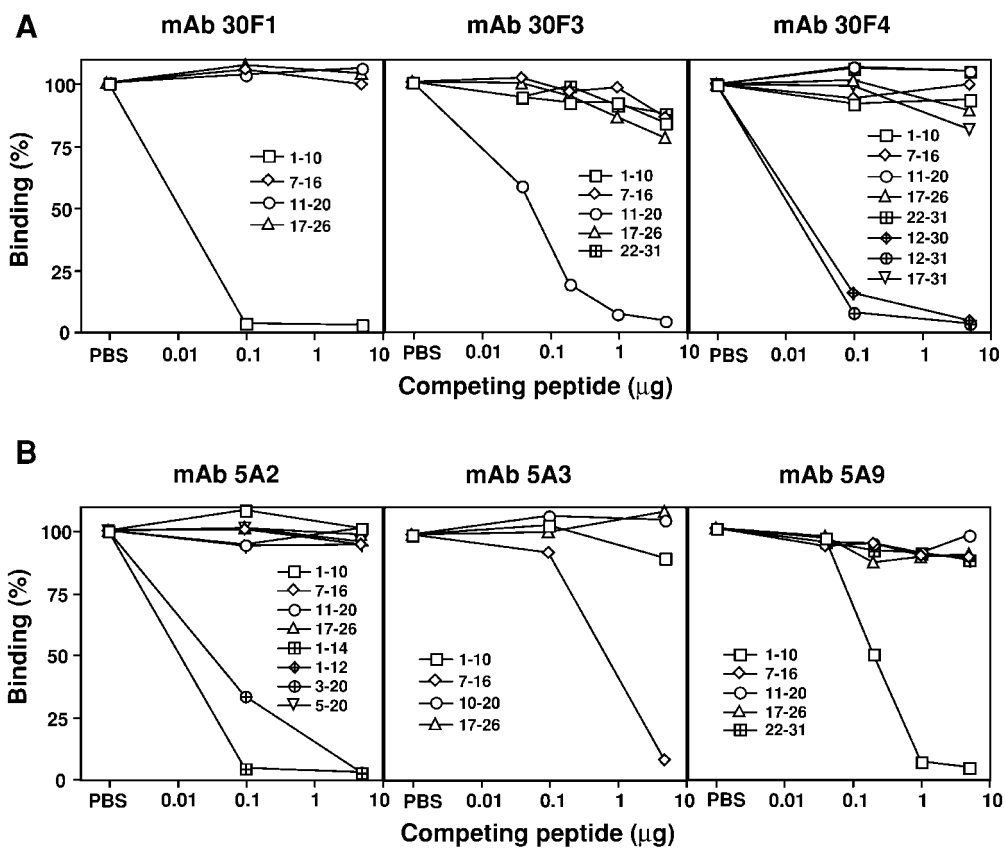

FIG. 1. Epitope mapping of mAbs by competitive ELISA. Binding reactivity of each indicated mAb to the solid-phase corresponding immunogens was inhibited by various competing liquid-phase peptides. Before addition to the ELISA plates, the mAbs at a certain concentration were preincubated with different competing peptides. The aa sequences of the competing peptides are shown in Table 1. The residual binding reactivity of mAbs is expressed as a percentage of the antibody binding activity of the respective mAb subject to the same experiment, except that the mAb was preincubated with PBS instead of competing peptides. (A) mAbs against HVR1-6-1; (B) mAbs against HVR1-7.

12–31 or aa 12–30 inhibited the reactivity (Fig. 1A). It is likely that the amino acids at both ends of the peptide, i.e., aa 12 to aa 16 and aa 21 to aa 30, played a critical role in the recognition of this mAb because neither peptide 17–31 nor peptide 11–20 inhibited the binding of the mAb to the immunogen (Fig. 1A). The epitope of mAb 5A2 was defined to be aa 3–14 in HVR1-7 because peptides 1–14 and 3–20 inhibited the binding of the mAb to the immunogen, whereas peptides 1–12 and 5–20 did not show significant inhibition (Fig. 1B). The epitope location of each of the six mAbs is summarized in Table 2. It seems that most subregions in HVR1 can induce a humoral immune response because the six epitopes of the six mAbs together covered the entire HVR1 sequence.

In our previous study, we found that the polyclonal antibodies against HVR1-6-1 and HVR1-7 induced in mice and in chimpanzees were mutually cross-reactive, and that both antibodies also cross-reacted with several other HVR1 peptides (Esumi *et al.,* 1998; Zhou *et al.,* 1999). Then, we attempted to examine whether the mAbs against these two HVR1 sequences were also reciprocally cross-reactive. All three mAbs against HVR1-6-1 were not reactive toward either 24-aa or 31-aa HVR1-7 peptide, and the three mAbs against HVR1-7 were not reactive toward either 24-aa or 31-aa HVR1-6-1 by ELISA (data not shown). However, one of the three mAbs against HVR1-6-1, 30F3, was cross-reactive to a different HVR1 sequence (HVR1-S1-1), and one mAb against HVR1-7, 5A9 was cross-reactive to another HVR1 sequence (HVR1-P33-1) (Zhou *et al.,* 1999). The remaining four mAbs were not reactive to each of the other seven HVR1 peptides that were cross-reactively positive for the mouse polyclonal antibodies (data not shown).

Virus capture by mAbs

Since an efficient *in vitro* replication system to propagate HCV to examine the neutralizing ability of the humoral immune response has not been developed, an alternative assay to evaluate the biological functions of the antibody was used to investigate the binding activity of the antibody to the virus (Hijikata *et al.,* 1993; Esumi *et al.,* 1998). We examined the binding activity of anti-HVR1-6-1 mAbs to homologous HCV-6 by adding a second antibody to immunoprecipitate the immune complexes. The cross-binding ability of anti-HVR1-7 mAbs to heterologous HCV-6 was also investigated because the polyclonal antibodies against HVR1-6-1 and those against HVR1-7 induced in animals were mutually cross-reactive (Esumi *et al.,* 1998; Zhou *et al.,* 1999). Figure 2 shows that two mAbs specific to HVR1-6-1, 30F1 and 30F3, completely captured the homologous viral isolate, since HCV RNA was detectable only in the precipitate and no HCV RNA remained in the supernatant (Fig. 2A). Another mAb specific to HVR1-6-1, 30F4, partially captured the virus qualitatively as some of the HCV RNA was detectable in

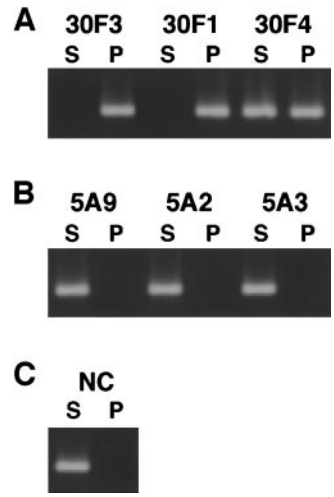

FIG. 2. PCR-based virus capture assays with mAbs. The mAbs were incubated with HCV-6, followed by the addition of anti-mouse IgG. The bound and unbound viral particles were separated by centrifugation into the supernatant (S) and precipitate (P), respectively. S and P were each tested for the presence of HCV RNA by RT-PCR. (A) mAbs specific to HVR1-6-1; (B) mAbs specific to HVR1-7; and (C) an unrelated mAb as a negative control. Two independent experiments were performed with the same results.

the supernatant (Fig. 2A). On the other hand, the three mAbs specific to HVR1-7 did not capture any of the HCV-6, as HCV RNA was detected only in the supernatant and not in the precipitate after HCV-6 was mixed with each of these three mAbs (Fig. 2B). The negative control mAb did not capture HCV-6, as only the supernatant was positive for HCV RNA as shown in Fig. 2C. We did not examine the binding activity of the anti-HVR1-7 mAbs to homologous HCV-7 nor the cross-binding of anti-HVR1-6-1 mAbs to HCV-7 since the viral isolate HCV-7 was no longer available.

We previously found that mAb 30F3, which is specific to HVR1-6-1, is cross-reactive to a different HVR1 sequence, HVR1-S1-1, and that mAb 5A9, which is specific to HVR1-7, is cross-reactive to a different HVR1 sequence, HVR1-P33-1 (Zhou *et al.,* 1999). We attempted to assess whether these two cross-reactive mAbs could capture the corresponding viral isolates. Unfortunately, both HCV-S1 and HCV-P33 existed in the immune-complex form in the serum after being subjected to the immune-complex assay (Hijikata *et al.,* 1993) and could not be used in these experiments (data not shown).

Inhibition of HCV absorption to cells by mAbs

Although an *in vitro* culture system for HCV is not available, an inhibition assay of viral absorption or attachment to presumed susceptible cells has been developed for assessing the neutralizing-related capacity of the antibodies (Shimizu *et al.,* 1994, 1996; Zibert *et al.,* 1995). In this study we used the HPBMa 10.2 cell line to examine the biological function of the mAbs. HPBMa

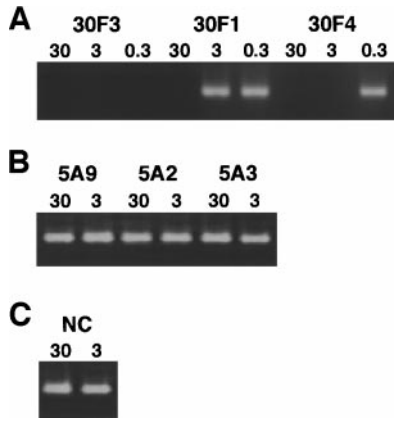

FIG. 3. Inhibition of HCV adsorption to HPBMa 10.2 cells by mAbs. A specific amount of HCV-6 was preincubated in 0.3, 3, or 30 μg/ml of the indicated mAb, and then the mixture was inoculated to HPBMa 10.2 cells. The quantity of cell-attached HCV was estimated by detecting HCV RNA with RT-PCR. (A) HCV-6 was preincubated in 0.3, 3, or 30 μg/ml of an mAb specific to HVR1-6-1. (B) HCV-6 was preincubated in 3 or 30 μg/ml of an mAb specific to HVR1-7. (C) HCV-6 was preincubated in 3 or 30 μg/ml of an unrelated mAb as a negative control. Each experiment was repeated twice and the same results were obtained.

10.2 cells are susceptible to *in vitro* infection of several HCV isolates, including HCV-6, and the infectivity of HCV in this system is correlated with the infectivity of HCV *in vivo* in chimpanzees (Shimizu *et al.,* 1993). Actually, the serum of a hyperimmune rabbit against HVR1, which neutralized *in vivo* the infection of HCV (Farci *et al.,* 1996), inhibited HCV absorption and infection *in vitro* to HPBMa 10.2 cells (Shimizu *et al.,* 1996).

A defined amount of HCV-6 was preincubated with different concentrations of each mAb. Each mixture was added to HPBMa 10.2 cells, and further incubated to allow the unneutralized HCV to adsorb to the cells. Figure 3 shows the adsorbed HCV to the cells which was detected by reverse transcription (RT) and nested-polymerase chain reaction (PCR). All three mAbs specific to HVR1-6-1 dose-dependently inhibited the absorption of HCV-6 to HPBMa 10.2 cells; HCV absorption to the cells was not detected in HCV that had been preincubated with higher concentrations of mAb (Fig. 3A). Even at an mAb concentration of 0.3 μg/ml, mAbs 30F3 showed the capacity to inhibit the binding of HCV-6 to HPBMa 10.2 cells (Fig. 3A). On the other hand, all three mAbs specific to HVR1-7 (Fig. 3B) and the negative control mAb (Fig. 3C) could not prevent the attachment of HCV-6 to HPBMa 10.2 cells even at a higher concentration of 30 μg/ml. Thus, mAbs against HVR1 had the isolate-specific absorption-inhibiting ability in an epitope position-independent manner.

DISCUSSION

HVR1 in HCV has been thought to contain neutralizing epitopes. In this study, we generated and characterized six mouse mAbs against two different HVR1 sequences and used them to investigate the neutralizing-related properties *in vitro*. We report for the first time that mAbs against HVR1 can capture HCV and inhibit the absorption of HCV to susceptible cells in an isolate-specific manner. These neutralizing-related activities were not dependent on the position of the epitope within HVR1; that is, antibody directed against many epitopes in HVR1 (i.e., antibody against the N-terminus, middle region, or C-terminus of HVR1) had the capacity to prevent HCV infection. This is in contrast with the observation by Zibert *et al.* (1997) that only anti-HVR1 antibodies directed against the epitope at the N-terminus were effective in eliminating HCV infection in HCV-infected patients, and in preventing HCV binding to human fibroblast cells *in vitro*. However, anti-HVR1 antibodies directed to the C-terminal region of HVR1 raised in a rabbit blocked HCV absorption to the cells (Shimizu *et al.,* 1996), and moreover proved to have neutralizing activity in chimpanzees challenged by the mixture of antibodies and homologous HCV (Farci *et al.,* 1996). In our HVR1 vaccine experiment, we also found that a high titer of anti-HVR1 antibodies that were directed mainly to the C-terminal region of HVR1, could protect a chimpanzee from the HCV challenge (unpublished data). Taken together, therefore, we consider that not only anti-HVR1 antibodies directed to the N-terminal region, but also antibodies to the other subregions of HVR1 have neutralizing activity to prevent the infectivity of HCV. This suggests that the entire HVR1 sequence of 31 aa at the N-terminus of the E2 protein is probably exposed outside and sterically free on the envelope of HCV, and that any of these differentially recognizing antibodies against HVR1 easily bind to the virion surface to inhibit the virus–host interaction. However, the true neutralizing activity of the mAbs remains to be further confirmed by experimental infection in chimpanzees because the methods we used were surrogate of the neutralizing assessment.

In addition, the epitopes of the three mAbs raised against HVR1-7 were also found to be in various locations within the HVR1 sequence (Fig. 1B). These data indicate that most subregions in the HVR1 sequence can induce a humoral immune response. This can be explained by the observation that HVR1 lacks a strongly predictive tertiary structure (Weiner *et al.,* 1992). Linear epitopes tend to be associated with less-structured regions of proteins, especially the ends of proteins. mAb 30F4, however, seemed to be induced not against a simple linear epitope, but against a semiconformational epitope because both short subregions within aa 12–16 and aa 21–30 were requisite for mAb recognition (Fig. 1A). This suggests that the combined structure of these two subregions was an epitope, or that the remaining subregion structure constructed by the interaction of the two subregions was the epitope.

HVR1 could be of interest for vaccine development. A

broadly reactive humoral immune response to HVR1 was inducible in mice and chimpanzees (Puntoriero *et al.,* 1998; Zhou *et al.,* 1999). However, it remains unclear whether a broadly neutralizing antibody response is inducible. We tried to examine such broadly neutralizing activity at the mAb level. Unfortunately, we did not produce anti-HVR1-7 mAbs cross-reactive to HVR1-6-1; therefore, such cross-neutralizing activity still remains to be clarified. We actually produced cross-reactive mAbs against different HVR1 (Zhou *et al.,* 1999); however, the corresponding HCV isolates were not available for the neutralizing assessment in this study. We previously found one broadly cross-reactive epitope located in the C-terminal subregion of HVR1, aa 24–31 (Zhou *et al.,* 1999). Unfortunately, in this study we did not produce mAbs that recognized aa 24–31 epitope. This may explain that the mAbs against HVR1-6-1 and HVR1-7 were not cross-reactive. The mAb against this subregion should be produced to assess the cross-neutralizing ability, to solve the challenging issue of how to overcome the high variability of the amino acids in HVR1 and induce a broadly neutralizing antibody response, for the development of an HCV vaccine using HVR1.

MATERIALS AND METHODS

Mouse mAbs and peptides

Monoclonal antibodies specific to HVR1-6-1, designated as 30F3, and mAbs specific to HVR1-7, designated as 5A9, were generated by immunizing BDF1 mice as previously described (Zhou *et al.,* 1999). Another mAb to HVR1-6-1, designated 30F4, was produced by immunizing BALB/c mice intralymphonodularly at both inguinal regions three times at an interval of 1–2 weeks with peptide 6(1–31) (Table 1). A monoclonal antibody specific to HVR1-6-1, designated as 30F1, in the C57BL/6 strain, and two monoclonal antibodies specific to HVR1-7 in the B6C3F1 strain, designated as 5A2 and 5A3, were generated by intraperitoneally immunizing mice four times at an interval of 1–2 weeks with HVR1 peptides of 24 aa in length in which the 7 aa at the C-terminus had been deleted, i.e., 6(1–24) or 7(1–24) (Table 1). Lymphonodular cells or spleen cells from the immunized mice were fused with myeloma cells P3-X63-Ag8-U1 (P3U1) from the BALB/c strain, and hybridomas were selected and maintained as previously described (Zhou *et al.,* 1999). A protein A column was used for affinity purification of mAbs from ascites fluid. The mAb isotype was determined with a Mouse-Hybridoma Subtyping Kit (Boehringer-Mannheim, Germany).

HVR1 peptides were synthesized using F-moc technology on a Shimadzu PSSM-8 Automated Synthesizer (Kyoto, Japan), purified by high-performance liquid chromatography (HPLC) using a SynProPep column (type RPC18, 20 × 150 mm, Shimadzu), and then coupled with an equal amount of maleimide-activated keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL) through a carboxyl-terminal cysteine residue as described previously (Esumi *et al.,* 1998). The aa sequences of the HVR1 peptides used in this study are shown in Table 1. In addition to the peptides used in the immunization, other overlapping peptides for each of HVR1-6-1 and HVR1-7 that span the full-length of HVR1 (Table 2) were synthesized for linear epitope mapping as previously described (Zhou *et al.,* 1999).

Affinity assessed by BIACORE

The antigen-binding property of each mAb was examined by biointeraction analysis using the BIACORE 2000 Biosensor (Biacore AB, Uppsala, Sweden). The purified synthetic HVR1 peptides, 6(1–31) and 7(1–31), to which a cysteine residue was added at the C-terminus, were immobilized on CM5 sensor chips by the use of the thiol coupling method according to the manufacturer's instructions. Various concentrations of each purified mAb were applied at a flow rate of 50 μl/min for 3 min in HBS running buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20), and the dissociation was measured with HBS running buffer for 30 min. The surface of the sensor chip was regenerated by 1-min pulses of 10 mM glycine–HCl buffer (pH 1.75). The rate constants of the association (k_a) and dissociation phases (k_d) were calculated using the BIAevaluation software. An apparent affinity constant (K_D) was calculated as the ratio of k_d to k_a.

ELISA and epitope mapping

The ELISA for screening antibodies to HVR1 and testing the cross-reactivity of the mAbs was performed with synthetic peptides essentially as described previously (Ahmed *et al.,* 1996). The epitope on the HVR1 sequence that was recognized by each mAb was determined by competitive ELISA as previously described (Zhou *et al.,* 1999). Briefly, the wells of microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μl of a 31- or 24-aa HVR1 peptide (Table 1) in 0.05 M carbonate buffer at a concentration of 1 μg/ml, washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), and blocked with 5% skim milk in PBS-T. Following a second wash, 100 μl of appropriately diluted mAbs that had been preincubated with different concentrations of competing peptides (Table 1) for 1 h at 37°C, was added and this was incubated for 1 h. Following the addition of peroxidase-conjugated goat anti-mouse polyvalent immunoglobulins (Sigma, St. Louis, MO) and color development, the absorbency at 450 nm was measured with a Multiskan Bichromatic System (Labsystems Inc., Helsinki, Finland). The remaining binding reactivity of the mAb was expressed as the percentage of the binding of the mAb measured under identical conditions, except that the competing peptides were replaced by PBS.

Virus capture

The virus capture assay of mAbs was performed essentially as described previously (Esumi *et al.,* 1998). Serum 6 (HCV-6) was obtained from a blood donor who was positive for both anti-HCV and HCV RNA, and was provided by a research group supported by Grants-in-Aid from the Japanese Ministry of Health and Welfare. The serum diluted by 10^{-2} times in PBS, was centrifuged at 16,000 rpm for 15 min at 4°C, and 50 μl of the supernatant was mixed with 5 μl of each mAb (1 mg/ml in PBS). The mixtures were incubated for 1 h at 4°C, and then 50 μl of 2 mg/ml of anti-mouse IgG goat F(ab′)2 (Organon Teknika, Durham, NC) was added. After further incubation for 1 h at 4°C, each mixture was centrifuged at 2800 rpm for 15 min at 4°C and separated into the supernatant and the pellet. Both the supernatant and the pellet were subjected to detection of HCV RNA by RT-nested PCR as described later. HCV was considered to be entirely captured by the mAb if HCV RNA was found only in the pellet and not in the supernatant. HCV was considered to be partially captured if both the supernatant and pellet contained HCV RNA. If HCV RNA was detected only in the supernatant, it meant that HCV was not captured.

Inhibition of HCV absorption to cultured cells

The HPBMa 10.2 cell line (Yoshikura, 1989) was used in the absorption of HCV isolates as previously described (Shimizu *et al.,* 1993, 1996). The cells were cultured in RPMI 1640 medium with 8% heat-inactivated fetal calf serum. One hundred microliters of undiluted or serial, 10-fold-diluted patient serum was added to 1 ml of the cell suspension of HPBMa 10.2 cells (5×10^5 cells). After incubation at 37°C for 2 h, the cells were washed twice with PBS by centrifugation at 1000 rpm at 20°C for 5 min. The pellet was subjected to detection of cell-adsorbed HCV RNA by RT-nested PCR as described later. The absorption titer of HCV to HPBMa 10.2 cells was estimated to be the sample of maximum dilution that was positive for HCV RNA. The absorption titer of HCV-6, whose genome titer in the serum was 10^5/ml, was 10^3/ml, which is approximately equal to the absorption titer of $10^{3.5}$/ml reported by Shimizu *et al.* (1993).

Inhibition of HCV absorption to HPBMa 10.2 cells was performed as previously described (Shimizu *et al.,* 1996) with some modification. One hundred microliters of the target virus corresponding to 100 absorption titers per milliliter, was incubated with an equal volume of different amounts of mAbs in PBS at 4°C overnight. Each mixture was inoculated to 1 ml of the cell suspension of HPBMa 10.2 cells (5×10^5 cells), and the following steps for determining the absorption titer are the same as those described earlier. The mAbs were inactivated at 56°C for 30 min before mixing with the target virus, and another mAb unrelated to HCV was used as the negative control.

RT-PCR

Total RNA was extracted with TRIzol Reagent (Life Technologies, Gaithersburg, MD), and was reverse-transcribed into cDNA with the RNA PCR Kit (Takara, Kyoto, Japan) using 50 pmol 9-mer random primer and 5 U Avian Myeloblastosis virus (AMV) reverse transcriptase in 20 μl of mixture. Half of the cDNA was subjected to nested PCR with the use of 1 U AmpliTaq Gold polymerase (PE Applied Biosystems, Foster City, CA) and 15 pmol of primers in a volume of 50 μl. One-tenth of the first PCR product was applied to a second round of PCR. The primers were the same as those previously reported (Esumi *et al.,* 1998; Zhou *et al.,* 1999). The thermal cycles of PCR were one cycle of 9 min at 95°C, 35 cycles of 30 s at 94°C, 60 s at 60°C, 60 s at 72°C, followed by extension at 72°C for 7 min.

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