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Recombinant antibody Fab against the hypervariable region 1 of hepatitis C virus blocks the virus adsorption to susceptible cells in vitro

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Abstract

Antibodies against hypervariable region 1 (HVR1) of hepatitis C virus (HCV) are putatively considered to be neutralizing. We previously found that monoclonal antibodies (mAbs) (30F1 and 30F3) against the HVR1 of HCV neutralize HCV in vitro. To develop potentially therapeutic molecules against HCV, we cloned cDNAs of antibody Fab fragments from the mouse hybridoma cells secreting these two mAbs. Fab fragments produced in *Escherichia coli* were purified by a single step of nickel-chelate affinity chromatography via a hexa-histidine tag. The specificity of the Fabs was confirmed by competition ELISA, BIAcore analysis, and N-terminal amino acid sequencing. The binding constant for the interaction with HVR1 was 1.39 nM for Fab 30F1 and 3.96 nM for Fab 30F3. The HCV capture assay and inhibition of HCV adsorption test demonstrated that both Fabs had neutralizing activity. The data may be useful for designing immunological therapy of HCV. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Hepatitis C virus; Hypervariable region 1; Immunoglobulin; Recombinant Fab; Antiviral effect

1. Introduction

Hepatitis C virus (HCV) infection is common, with an estimated 400 million chronic HCV carriers worldwide. Currently there is no specific means to prevent HCV infection, and interferon therapy benefits only 10–30% of HCV-infected patients. HCV is an enveloped RNA virus and possesses two putative envelope proteins E1 and E2. At the N-terminus of the E2 protein, there is a specific region of approximately 27–31 amino acids in length, termed hypervariable region 1 (HVR1) (Hijikata et al., 1991) because of its high degree of variation. The HVR1 may encode neutralizing B-cell epitopes (Weiner et al., 1992). Hyperimmune serum induced in a rabbit immunized by HVR1 peptide neutralized homologous HCV in chimpanzees (Farci et al., 1996) and blocked HCV adsorption to susceptible cells in

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vitro (Shimizu et al., 1996). A chimpanzee which had maintained high levels of anti-HVR1 antibodies after immunization with synthetic HVR1 peptides was protected against repeated homologous HCV challenge; moreover, the antisera from this chimpanzee neutralized homologous HCV in two other chimpanzees (Esumi et al., 1999; Goto et al., 2001). Therefore, anti-HVR1 antibodies induced by active immunization may be useful in developing prophylactic and therapeutic agents for HCV in humans.

Production of recombinant antibody Fab fragments has become an attractive means for developing therapeutic and other useful agents. It has been shown that recombinant human Fabs neutralize human immunodeficiency virus in vitro (Barbas et al., 1992). Multimeric humanized antibody fragments against the fusogen of varicella-zoster virus neutralize the virus in vitro (Drew et al., 2001). In a mouse model, human non-small-cell lung carcinoma was significantly reduced in mass and occurrence after treatment with recombinant products containing a Fab antibody to a superantigen (Forsberg et al., 2001).

We previously found that mouse monoclonal antibodies (mAbs) directed to the HVR1 of HCV neutralize homologous HCV in vitro in an isolate-specific manner (Zhou et al., 2000). To develop potentially therapeutic molecules against HCV, here, we produced and characterized Fab fragments from two lines of hybridoma cells, which secreted mAbs 30F1 and 30F3, respectively, by using a novel vector system for cloning and expressing immunoglobulin variable genes (Maeda et al., 1999). The antiviral activity of the recombinant Fabs was evaluated by an HCV capture assay and by inhibition of HCV adsorption to susceptible cells.

2. Materials and methods

2.1. Hybridoma cell lines

Two mouse hybridomas, 30F1 and 30F3, which produce mAbs 30F1 (IgG2b κ) and 30F3 (IgG2b κ), respectively, were used in this study. These two mAbs recognize epitopes in amino acid residues 1–10 and 11–20 in HVR1 of HCV-#6, respectively (Zhou et al., 2000).

2.2. Expression cloning of Fab cDNA

One microgram of total RNA, which was extracted from the hybridoma cells with TRIzol Reagent (Life Technologies, Gaithersburg, MD), was reverse transcribed into cDNA using the RNA PCR Kit (Takara, Kyoto, Japan) with 50 pmol of 9-mer random primer and 5 U of Avian Myeloblastosis virus reverse transcriptase in 20 μl of mixture. The cDNA was subjected to touch-down PCR (Don et al., 1991) using 20 pmol of degenerate primers in a volume of 100 μl for amplifying immunoglobulin H and L chains, respectively. The thermal cycles were the same as previously reported (Maeda et al., 1999). The primer sets from an Immunogene M Kit (Nisshinbo, Tokyo, Japan) were used. The products amplified with these primers covered VH-CH1 and VL-CL of the antibody mRNA. After purification, the L and H chains were, respectively, inserted into the expression vector pFab1-His2 as described previously (Maeda et al., 1999). The pFab1-His2 vector containing both L and H chains was used to transform *Escherichia coli* (*E. coli*) JM109. Sixty independent transformants for each Fab were cultured and expression was induced by 0.1 mM of isopropyl-B-D-thiogalactopyranoside (IPTG). Crude *E. coli* lysates, prepared by freeze–thawing, were used to screen for clones producing Fab reactive to HVR1 by ELISA. The H and L chain genes from the reactive Fab-producing plasmids were, respectively, recloned into the sequencing vectors CV-1 and CV-2 for analyzing the sequences of the Fab fragments as described (Maeda et al., 1999) on a PRISM 310 automated DNA sequencer (Applied Biosystems, Foster City, CA). The comparisons of murine germ line genes were carried out using DNAPLOT (http://www.genetik.uni-koeln.de/dnaplot).

2.3. ELISA

The ELISA for screening Fab fragments positive against HVR1 was performed essentially as described previously (Zhou et al., 1999). The

bacterial lysates were 2-fold diluted with 5% skim milk before being added to microtiter plates. After color development, the absorbance at 450 nm was measured with a Multiskan Bichromatic System (Labsystems Inc., Helsinki, Finland). The competition ELISA for confirming Fab epitope specificity was carried out as previously described (Zhou et al., 1999).

2.4. Production and purification of Fab fragments

JM109 transformants containing Fab-producing plasmids, pFab-30F1-1 and pFab-30F3-18, were cultured in 1 l of SB with 20 mM of $MgCl_2$ to produce recombinant Fabs as described elsewhere (Maeda et al., 1999). The bacterial lysate, prepared by sonicating *E. coli* pellets, was filtered through a 0.45 μm filter and subsequently subjected to affinity purification using HisTrap Columns (Pharmacia Biotech, Sweden). The imidazole concentration was 10 mM in both the lysate supernatant and wash buffer, and 500 mM in the elution buffer. Fractions containing the highest reactive Fab were combined and dialyzed against PBS. SDS-PAGE was performed to establish the molecular weight and purity of the purified Fabs. The protein concentration was determined with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

2.5. Binding analysis of purified Fab fragments

The antigen-binding property of each Fab was examined by biointeraction analysis using the BIAcore 2000 Biosensor (Biacore AB, Uppsala, Sweden) as described (Zhou et al., 2000). The rate constants of the association (k_a) and dissociation (k_d) phases were calculated using the BIAEVALUATION Software 2.1. An apparent affinity constant (K_D) was calculated as the ratio of k_d to k_a.

2.6. N-terminal amino acid sequencing

The H and L chains of bacterially synthesized Fab fragments were separated by SDS-PAGE and transferred to PVDF membranes by semi-dry blotting. The H and L chain bands were cut and sequenced on a PSQ10 amino acid sequencer (Shimadzu, Kyoto, Japan). The N-terminal ten amino acid residues of each chain of Fab were determined. For comparison, the N-terminal residues of each chain of the parent mAbs were also analyzed.

2.7. Virus capture activity of purified Fab fragments

The virus capture activity of the purified Fabs was tested as described previously (Esumi et al., 1998). A recombinant human Fab against human cytomegalovirus (Takekoshi et al., 1998) was included as a negative control. Briefly, 50 μl of 100-fold diluted serum #6 (HCV-#6) was incubated with 1–5 μl of each Fab (1 mg ml^{-1} in PBS) at 4 °C for 1 h, and then 50 μl of 2 mg ml^{-1} of anti-mouse or anti-human IgG goat $F(ab')_2$ was added. The mixtures were further incubated at 4 °C for 1 h. After centrifugation, HCV RNA in the immunoprecipitate and supernatant was detected by RT-nested PCR, respectively. HCV was considered to have been captured by the Fab if HCV RNA was found only in the precipitate and not in the supernatant and was considered to have been partially captured if both the supernatant and precipitate were positive for HCV RNA. If HCV RNA was detected only in the supernatant, this indicated that HCV had not been captured.

2.8. Inhibition of virus adsorption in vitro

Inhibition of HCV-#6 adsorption to HPBMa 10.2 cells by the purified Fabs was assayed as described (Shimizu et al., 1996; Zhou et al., 2000). Briefly, ten adsorption titers of HCV-#6 were incubated with 0.3–3 μg of each purified Fab in 200 μl of PBS at 4 °C overnight. Each mixture was inoculated into 1 ml suspension cultures of HPBMa 10.2 cells (5×10^5 cells). After incubation at 37 °C for 2 h, the cells were washed twice with PBS. After centrifugation, cell-adsorbed HCV RNA was detected in the cell pellet by RT-nested PCR. If HCV RNA was not detected, this indicated that adsorption of the virus to the cells had been blocked. If HCV RNA was detected in the cell pellet, it suggested that the virus had not been inactivated and could still adsorb to the cells.

3. Results

3.1. Recombinant Fab fragments produced in E. coli

We isolated four reactive clones from pFab-30F3 based on screening 60 transformants. For expressing Fab cDNA of 30F1, we selected the correct sequences to be inserted into the expression vector, resulting in all 60 screened transformants being positive for expression. From these Fab-producing clones, pFab-30F1-1 and pFab-30F3-18 were selected for production of Fab fragments in *E. coli*. The Fabs 30F1 and 30F3, purified by a single step of nickel affinity chromatography, showed two bands on SDS-PAGE with molecular weights of about 29 and 26 kDa, respectively (Fig. 1). The amounts of the polypeptides in the two bands appeared to be equal. Eluted fractions from the HisTrap Columns loaded with periplasmic extract of bacteria mock-transformed by the pFab1-His2 plasmid showed no bands at corresponding positions. This indicated that the 29- and 26-kDa polypeptides were the VH-CH1 and VL-CL of the antibody, respectively. The yields of purified Fabs for 30F1 and 30F3 were 1.2 and 1.5 mg proteins per liter culture, respectively.

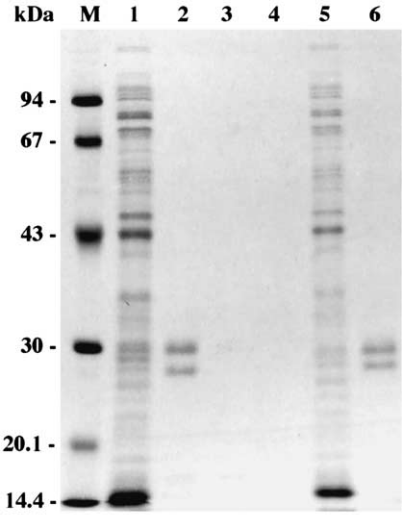

Fig. 1. SDS-PAGE of purified Fabs. Molecular mass markers (M) in kDa are shown on the left. Lanes 1–3, 30F1; lanes 4–6, 30F3; lanes 1 and 5, crude *E. coli* lysates; 2 and 6, purified recombinant Fabs; 3 and 4, eluted fractions of mock-transformed bacteria.

3.2. Specificity and binding characteristics of Fab fragments

To confirm the specificity of the Fabs synthesized in bacteria, we employed a competition ELISA. The reactivity of the purified recombinant Fabs 30F1 and 30F3 to HVR1-#6 was significantly inhibited by the peptides spanning residues 1–10 and 11–20, respectively (Fig. 2). This demonstrated that the epitopes of Fabs 30F1 and 30F3 were, respectively, the same as their parent mAbs (Zhou et al., 2000), confirming that the Fabs produced in bacteria were specific for HVR1-#6.

In a surface plasmon resonance analysis using a BIAcore instrument, with the antigen immobilized on the sensor chip, we found that the resonance units (RU) of both Fabs′ binding to HVR1-#6 increased with increasing Fab concentrations (Fig.

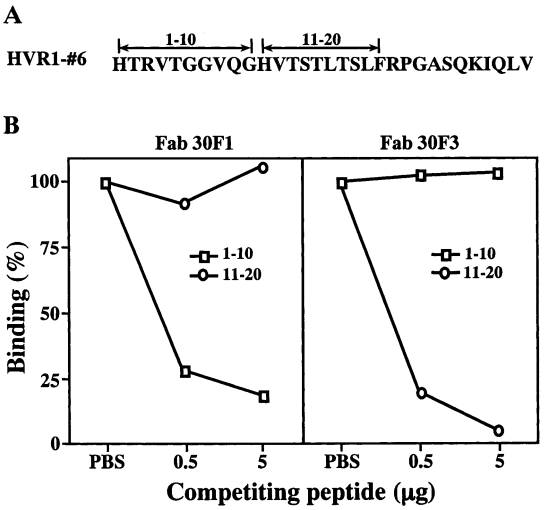

Fig. 2. Epitope confirmation of the Fab fragments by competition ELISA. Before addition to the ELISA plate, each Fab was preincubated with different competing peptides. The binding activity of Fab in PBS, in which no competitive peptide was added, served as a reference. The residual binding activity of the Fabs to HVR1-#6 is expressed as a percentage of the binding activity after preincubation only with PBS. (A) HVR1-#6 amino acid and the competing peptides 1–10 and 11–20; (B) inhibition of binding of Fabs 30F1 and 30F3.

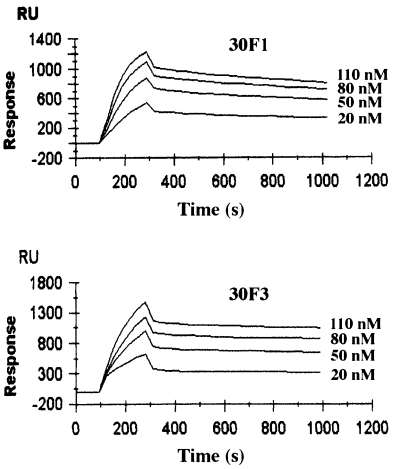

Fig. 3. BIAcore analysis of the Fab fragments. HVR1-#6 was immobilized on a sensor chip by thiol coupling. The surface plasmon resonance (RU) of each purified Fab at different concentrations from 20 to 110 nM was detected by BIAcore 2000.

3), while the RU of both Fabs′ binding to unrelated HVR1-#7 did not (data not shown). This also confirmed the specificity of the Fabs. Table 1 shows the binding kinetics of each purified Fab to HVR1-#6. Compared with the original mAbs, the binding affinities of both recombinant Fabs were reduced 5–7-fold. Quantitative titration of the purified Fabs in ELISA (data not shown) also indicated that the binding activity of both Fabs to HVR1 was lower than that of the original mAbs.

3.3. Sequences of Fab fragments

The amino acid residues of the variable regions of Fabs 30F1 and 30F3 were predicted from the nucleotide sequences (Fig. 4). The assignment of complementarity-determining regions (CDRs) was based on the Kabat numbering scheme. The VH genes of 30F1 and 30F3 utilized the germ lines of the VH1 and VH9 families, respectively; while the 30F1 VL gene was assigned to the Vκ1 family and 30F3 VL to the Vκ2 family. Within the family, the VH and Vκ genes of 30F1 were most similar to clone J558.51 (accession number AF303882) with 90.7% homology and to an anti-DNA antibody (accession number Z22135) with 98.2% homology, respectively; while the VH and Vκ genes of 30F3 were most similar to an antibody against a carcinoma-associated antigen (accession number AB016619) with 84.4% homology and to GB-1 antibody (accession number L22888) with 97.3% homology, respectively.

Table 1
Kinetic constants of Fabs and their parent mAbs

Clone	k_a ($\times 10^5$) (s^{-1} M^{-1})	k_d ($\times 10^{-4}$) (s^{-1})	K_D ($\times 10^{-9}$) (M)
Fab 30F1	1.81	2.50	1.39
MAb 30F1	3.71	0.77	0.21
Fab 30F3	1.89	7.48	3.96
MAb 30F3	0.81	0.16	0.20

Affinity constant (K_D) is the ratio of dissociation constant (k_d) to association constant (k_a).

The direct amino acid sequencing showed that the N-terminal ten residues of each chain of the Fab fragments were identical to the residues of each chain of the parent mAbs. The amino acid residues were also identical to those deduced from the nucleotide sequences as shown in Fig. 4.

3.4. Biological activity of Fab fragments

Since there is currently no in vitro replication system to propagate HCV for evaluating antiviral activity of the antibodies and because the only animal model, the chimpanzee, is not readily available, we employed two surrogate assays, virus capture and inhibition of virus adsorption to cells, to assess the potential antiviral activity of the purified Fabs directed to HVR1. Fig. 5A shows that Fabs 30F1 and 30F3 at a dose of 5 μg both captured the homologous HCV completely because there was no HCV RNA remaining in the supernatant. In contrast, unrelated Fab at the same dose failed to capture the HCV because HCV RNA was only detected in the supernatant. When 1 μg of Fab fragments was used, Fab 30F3 still captured HCV and Fab 30F1 partially captured the virus. These data show that the purified Fabs against HVR1 could capture HCV in a dose-dependent manner.

An inhibition assay of HCV adsorption to susceptible cells would be helpful to assess neutralizing activity of the recombinant Fabs. Fig. 5B

κ chain

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     1                                  CDR1                                CDR2
30F1 DVVMTQTPLSLPVSLGDQASISC RSSQSIVHDNGNTYLE WYLQKPGQSPKLLIY KVSSRFS GVPDRFSGS
30F3 ---------T-S-TI-QP----- K----LLDST-T---N -L--R------R--- L--KLD- ------T--

    61                          CDR3
    GSGTDFTLKISRVEAEDLGVYYC FQGSHVPF TFGSGTKLEIK
    ---------------------- W--T-F-P ---A-----L-
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γ chain

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     1                                  CDR1                                CDR2
30F1 EVQLQQSGAELVRPGASVTLSCKASDYTFT DSDMH WVKQTPVHGLEWIG AIDPETGNTAYNQKFKG KAIV
30F3 --------P--KK--ET-KI---T-G-K-- -NPI- ----A-GK-FR-M- M-F-D--EAT-AED-R- RFAL

    61                                  CDR3
    TADKSSSTAYLELRSLTSEDSAVYYCSK DFYYGSNSYSFAY WGQGTSLTVSS
    FLET-D----VQINN-KN--T-K---VS EAD********** -----LVT--A
```

Fig. 4. Amino acid sequences of heavy- and light-chain variable regions of the Fab fragments. The amino acid residues were predicted from the nucleotide sequences. CDRs are indicated for each chain by comparison with the Kabat database. Dashes indicate the same residues as those at the top. Asterisks indicate the gap added to permit alignment. The nucleotide sequences of the Fab fragments have been deposited in GenBank/EMBL/DDBJ under accession numbers AB073321 ~ AB073324.

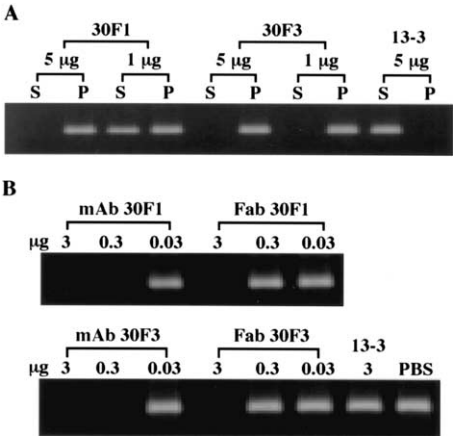

Fig. 5. Biological activity of the Fabs against HCV in vitro. (A) PCR-based HCV capture with the Fabs. The captured and uncaptured viral particles were separated by centrifugation into the precipitate (P) and supernatant (S), respectively. P and S were examined for HCV RNA by RT-PCR. Fab 13-3, a human Fab against human cytomegalovirus, served as a negative control. (B) Inhibition of HCV adsorption to HPBMa 10.2 cells by the Fabs. The cell-adsorbed HCV was assessed by detecting HCV RNA with RT-PCR. For comparison, the parental mAb were included. Fab 13-3 and PBS served as negative controls.

shows that Fabs 30F1 and 30F3 both inhibited HCV adsorption at a dose of 3 μg while the same amount of unrelated Fab and PBS did not block HCV adsorption to the cells. Compared with the original mAbs, both recombinant Fabs showed relatively lower inhibitory activity in that 0.3 μg of mAbs inhibited HCV adsorption whereas the same amount of the Fabs did not (Fig. 5B). These data indicate that inhibition of HCV adsorption to the cells by the Fabs was also dose-dependent.

4. Discussion

In this study, we cloned and expressed in bacteria cDNAs coding for the VH-CH1 and VL-CL fragments of the mouse anti-HVR1 mAbs 30F1 and 30F3. Both purified recombinant antibody Fabs captured homologous HCV and inhibited homologous HCV adsorption to susceptible cells.

The successful synthesis of recombinant antibody Fab fragments in bacteria may be attributed to the structure of the expression vector. The expression plasmid pFab1-His2 used in this study contained a hexa-histidine tag only downstream of the VH gene (Maeda et al., 1999); however, the Fab fragments purified by HisTrap nickel columns comprised approximately equal amounts of both L and H chains, as shown by SDS-PAGE (Fig. 1). This indicates that the H and L chains synthesized in the bacteria correctly folded to form Fab fragments by the formation of interchain disulfide

bonds via cysteine residues between the chains. This process is crucial for producing recombinant antibody because inefficient folding would result in lower production of recombinant Fab or production of dysfunctional Fab.

The formation of interchain disulfide bonds may ensure the high binding activity of recombinant Fab fragments. The Fab fragments synthesized in this study had high affinity, with a K_D of 1.39 nM for Fab 30F1 and 3.96 nM for Fab 30F3. Compared with those of the parent mAbs, the affinity constants of their Fabs were reduced only 5–7-fold (Table 1). Although the single chain fragment of variable regions VL and VH (scFv) is simpler and more popular in antibody engineering, the low binding affinity of this molecule to the target antigen is a significant problem for the development of biological applications of these recombinant molecules. For example, the scFv against the NS3 protease of HCV had significantly lower affinity compared with the parent mAb, with the K_D being reduced more than 60-fold (Kasai et al., 2001). Thus, Fab fragments appear to be superior in antibody engineering regarding their biological and immunological activities.

The Fabs 30F1 and 30F3 showed to be biologically functional. We used two in vitro surrogate assays, HCV-capture and inhibition of HCV adsorption to susceptible cells, to evaluate the antiviral property of the recombinant Fab fragments. The results of HCV-capture (Fig. 5A) suggested that the bacterially synthesized Fab fragments against HVR1 could efficiently bind HCV. Such binding further abolished the infectivity of HCV because the purified Fab fragments inhibited the adsorption of HCV to HPBMa 10.2 cells (Fig. 5B). The high affinity of the recombinant Fabs generated in this report may favor their antiviral activity against HCV. HPBMa 10.2 cells are susceptible to in vitro infection with several HCV isolates, including HCV-#6, and the infectivity of HCV in this system correlates with the infectivity of HCV in vivo in chimpanzees (Shimizu et al., 1993). The inhibition of the HCV adsorption assay in vitro correlates with the neutralization test in vivo (Shimizu et al., 1994). Therefore, the present data suggest that the Fab fragments against HVR1 may neutralize homologous HCV. The specificity of the Fabs synthesized in this study was validated by the epitope confirmation assay (Fig. 2) and BIAcore analysis (Fig. 3). Hence, only the purified Fabs were responsible for the HCV-capture and the inhibition of HCV adsorption to HPBMa 10.2 cells.

Compared with the parental mAbs, the Fab fragments generated in this study had 5–7-fold lower affinity (Table 1) and around 10-fold lower activity of inhibition HCV adsorption. The practical application of the recombinant Fab fragment may be limited because of the reduced affinity. Strategies such as chain shuffling (Marks et al., 1992) and mutation in CDR (Jackson et al., 1995) may be used to raise the affinity of recombinant antibodies. The second concern in the practical utility of the Fab fragments against HVR1 in this study is the narrow specificity to HCV isolates. Although HVR1 is highly mutable and heterogeneous, a broad antibody response to it can be induced. Immunization of mice or chimpanzees with a single HVR1 peptide can induce multiple sequence-reactive anti-HVR1 antibodies (Zhou et al., 1999). HVR1 mimotopes may induce more rigorously cross-reactive antibody responses (Puntoriero et al., 1998). More recently, it was reported that most of the mAbs developed from mice immunized with HVR1 mimotopes recognized correctly folded HVR1 and captured HCV particles as well as recombinant HCV-like particles (Cerino et al., 2001). Therefore, the obstacle of high mutation in HVR1 may be overcome by a mechanism of inducing broadly cross-reactive anti-HVR1 antibodies.

The HCV-neutralizing activity of the Fab fragments from mouse mAbs produced in this study implies that human Fab against HVR1 would also block HCV infectivity. Work is currently ongoing to clone and express Fab genes of human antibody against HVR1. This will be helpful for developing immunological therapeutic molecules against HCV.

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