The Journal of Immunology

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J Immunol 2001; 167:3878-3886; ;
doi: 10.4049/jimmunol.167.7.3878
http://www.jimmunol.org/content/167/7/3878

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Monoclonal Antibodies with Broad Specificity for Hepatitis C Virus Hypervariable Region 1 Variants Can Recognize Viral Particles[1]

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The hypervariable region 1 (HVR1) of the E2 protein of hepatitis C virus (HCV) is a highly heterogeneous sequence that is promiscuously recognized by human sera via binding to amino acid residues with conserved physicochemical properties. We generated a panel of mAbs from mice immunized with HVR1 surrogate peptides (mimotopes) affinity-selected with sera from HCV-infected patients from a phage display library. A high number of specific clones was obtained after immunization with a pool of nine mimotopes, and the resulting mAbs were shown to recognize several 16- and 27-mer peptides derived from natural HVR1 sequences isolated from patients with acute and chronic HCV infection, suggesting that HVR1 mimotopes were efficient antigenic and immunogenic mimics of naturally occurring HCV variants. Moreover, most mAbs were shown to bind HVR1 in the context of a complete soluble form of the E2 glycoprotein, indicating recognition of correctly folded HVR1. In addition, a highly promiscuous mAb was able to specifically capture bona fide viral particles (circulating HCV RNA) as well as rHCV-like particles assembled in insect cells expressing structural viral polypeptides derived from an HCV 1a isolate. These findings demonstrate that it is possible to induce a broadly cross-reactive clonal Ab response to multiple HCV variants. In consideration of the potentially important role of HVR1 in virus binding to cellular receptor(s), such a mechanism could be exploited for induction of neutralizing Abs specific for a large repertoire of viral variants. *The Journal of Immunology,* **2001, 167: 3878–3886.**

Hypervariable region 1 (HVR1)[3] is a highly heterogeneous sequence located at the N terminus of hepatitis C virus (HCV) E2 envelope glycoprotein that is responsible for significant inter- and intraindividual variation of the infecting virus, possibly leading to escape and persistent infection. Several lines of experimental evidence argue in favor of a dominant role of positive selection for amino acid changes in driving the pattern of HVR1 genetic diversification (1, 2). Prospective studies of serological responses to synthetic oligopeptides derived from HVR1 sequences of patients with acute and chronic HCV infection showed apparently extensive serological cross-reactivity for unrelated HVR1 peptides in the majority of the patients (3–9). A significant correlation was found between HVR1 sequence variation and the intensity and cross-reactivity of humoral immune responses, strongly supporting the contention that HCV variant selection is driven by the host immune response (9). Additional data in support of Ab-mediated HVR1 variant selection derive from studies reporting that persistent viremia was associated with higher intersample antonymous vs synonymous substitutions, suggesting that HVR1 can function as an immunological decoy, stimulating a strong immune response that would be responsible for variant selection but would be ineffective to clear HCV (10). This last aspect may be a function of CD4 T cells, which appeared to exhibit stronger HVR1-specific responses in patients who cleared the virus after successful antiviral therapy (11).

The importance of the environmental selection pressure has been recently confirmed by comparing the stability of HVR1 as a function of time in humans and primates. In agreement with the weak anti-HVR1 responses detected in chimpanzees, HVR1 sequence diversity was significantly lower in this setting compared with those in humans (12)

That being stated, the biological relevance of HVR1 is still unclear. Several studies identified binding sites for allegedly neutralizing Ab in this region (13, 14), and it is therefore conceivable that HVR1 expressed on integral HCV particles could represent a major target for immune responses that may play an important role in the outcome of HCV infection. In this study, we have generated mAbs by immunizing mice with peptides derived from an HVR1 consensus profile, accounting for ~80% of the total sequence variability (15), and we characterized such mAbs in terms of fine specificity and ability to bind to HVR1 sequences expressed in the context of correctly folded chimeric E2-HVR1 proteins and on recombinant and natural bona fide viral particles. The long-term

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Received for publication April 5, 2001. Accepted for publication July 9, 2001.

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[1] This work was supported by project grants from the Italian Ministry of Health to IRCCS Policlinico San Matteo, Progetto Strategico no. 015/RFM/00/01 "Risposta immunitaria dell'ospite, eventi cellulari e variabilità virale nell'infezione da HCV," Progetto di Ricerca Finalizzata ICS120.5/RF99.96

"Modelli per lo sviluppo di vaccini contro il virus dell'epatite C ed il micobatterio della tubercolosi," and Progetto di Ricerca Corrente 3180/98. Grant support was also provided by Schering-Plough (Italy), Fondazione Oretta Bartolomei Corsi (Firenze, Italy), and Ministero dell'Università e della Ricerca Scientifica e Technologica-Cofin no. MM06261448_003.

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[3] Abbreviations used in this paper: HVR, hypervariable region; HCV, hepatitis C virus; MAP, multiple antigenic peptide; GNA, *Galantus nivalis* lectin; -LP, -like particles.

0022-1767/01/$02.00

objective was to analyze in detail the features of the humoral immune responses elicited by peptides that are thought to represent efficient antigenic and immunogenic mimics of natural HVR1 sequences (15) and that may be considered as potential components of a synthetic vaccine inducing broad specificity for several HCV variants.

Materials and Methods

HVR1 peptides

HVR1 surrogate peptides (mimotopes) were obtained by constructing an HVR1 library by back-translation of an HVR1 consensus profile, accounting for ~80% of the total sequence variability, into the corresponding nucleotide sequence (15). A degenerate synthetic oligonucleotide was cloned as a fusion to the gene encoding for the major coat protein (pVIII) in a phagemid vector for display on M13 bacteriophage. Phage were subjected to two rounds of affinity selection using sera from anti-HCV-positive patients. A number of clones reacting exclusively with HCV sera were identified, with the best clones reacting with ~80% of the sera (15). Nine HVR1 mimotopes were synthesized as multiple antigenic peptides (MAPs) (16) and used for immunization as described below (Table I).

To examine binding to naturally occurring HVR1 isolates, two panels of previously validated biotinylated peptides of 16- and 27-aa residues (9, 15) were used on streptavidin-coated ELISA plates. Similarly, to determine the fine specificity of murine hybridomas, 14 overlapping biotinylated 14-mers scaled by one residue and covering the entire HVR1 region (amino acids 384–410) were synthesized based on the sequence of the HVR1 consensus profile reported previously (15).

Immunization

Four- to 7-wk-old female BALB/c mice (Charles River Italia, Como, Italy) were immunized i.p. with 100 μl immunogen at wk 0, 3, and 7 and bled on day 0 (prebleed) and 10 days after each injection. MAPs used as immunogens were dissolved in PBS at a concentration of 400 μg/ml and injected as a 1/2 dilution in either CFA (first injection) or IFA (booster).

Splenocytes were taken from the immunized mice with the highest titers 3 days after a booster injection (i.v., without any adjuvant) at wk 10. Two mice, M18 and M22, were used for the production of mAbs. M18 was a control mouse immunized with MAP 313, which in preliminary experiments was found to be reactive with <15% of anti-HCV positive sera. M22 was instead immunized with a pool of all nine MAPs (Table I), one of which (MAP 455) was recognized by >65% of anti-HCV-positive sera, to maximize the repertoire of the Ab response to HVR1.

Construction and expression of chimeric glycoproteins

Natural HVR1 sequences were selected from databases as previously described (15). They were cloned into V1JnsTPA-ΔE2 plasmid, containing the E2 coding region of a genotype 1b isolate (N strain) (17) devoid of HVR1 (amino acids 411–684) as previously described (18). Briefly, for each clone, a forward primer (with a *Pac*I site at the 5′ end) and a reverse primer were synthesized. Together, these primers encompassed all the HVR1 sequence to be cloned and had a central overlapping region of 19 nucleotides. The forward and reverse primers were diluted in 1× Klenow buffer (Biolabs, Northbrook, IL) at a final concentration of 20 pmol/μl. For the annealing, the mixture of primers was boiled for 2 min and then left at room temperature until cool. Fill-in was performed by adding 1 U DNA polymerase I Klenow fragment (Biolabs, 210S) per microgram of DNA and incubating for 20 min at room temperature. The fragment was purified, digested with *Pac*I, and cloned into the *Pac*I site of V1JnsTPA-ΔE2 vector.

Table I. *Mimotopes used for immunization*

Peptide	Sequence
313	TTTTTGGVQGHTTRGLVRLFSLGSKQN
316	TTTTTGGQVGHQTSGLTGLFSPGAQQN
320	QTTTTGGQVSHATHGLTGLFSLGPQQK
440	QTTVVGGSQSHTVRGLTSLFSPGASQN
441	QTHTTGGVVGHATSGLTSLFSPGPSQN
442	QTHTTGGVVSHQTRSLVGLFSPGPQQN
443	TTHTVGGSVARQVHSLTGLFSPGPQQK
444	QTTTTGGSASHAVSSLTGLFSPGSKQN
455	QTHTTGGQAGHQAHSLTGLFSPGAKQN

Large quantities of DNA were prepared using Qiagen 2500-Tip columns (Plasmid Mega kit, catalog no. 12183; Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The different clones were expressed by transient transfection of human embryonal kidney 293 cells using the calcium phosphate method (Calcium Phosphate Mammalian Cell Transfection kit, catalog no. 2-463335; 5′→3′, Boulder, CO). Cell extracts were harvested, and the amount of E2 was evaluated as described by Yagnik et al. (19).

Production of HVR1-specific mAbs

The mAbs were essentially produced as originally described by Köhler and Milstein (20) by fusing splenocytes from immunized animals with the non-Ig-secreting, hypoxanthine guanine phosphoribosyltransferase-deficient murine myeloma cell line Sp2/0-Ag14. Following selection on hypoxanthine-aminopterin-thymidine, cells were expanded, and anti-HVR1 secreting hybrids were cloned by limiting dilution at 5, 1, and 0.5 cell/well in complete medium (RPMI 1640, 4 mM L-glutamine, 2 mM sodium pyruvate, 1% nonessential amino acids, and 10% FBS) containing 20% hybridoma cell growth supplement (ICN Biomedicals, Irvine, CA). After positive identification of specific Ab-secreting cultures, hybridomas were subjected to at last two subcloning cycles at 0.5 cell/well and further characterized in terms of Ig production, subclass, and fine specificity as described below.

ELISA

Two different MAPs were used for screening hybrid cultures. MAP 313 was used to screen cultures derived from M18 (immunized with the same MAP), whereas MAP 455, which was previously shown to be highly cross-reactive (15), was used for screening M22 (immunized with a pool of MAPs including 455). Cultures testing negative upon screening with MAP 455 were also examined for binding to the remaining eight mimotopes not used for preliminary analysis. Assay conditions were essentially as previously described (9) with modifications as a function of the Ag used. Specifically, MAPs were coated onto 96-well microplates at a concentration of 10 μg/ml in 50 mM bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. To detect binding to peptides derived from natural HVR1 isolates or to determine the fine specificity of mAb binding, biotinylated 27-, 16-, and 14-mers were added to streptavidin-coated ELISA plates exactly as previously described (9). Coating of ELISA plates with *Galantus nivalis* lectin (GNA; Sigma, St. Louis, MO) was required for adsorption of E2 chimeric glycoproteins. Briefly, GNA (1 μg/well in PBS) was incubated overnight at 4°C, and after saturation of nonspecific binding sites with PBS, 2.5% BSA, and 0.1% Tween 20 followed by several washing steps, 5 μl supernatant from cells transiently transfected with the V1JnsTPA-ΔE2 vector described above was added in a 1/20 dilution to GNA-coated wells. E2 chimeras were allowed to bind for 2 h at room temperature under agitation.

After washing, 100 μl hybridoma culture supernatants were added to Ag-coated microplate wells and incubated for 2 h at room temperature. After several washing steps with PBS-0.05% Tween 20 and an additional 1-h incubation at room temperature with appropriately diluted HRP-conjugated goat anti-mouse Ig (Sigma) followed by washing, the reaction was developed with *o*-phenylenediamine (DAKO, Copenhagen, Denmark) as substrate. Absorbance values were read at 492 nm.

Characterization of Ig secreted by HVR1-specific hybridomas

To determine the Ig class secreted by hybridoma cultures, mAbs were captured with HVR1 MAPs bound to solid phase and identified with anti-IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 H chains and anti-κ and anti-λ L chains (Sigma) as previously described (21). Ig concentrations in hybridoma supernatants were determined by quantitative ELISA as previously described (21). Standards were obtained from commercially available mouse myeloma cell lines (Sigma).

Competitive inhibition of mAb binding to HVR1 peptides by anti-HCV-positive human sera

Hybridoma supernatants were diluted in PBS-BSA to adjust the specific mAb concentration to give an A_{492} reading of ~1.0. Streptavidin-coated ELISA plates were incubated with a number of biotinylated 16-mers derived from naturally occurring HVR1 sequences as previously described (9). Human sera (n = 4) from patients with chronic HCV infection were diluted 1/10 in PBS containing 0.05% Tween 20 and added to the wells for 5 min at 37°C as previously described (22). Murine mAb 3C7-C3, which displayed promiscuous binding to several HVR1 peptides (this article), was subsequently added and incubated for 1 h at 37°C. Following extensive washing, goat anti-mouse Ig was dispensed in each well, and the reaction was developed as described above. Twelve sera from HCV-seronegative healthy subjects served as controls. Competition was considered significant

if reduction of mAb binding to HVR1 peptides was >28%, 3 SD above the mean (9%) percentage of inhibition exerted by control sera.

Binding of mAbs to rHCV-like particles (LP)

HCV-LP were synthesized in insect cells using a recombinant baculovirus containing the cDNA of the HCV structural region, including core, E1, and E2, and partially purified as previously described (23). HCV-LP were prepared from strains H (genotype 1a) (24) and J (genotype 1b) (25). The binding of mAbs to HCV-LP was determined by ELISA as previously described (26) with minor modifications. Briefly, HCV-LP were incubated overnight at 4°C on GNA-coated 96-well microplate (Maxisorp, Nalge-Nunc International, Roskilde, Denmark) prepared as described above at a concentration of 100 μg/ml in PBS and 10% glycerol to avoid possible denaturation. In some experiments, HCV-LP were denatured by boiling for 5 min in PBS-glycerol containing 0.1% SDS. Wells were blocked with PBS containing 4% goat serum (Sigma), 10% glycerol, and 5% skimmed dry milk (Sigma) for 3 h at room temperature. After several washing steps with PBS and 10% glycerol, mAb supernatants were added undiluted to the wells and incubated at 37°C for 1 h. After being washed with PBS glycerol, the wells received 100 μl HRP-conjugated anti-mouse Ig (Sigma) diluted 1/10,000 in PBS-glycerol-skimmed dry milk. The reaction was then developed with *o*-phenylenediamine, and OD was determined at 492 nm.

Binding of HVR1-specific mAbs to bona fide HCV particles

Magnetic beads coated with rat anti-mouse IgG1 mAb (Dynabeads M-450, Dynal, Oslo, Norway) were used to capture circulating bona fide HCV particles as follows. Beads were washed three times with PBS and 0.1% BSA, and 2 mg was incubated with 2 μg purified mAb 3C7-C3 in 2 ml PBS-BSA. Control beads received buffer alone. Following incubation for 45 min at 4°C, beads were exposed to a magnet for 10 min, and excess mAb was removed by aspiration and washing five times with 3 ml PBS-BSA after exposure to a magnet each time for 7 min. Serum from a viremic (HCV RNA titer, 3.7×10^7 GEq/ml by branched chain DNA assay; Quantiplex HCV RNA 2.0 assay; Chiron, Emeryville, CA) immunosuppressed patient infected with genotype 2c and containing no detectable Abs to several HVR1 peptides was diluted 1/20 in PBS-BSA and incubated with the beads at 37°C for 90 min under rotation. Beads were again washed five times with PBS-BSA by exposing the mixture each time to 1 ml PBS-BSA, transferred to an Eppendorf tube, and centrifuged at 10,000 $\times$ *g* for 20 min at room temperature. The supernatant was discarded, and the pellet containing magnetic beads was processed for HCV RNA detection by PCR as follows. Viral RNA was extracted using the QIAmp Viral RNA kit (Qiagen) with minor modifications. Specifically, after addition of AVL buffer (containing guanidinium thiocyanate), samples were left on the bench for 10 min at room temperature and then vortexed. The procedure was repeated three times. After centrifugation at 10,000 $\times$ *g* for 1 min, the supernatant was subjected to a standard extraction procedure and amplification by a two-round nested RT-PCR as reported previously (27). First- and second-round PCR products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide. The lengths of the first and second round PCR products were 289 and 235 bp, respectively.

Results

Immunization of M18 and M22 yielded profoundly different results. Thus, the titer of the serum from M18 (1/6,400) was significantly lower than that of M22 (1/25,600) with the respective immunogen. Moreover, of the 544 wells derived from M18 showing significant hybrid growth, 13 (2.4%) secreted Ab that specifically reacted with the immunizing mimotope (313), and of these only two Ag-specific hybridomas could be stabilized in continuous culture. One of the clones secreted IgM, whereas the Ig isotype could not be characterized in the other. Instead, 54 (33%) of the 165 wells derived from M22 secreted Ab that specifically bound to the highly cross-reactive MAP 455 included in the pool of nine mimotopes used for immunization. Twenty-six clones (48%) of the initial 54 hybrid cultures could eventually be stabilized in continuous culture: 19 secreted IgG1κ, four secreted IgM, two secreted IgA, and in one, the Ig isotype could not be characterized. To avoid the possibility that screening with a single mimotope (455) could select for a dominant epitope present on HVR1, cultures negative upon initial screening with MAP 455 were tested for binding to the remaining eight MAPs included in the immunizing pool. Only two IgM-secreting hybridomas showing weak or no significant reactivity for MAP 455 were reactive with MAP 441 and 443, further attesting to the promiscuous reactivity of MAP 455 synthesized on the basis of the HVR1 consensus profile (15). MAP 313 was only weakly reactive with the majority of mAbs as previously described for human sera (15). The mAb titer was determined by end-point dilution for selected IgG- and IgA-secreting hybridomas after normalizing the Ig concentration to 0.5 μg/ml. Typical titers determined on MAP 455 varied from 1/8 to 1/65,000. Although there was a trend for low titer mAbs to show limited reactivity with the different 16- and 27-mers used in the experiments, there was no correlation between mAb titers and cross-reactivity for HVR1 variants (data not shown).

Murine mAb reactivity with peptides derived from natural HVR1 isolates

To evaluate the potential of HVR1 mimotope-specific mAbs to recognize natural HVR1 sequences we used panels of biotinylated peptides synthesized from the deduced amino acid sequences of several HCV isolates obtained from patients with acute and chronic HCV infection (9, 15). The analysis was restricted to IgG- and IgA-secreting hybridomas, because IgM showed high background binding to irrelevant peptides (data not shown). As shown in Fig. 1, mAbs showed significant cross-reactivity with a number of 16- and 27-mers, confirming the findings reported previously using polyclonal sera from patients (9) and animals (3) and providing additional evidence in favor of recognition of residues with conserved hydropathicity profile that confer a remarkable conformational conservation to HVR1, as recently suggested (28). The relevance of the sequence recognized by our murine mAb panel was further supported by competitive inhibition studies in which mAb binding to HVR1 peptides was almost always significantly reduced by preincubation with sera from patients with HCV infection, but not with control sera (Fig. 2).

Fine mapping of HVR1-specific mAbs

To determine the fine specificity of our mAbs, we used a panel of overlapping 14-mers, scaled by one amino acid residue, and covering the entire HVR1 sequence (amino acids 384–410). Peptides were synthesized on the basis of the HVR1 consensus profile previously identified (15). As shown in Fig. 3, all but one of those mAbs for which a PEPSCAN analysis could be performed (15 IgG and two IgA) recognized a C-terminal subregion of HVR1 broadly defined by amino acid residues 390–410. One mAb (1D5-F10) showed restricted binding to residues 384–398 at the amino-terminal end. These findings are entirely compatible with previous studies from our group suggesting the existence of an immunodominant epitope on the HVR1 C-terminal subregion recognized by human sera (9). The inability to map the fine specificity of four of our mAbs may have several explanations. First, epitope mapping of these mAbs may be difficult using shorter peptides. Second, the overlapping 14-mers were designed on the basis of a highly cross-reactive HVR1 sequence expressed in MAP 455 and linear 27-mer 539, which, however, cannot be representative of all HVR1 variants. Third, the peptides showing little or no reactivity in the fine-mapping experiments were also poorly reactive with the panel of 16- and 27-mers, and this may be the most plausible explanation.

The mAbs recognize HVR1 in the context of a correctly folded E2 glycoprotein

To examine whether our mAbs could bind to HVR1 expressed in its correct conformation as part of the complete E2 envelope glycoprotein we constructed chimeric E2-HVR1 glycoproteins in which the HVR1 moiety was identical with that expressed as linear

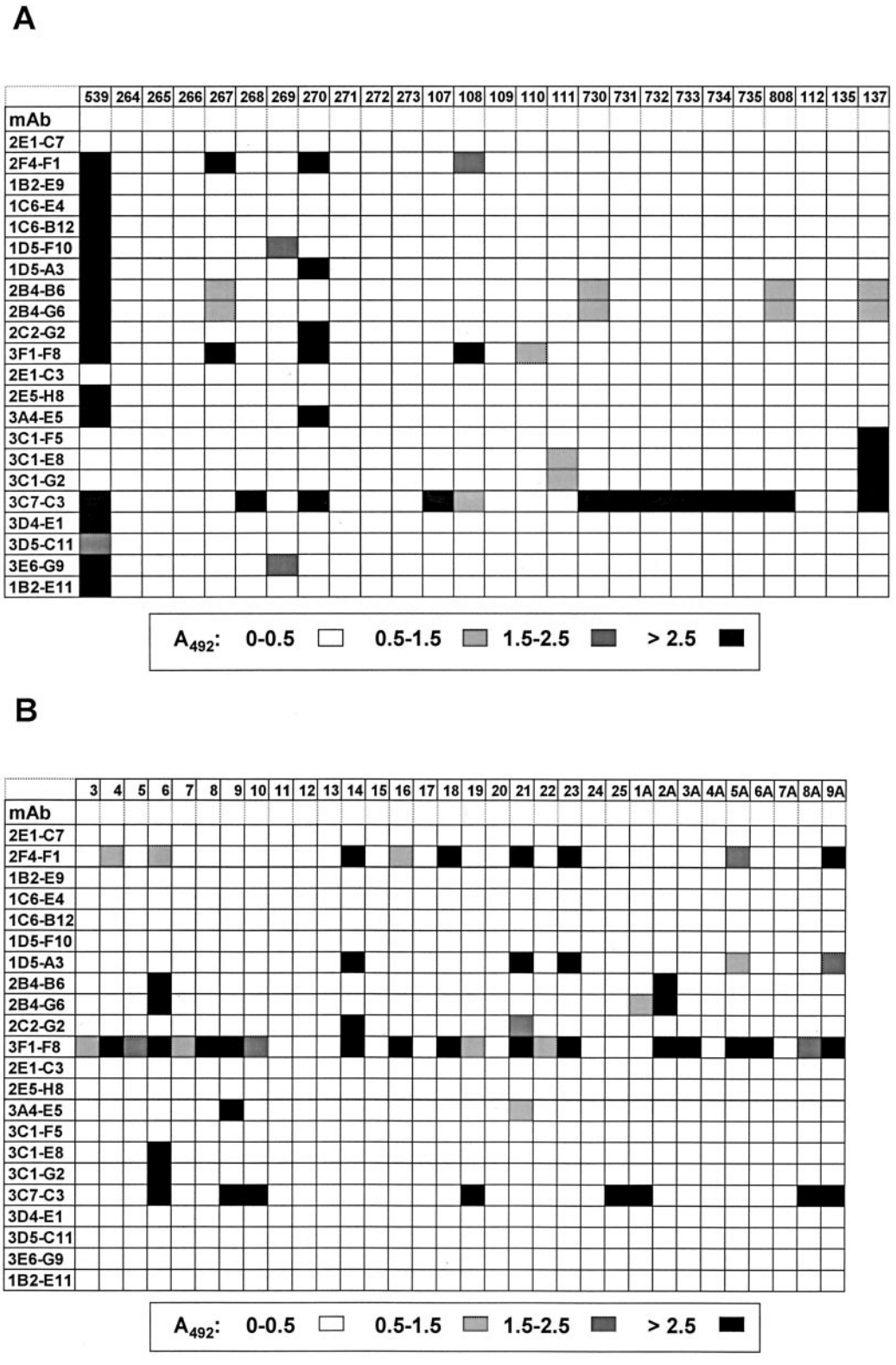

FIGURE 1. Murine mAb reactivity with 27-mers (*A*) and 16-mers (*B*) derived from natural HVR1 sequences. The columns corresponding to HVR1 peptides are identified in the *top row* by a number. Listed on the *left side* in each row are the mAbs examined for binding to each peptide. A_{492} values >0.5 (well over the 5 SD above mean control values that was arbitrarily defined as the upper limit of normal) were considered significant. Different patterns identify A_{492} intervals, as indicated at the *bottom*. The mAb isotype was IgG1, except for IgA-secreting hybridomas 3F1-F8 and 3D5-C11.

peptide (27-mer). In these constructs the E2 moiety was derived from the HCV N strain (genotype 1b). In addition, several other soluble E2 proteins derived from natural isolates containing HVR1 and truncated at position 661 (Table II) were used in binding experiments as described above. As illustrated in Fig. 4, virtually all mAbs were able to bind to peptide 539 and its matched chimera E2-F78. This was not unexpected, because 539 is a broadly reactive linear peptide version of MAP 455 used for the immunization

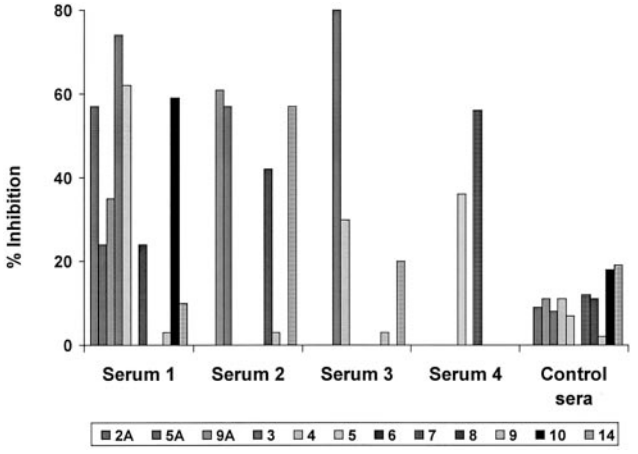

FIGURE 2. Competitive inhibition of mAb 3C7-C3 binding to peptides derived from natural HVR1 sequences by sera from patients with chronic HCV infection. A cut-off value of 28% was obtained by adding 3 SD to the mean percentage of inhibition obtained with sera from 12 healthy HCV-seronegative controls. Different patterns identify different HVR1 peptides.

Table II. *Linear peptides and corresponding E2-HVR1 chimeric proteins used in mAb-binding experiments*[a]

HVR1 Linear Peptides (27-mers)	E2-HVR1 Chimeras or Complete E2 Glycoproteins from Natural Isolates
267	E2-267
268	E2-268
269	E2-269
107	E2-275
730	E2-304
735	E2-21
732	E2-103
731	E2-163
539	E2-F78
730	H661[b]
135	BK661[b]
137	N[b]
NA	J[b]

[a] All chimeric and natural E2-HVR1 proteins, with the exception of H661 which derived from a genotype 1a isolate, were from a genotype 1b isolate.
[b] Natural HCV isolates.
NA, Not available.

protocol. One mAb (3C7-C3) efficiently bound to several E2-HVR1 chimeras/linear peptides pairs, including peptide 730 and H661 (a native truncated E2 protein derived from HCV strain H), suggesting promiscuous recognition of a conformation-independent, conserved sequential epitope expressed in the context of a correctly folded E2 glycoprotein (Fig. 4). Such an epitope was also present on the natural E2 glycoprotein H661 derived from HCV strain H (24) (genotype 1a), but not on E2 from genotype 1b strains BK (29), J (25), and N (17) (a linear peptide from N was recognized by mAbs 2B4-B6 and 2B4-G6). Recognition was limited to the linear peptide only in three instances, whereas three chimera/peptide pairs were nonreactive. Three additional mAbs showed only limited reactivity for the 267/E2–267 peptide/chimera pair.

E2-HVR1 chimera/peptide pairs could also be recognized by polyclonal human sera from HCV-infected patients, attesting to the correct conformation of the recombinant E2 glycoproteins (Fig. 5).

Binding of HVR1-specific mAbs to rHCV-LP

To determine whether our mAbs could recognize HVR1 expressed on viral particles we used HCV-LP, constituted by HCV structural

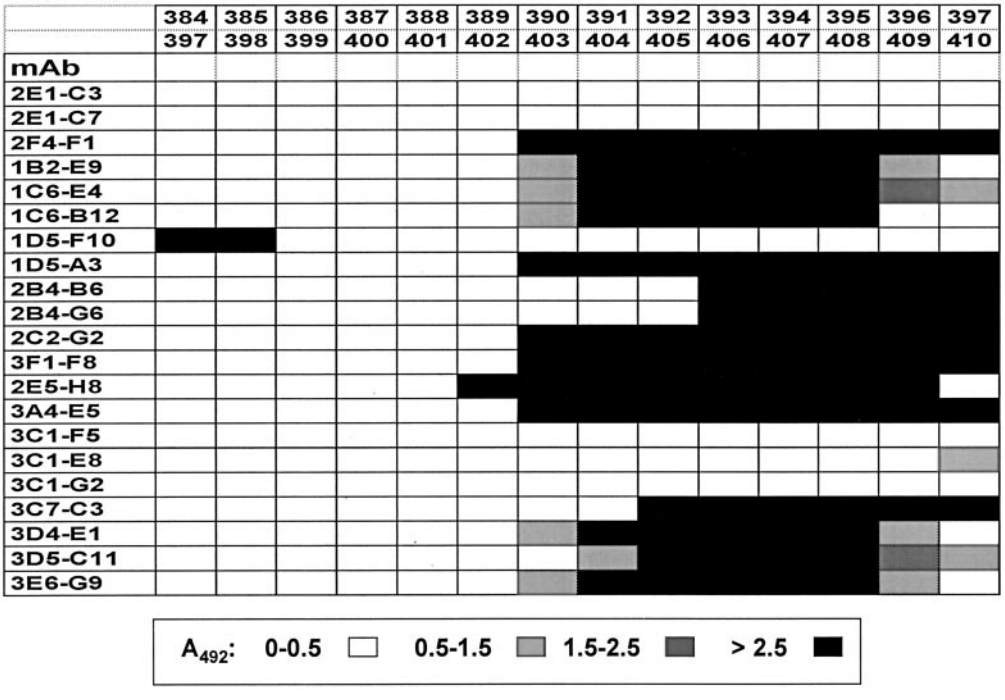

FIGURE 3. Fine mapping of HVR1-specific mAbs by PEPSCAN analysis. Fourteen overlapping 14-mers were used in direct binding experiments as shown in *Materials and Methods*. Each column corresponds to a peptide whose coordinates are indicated by the amino acid positions. The mAb identifications (rows) are listed in the *left column*. A_{492} values >0.5 (well over the 5 SD above mean control values that was arbitrarily defined as the upper limit of normal) were considered significant. Different patterns identify A_{492} intervals as indicated at the *bottom*. The mAb isotype was IgG1, except for IgA-secreting hybridomas 3F1-F8 and 3D5-C11. Notice that 1D5-F10 was the only mAb among those tested that recognized the N terminus of HVR1. Each experiment was performed in duplicate and repeated at least three times.

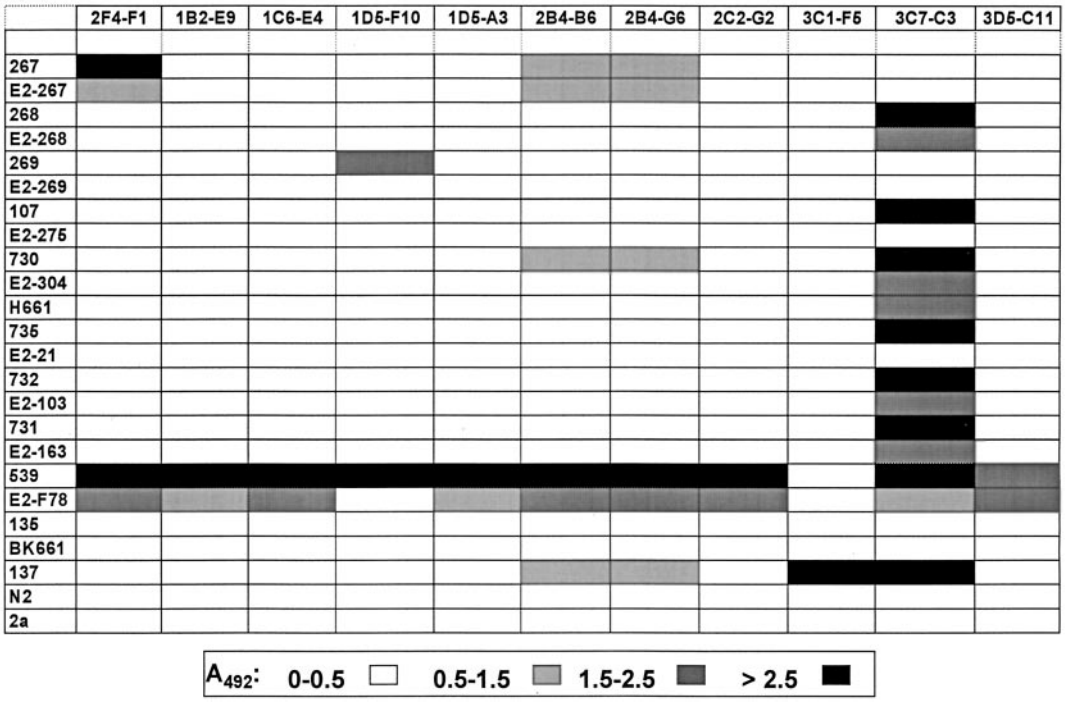

FIGURE 4. HVR1 peptide-specific murine mAbs recognize HVR1 sequences expressed in the context of correctly folded chimeric E2 glycoproteins. Each column corresponds to a mAb (*top*), whereas linear and chimeric HVR1 sequences are listed in each row on the *left*. The E2 prefix identifies a chimeric protein whose corresponding linear peptide is shown in the row immediately above it. A_{492} values >0.5 (well over the 5 SD above mean control values that was arbitrarily defined as the upper limit of normal) were considered significant. The mAb isotype was IgG1, except for IgA-secreting hybridoma 3D5-C11.

proteins assembled in insect cells, which provide a good surrogate model of native HCV particles. Care was applied to avoid denaturing of such particles, which could expose hidden epitopes that would normally not be accessible to Ab. The entire panel of IgG- and IgA-secreting mAbs was screened for binding to HCV-LP derived from genotype 1a and 1b isolates. The mAb 3C7-C3, which showed promiscuous binding to several E2-HVR1 glycoproteins, showed consistent and reproducible binding to HCV-LP from genotype 1a, but not to HCV-LP from genotype 1b (Fig. 6). Binding was completely abolished by preabsorption of 3C7-C3 to magnetic beads coated with linear 27-mer 539 derived from the HVR1 consensus sequence but not when beads were coated with an irrelevant 27-mer. A control mAb (7C2) specific for anti-apolipoprotein A1 (a gift from Dr. V. Bellotti, University of Pavia, Pavia, Italy) failed to bind to HCV-LP. None of the remaining mAbs were able to bind to either HCV-LP 1a or 1b. Interestingly, denaturation significantly increased the binding efficiency of 3C7-C3 to HCV-LP 1a, probably as a result of better exposure of the relevant epitope, but had no effect on binding to HCV-LP 1b (Fig. 6). None of the mAbs that failed to bind to integral HCV-LP was able to recognize HCV-LP 1a or 1b under denaturing conditions (data not shown). Collectively, these findings indicate that HVR1 is expressed on rHCV particles and that such region may be accessible to Ab, although the relevant epitope may often not be completely exposed on integral viral particles in a conformation recognizable by Ab.

Binding of HVR1-specific mAbs to bona fide native HCV particles

As an extension of the experiment described above, we asked whether mAb 3C7-C3 could capture bona fide circulating HCV particles by determining the presence of HCV RNA bound to magnetic beads coated by HVR1-specific mAbs or control mAbs. As shown in Fig. 7, only mAb 3C7-C3 could efficiently capture HCV RNA, whereas control beads coated with control mAb 7C2 showed no specific signals, providing further evidence in support of the contention that HVR1 is indeed expressed on native HCV particles.

Discussion

Several previous studies indicated that circulating Abs from patients with acute and chronic HCV infection show broad specificity for a large number of HVR1 variants. This finding may appear anomalous when confronted with the propensity of HVR1 to undergo extensive sequence variation, particularly in its central subregion (1). Subsequent studies from our group detected the simultaneous presence of variant-specific and cross-reactive Ab responses in the same serum, the latter being predominantly directed at the C terminus (9). Here we show that it is possible to induce a cross-reactive monoclonal B cell response to HVR1 following immunization with peptides mimicking natural HVR1 isolates. The interpretation of such a finding is not immediately evident, because HVR1 has been traditionally considered structurally flexible and antigenically variable (30); however, little attention has been devoted to characterize its conformation and physicochemical properties. Indeed, close inspection and comparison of a large number of HVR1 sequences indicated either a broad amino acid repertoire at each position despite a remarkable residue conservation in specific sites or replacements with amino acids with similar physicochemical properties, usually positively charged basic residues, in the most variable segments (28). The very similar hydropathy and antigenicity profiles of HVR1 variants revealed a substantial conformational conservation, thus providing a plausible explanation for the extensive cross-reactivity demonstrated by Ab and confirming the existence of an active selection process.

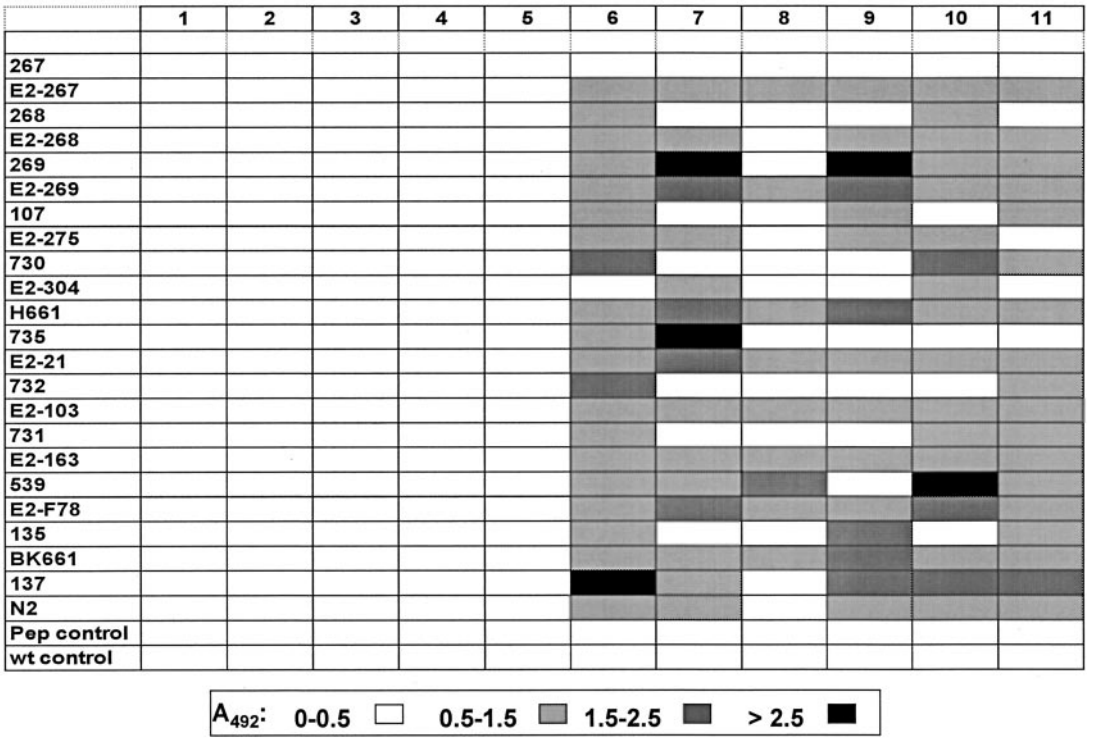

FIGURE 5. Binding of sera from patients with HCV infection to E2-HVR1 chimeras/linear peptides pairs. *Columns 1–5*, Sera from HCV-seronegative healthy controls; *columns 6–11*, sera from HCV-infected patients. Linear and chimeric HVR1 sequences are listed in each row on the *left*. The E2 prefix identifies a chimeric E2 glycoprotein, and the corresponding linear peptide is shown in the row immediately above it. N2, BK661, and H661 indicate proteins from natural strains. A_{492} values >0.5 (well over the 5 SD above mean control values arbitrarily defined as the upper limit of normal) were considered significant. Different patterns identify A_{492} intervals, as indicated at the *bottom*.

Interestingly, two sites with a high antigenicity score were identified at positions 1–13 and 19–24, a pattern that is predicted also with philogenetically distant variants (28). The latter site contains the immunodominant B cell epitope(s) described previously (3, 9), whereas the N-terminal epitope recognized by mAb 1D5-F10 is reportedly less immunoreactive, although it may be preferentially recognized by sera from patients with acute hepatitis (31).

The possibility to generate broadly reactive Abs may represent a useful approach to overcome the natural diversity of a virus such

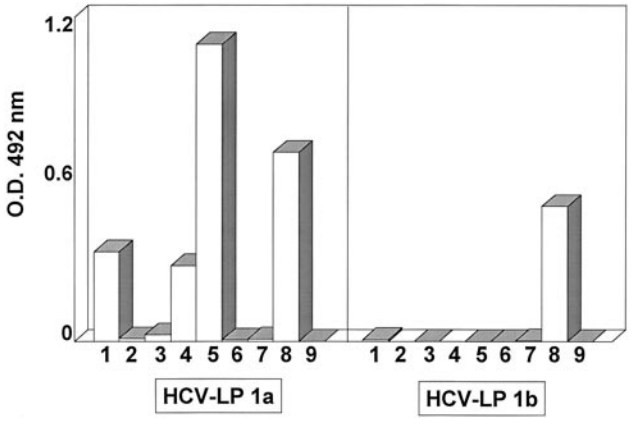

FIGURE 6. Binding of HVR1-specific murine mAbs to rHCV-LP synthesized in insect cells. HCV-LP were obtained from genotypes 1a and 1b. Assay conditions were as described in *Materials and Methods*. *Lane 1*, mAb 3C7-C3; *lane 2*, mAb 3C7-C3 and HVR1 27-mer 539; *lane 3*, control mAb 7C2 (anti-apolipoprotein A1); *lane 4*, mAb 3C7-C3 and irrelevant 27-mer; *lane 5*, mAb 3C7-C3 on denatured HCV-LP 1a; *lane 6*, mAb 3F1; *lane 7*, mAb 1D5; *lane 8*, anti-HCV positive serum diluted 1/100 in PBS and 10% glycerol, used as a positive control; and *lane 9*, anti-HCV-negative control serum. Values represent means of duplicate wells obtained from three experiments.

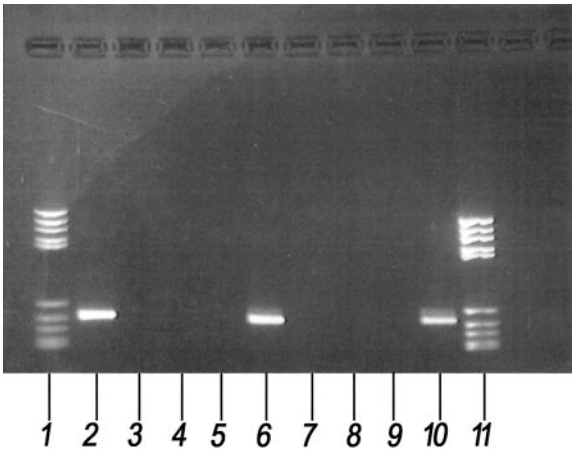

FIGURE 7. Binding of bona fide HCV particles to magnetic beads coated with mAb 3C7-C3. Assay conditions for nested RT-PCR were described in *Materials and Methods*. *Lane 1*, The m.w. markers; *lane 2*, HCV RNA-positive serum, no beads; *lane 3*, HCV RNA-positive serum and beads; *lane 4*, buffer and beads; *lane 5*, beads coated with mAb 3C7-C3; *lane 6*, HCV RNA-positive serum and beads coated with anti-HVR1 mAb 3C7-C3; *lane 7*, beads coated with control mAb 7C2 (anti-apolipoprotein A1); *lane 8*, HCV RNA-positive serum and beads coated with control mAb 7C2; *lane 9*, negative control; *lane 10*, positive control (HCV RNA extracted from the serum of a patient with chronic HCV infection); and *lane 11*, m.w. markers.

as HCV, suggesting that mimotope-based vaccines can be used as potentially effective HCV immunogens. This assumption is based on evidence indicating that Abs to HVR1 can prevent HCV infection in the chimpanzee model (13, 14). However, exposure of HVR1 on complete viral particles has not been formally proven, and in principle, the role of this sequence in binding neutralizing Abs is far from being established. A recent preliminary study suggested that mAbs obtained by immunization with peptides derived from natural HVR1 isolates were able to capture bona fide viral particles only from homologous HCV isolates and could also prevent infection of an allegedly susceptible cell line in vitro (32). These findings are in partial agreement with our data, in that one of our mAbs was also able to capture bona fide and recombinant viral particles, although there was no apparent genotype- or isolate-specific recognition. Indeed, mAb 3C7-C3 bound to rHCV-LP 1a and was able to specifically capture HCV RNA-positive material from serum of a patient infected with genotype 2c. Although the HVR1 sequence(s) could not be determined in this patient, this finding strongly suggests that the HVR1 sequence(s) from the 2c isolate shared significant homology with the HCV-H (genotype 1a) sequence recognized by the 3C7-C3 capture Ab. This observation fits with the idea of a significant structural conservation of HVR1 as discussed above. Instead, we found that mAb binding to serum HCV particles was difficult to demonstrate using sera from immunocompetent patients with persistent HCV infection, in whom anti-HVR1 Abs are invariably detected (3), presumably as a result of competitive inhibition by circulating Ab.

It may be argued that mAbs raised against HVR1 peptides are unable to recognize the same sequence when expressed in the context of a correctly folded complete E2 glycoprotein that included HVR1. The correct conformation of E2-HVR1 chimeras was previously demonstrated by binding to a conformation-sensitive mAb specific for an epitope expressed on a native prebudding form of the HCV envelope (18, 33), by the presence of a small quantity of disulfide-bridged aggregates (18), and by binding to CD81, which is known to be conformation dependent (18, 34). In this study, we showed that the overwhelming majority of our mAbs could recognize a chimeric E2 polypeptide that expressed the same HVR1 sequence synthesized as linear peptide, which was promiscuously recognized by several human sera and murine mAbs. However, cross-reactivity was confined to a limited number of E2-HVR1 chimeric proteins, and in some instances it was restricted to the homologous linear peptide only, suggesting recognition of a sequential epitope. To this effect, it is interesting to note that only one mAb of our entire panel bound to HCV-LP, and that recognition of the HVR1 region could be improved under denaturing conditions. This finding corroborates the hypothesis of the existence of a major sequential epitope at the C terminus of HVR1, which may, under certain circumstances, be exposed on integral viral particles. The binding pattern of mAb 3C7-C3, which, among other proteins, recognized a truncated natural E2 protein (H661) as well as HCV-LP 1a, both derived from strain H, suggests a possible role for HVR1 in virus neutralization. Evidence in support of this hypothesis comes from experiments demonstrating that a hyperimmune serum raised against an HVR1 peptide from strain H was able to prevent HCV infection in chimpanzees following either in vitro incubation with a pedigreed HCV inoculum (13) or passive immunization in vivo (14).

The mechanism by which anti-HVR1 can modulate HCV infection is a matter of speculation. Current evidence suggests that mammalian cell-derived E2 glycoproteins (35) and, to a lesser extent, HCV-LP 1a (M. Triyatni and T. J. Liang, unpublished observations) can bind human CD81, a candidate receptor molecule for HCV. Yet HCV-LP can penetrate into HepG2 cells via alternative receptor(s) which are constitutively expressed on hepatocytes (M. Triyatni and T. J. Liang, unpublished observations), supporting the hypothesis that HCV requires a second receptor molecule for internalization (35). Preliminary evidence suggests that HVR1 is not involved in binding to CD81 (36, 37), although no information is yet available on HCV envelope regions interacting with the putative alternative receptor(s) expressed on susceptible cells. The unique properties of the structure of HVR1, which contains several positively charged conserved residues, suggest that it is most likely involved in binding to negatively charged compounds, such as glycoaminoglycans, of which cell surface receptors are particularly rich, and/or phospholipids. Therefore, high affinity anti-HVR1 Ab elicited by immunization could modulate HCV infection by inhibiting binding of viral particles to a cellular receptor(s) and/or preventing interaction with plasma low density lipoproteins, which have been shown to bind HCV (38). This approach may have important implications for immunotherapy or prophylaxis of HCV infection.

Acknowledgments

We thank Vittorio Bellotti (Department of Biochemistry, University of Pavia) for providing mAb 7C2, Marta Gatti (Virology Service, Policlinico San Matteo) for help with PCR assays, and Lara Firmo for editorial assistance. We also thank Jean-Michel Pawlotsky for providing us with data before publication.

References

1. Brambilla, S., G. Bellati, M. Asti, A. Lisa, M. E. Candusso, M. D'Amico, G. Grassi, M. Giacca, A. Franchini, S. Bruno, et al. 1998. Dynamics of hypervariable region 1 (HVR1) variation in hepatitis C virus infection and correlation with clinical and virological features of liver disease. *Hepatology 27:1678.*
2. Manzin, A., L. Solforosi, M. Debiaggi, F. Zara, E. Tanzi, L. Romanò, A. R. Zanetti, and M. Clementi. 2000. Dominant role of host selective pressure in driving hepatitis C virus evolution in perinatal infection. *J. Virol. 74:4327.*
3. Scarselli, E., A. Cerino, G. Esposito, E. Silini, M. U. Mondelli, and C. Traboni. 1995. 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. *J. Virol. 69:4407.*
4. da Silva-Cardoso, M., K. Siemoneit, V. Nemecek, S. Epple, K. Koerner, and B. Kubanek. 1995. The serology of hepatitis C virus infection: antibody cross-reaction in the hypervariable region 1. *Arch. Virol.140:1705.*
5. Jackson, P., J. Petrik, G. J. M. Alexander, G. Pearson, and J.-P. Allain. 1997. Reactivity of synthetic peptides representing selected sections of hepatitis c virus core and envelope proteins with a panel of hepatitis C virus-seropositive human plasma. *J. Med. Virol. 51:67.*
6. Zibert, A., E. Schreier, and M. Roggendorf. 1995. Antibodies in human sera specific to hypervariable region 1 of hepatitis C virus can block viral attachment. *Virology 208:653.*
7. Hattori, M., K. Yoshioka, T. Aiyama, K. Iwata, Y. Terazawa, M. Ishigami, M. Yano, and S. Kakumu. 1998. Broadly reactive Abs to hypervariable region 1 in hepatitis C virus-infected patient sera: relation to viral loads and response to IFN. *Hepatology 27:1703.*
8. Yoshioka, K., T. Aiyama, A. Okumura, M. Takayanagi, K. Iwata, T. Ishikawa, Y. Nagai, and S. Kakumu. 1997. Humoral immune response to the hypervariable region of hepatitis C virus differs between genotypes 1b and 2a. *J. Infect. Dis. 175:505.*
9. Mondelli, M. U., A. Cerino, A. Lisa, S. Brambilla, L. Segagni, A. Cividini, M. Bissolati, G. Missale, G. Bellati, A. Meola, et al. 1999. Ab responses to hepatitis C virus hypervariable region 1: evidence for cross-reactivity and immune-mediated sequence variation. *Hepatology 30:537.*
10. Ray, S. C., Y. M. Wang, O. Laeyendecker, J. R. Ticehurst, S. A. Villano, and D. L. Thomas. 1999. Acute hepatitis C virus structural gene sequences as predictors of persistent viremia: hypervariable region 1 as a decoy. *J. Virol. 73:2938.*
11. Del Porto, P., G. Puntoriero, C. Scottà, A. Nicosia, and E. Piccolella. 2000. High prevalence of hypervariable region 1-specific and cross-reactive $CD4^+$ T cells in HCV-infected individuals responsive to IFN-α treatment. *Virology 269:313.*
12. Ray, S. C., Q. Mao, R. E. Lanford, S. Bassett, O. Laeyendecker, Y. M. Wang, and D. L. Thomas. 2000. Hypervariable region 1 sequence stability during hepatitis C virus replication in chimpanzees. *J. Virol. 74:3058.*
13. Farci, P., H. J. Alter, D. C. Wong, R. H. Miller, S. Govindarajan, R. Engle, M. Shapiro, and R. H. Purcell. 1994. Prevention of hepatitis C virus infection in chimpanzees after Ab-mediated in vitro neutralization. *Proc. Natl. Acad. Sci. USA 91:7792.*
14. Farci, P., A. Shimoda, D. Wong, T. Cabezon, D. De Gioannis, A. Strazzera, Y. Shimizu, M. Shapiro, H. J. Alter, and R. H. Purcell. 1996. Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc. Natl. Acad. Sci. USA 93:15394.*

15. Puntoriero, G., A. Meola, A. Lahm, S. Zucchelli, B. Bruno, R. Tafi, M. Pezzanera, M. U. Mondelli, R. Cortese, A. Tramontano, et al. 1998. Toward a solution for hepatitis C virus hypervariability: mimotopes of the hypervariable region 1 can induce Abs cross-reacting with a large number of viral variants. *EMBO J. 17:3521.*
16. Pessi, A., E. Bianchi, F. Bonelli, and L. Chiappinelli. 1990. Application of the continuous-flow polyamide method to the solid-phase synthesis of a multiple Ag peptide (MAP) based on the sequence of a malaria epitope. *J. Chem. Soc. Chem. Commun. 1:8.*
17. Nishihara, T., C. Nozaki, H. Nakatake, K. Hoshiko, M. Esumi, N. Hayashi, K. Hino, F. Hamada, K. Mizuno, and T. Shikata. 1993. Secretion and purification of hepatitis C virus NS1 glycoprotein produced by recombinant baculovirus-infected insect cells. *Gene 129:207.*
18. Zucchelli, S., R. Roccasecca, A. Meola, B. Bruni Ercole, R. Tafi, J. Dubuisson, G. Galfré, R. Cortese, and A. Nicosia. 2001. Mimotopes of the hepatitis C virus hypervariable region 1, but not the natural sequences induce cross-reactive Ab response by genetic immunization. *Hepatology 33:692.*
19. Yagnik, A. M., A. Lahm, A. Meola, R. M. Roccasecca, B. Bruni Ercole, A. Nicosia, and A. Tramontano. 2000. A model for the hepatitis C virus envelope glycoprotein E2. *Proteins 40:355.*
20. Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting Ab of predefined specificity. *Nature 256:495.*
21. Cerino, A., and M. U. Mondelli. 1991. Identification of an immunodominant B cell epitope on the hepatitis C virus non structural region defined by human mAbs. *J. Immunol. 147:2692.*
22. Mondelli, M. U., A. Cerino, P. Boender, P. Oudshoorn, J. Middeldorp, C. Fipaldini, N. La Monica, and W. Habets. 1994. Significance of the immune response to a major, conformational B cell epitope on the hepatitis C virus NS3 region defined by a human monoclonal Ab. *J. Virol. 68:4829.*
23. Baumert, T. F., S. Ito, D. T. Wong, and T. J. Liang. 1998. Hepatitis C virus structural proteins assemble into virus-like particles in insect cells. *J. Virol. 72: 3827.*
24. Choo, Q.-L, K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, A. Medina-Selby, P. J. Barr, et al. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA 88:2451.*
25. Okamoto, H., N. Kanai, and S. Mishiro. 1992. Full-length nucleotide sequence of a Japanese hepatitis C virus isolate (HC-J1) with high homology to USA isolates. *Nucleic Acids Res. 20:6410.*
26. Baumert, T. F., S. Wellnitz, S. Aono, J. Satoi, D. Herion, J. Tilman Gerlach, G. R. Pape, J. Y. Lau, J. H. Hoofnagle, H. E. Blum, et al. 2000. Abs against hepatitis C virus-like particles and viral clearance in acute and chronic hepatitis C. *Hepatology 32:610.*
27. Stuyver, L., R. Rossau, A. Wyseur, M. Duhamel, B. Vanderborght, H. Van Heuverswyn, and G. Maertens. 1993. Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. *J. Gen. Virol. 74:1093.*
28. Penin, F., C. Combet, G. Germanidis, P.-O. Frainais, G. Deléage, and J.-M. Pawlotsky. 2001. Conservation of the conformation and positive charges of hepatitis C virus E2 glycoprotein hypervariable region 1 points to a role in cell attachment. *J. Virol. 75:5703.*
29. Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama. 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. *J. Virol. 65:1105.*
30. Taniguchi, S., M. Okamoto, M. Sakamoto, M. Kojima, F. Tsuda, T. Tanaka, E. Munekata, E. E. Muchmore, D. A. Peterson, and S. Mishiro. 1993. A structurally flexible and antigenically variable N-terminal domain of the hepatitis C virus E2/NS1 protein: implication for an escape from Ab. *Virology 195:297.*
31. Zibert, A., W. Kraas, H. Meisel, G. Jung, and M. Roggendorf. 1997. Epitope mapping of Abs directed against hypervariable region 1 in acute self-limiting and chronic infections due to hepatitis C virus. *J. Virol. 71:4123.*
32. Zhou, Y. H., Y. K. Shimizu, and M. Esumi. 2000. Monoclonal Abs to the hypervariable region 1 of hepatitis C virus capture virus and inhibit virus adsorption to susceptible cells in vitro. *Virology 269:276.*
33. Deleersnyder, V., A. Pillez, C. Wychowski, K. Blight, J. Xu, Y. S. Hahn, C. M. Rice, and J. Dubuisson. 1997. Formation of native hepatitis C virus glycoprotein complexes. *J. Virol. 71:697.*
34. Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, et al. 1998. Binding of hepatitis C virus to CD81. *Science 282:938.*
35. Petracca, R., F. Falugi, G. Galli, N. Norais, D. Rosa, S. Campagnoli, V. Burgio, E. di Stasio, B. Giardina, M. Houghton, et al. 2000. Structure-function analysis of hepatitis C virus envelope-CD81 binding. *J. Virol. 74:4824.*
36. Flint, M., C. Maidens, L. D. Loomis-Price, C. Shotton, J. Dubuisson, P. Monk, A. Higginbottom, S. Levy, and J. A. McKeating. 1999. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. *J. Virol. 73:6235.*
37. Patel, A., J. Wood, F. Penin, J. Dubuisson, and J. McKeating. 2000. Construction and characterization of chimeric E2 glycoprotein of hepatitis C virus: analysis of E2 regions interacting with CD81. *J. Gen. Virol. 81:2873.*
38. Agnello, V., G. Abel, M. Elfahal, G. B. Knight, and Q. X. Zhang. 1999. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA 96:12766.*