Hepatitis C virus epitope-specific neutralizing antibodies in Igs prepared from human plasma

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Neutralizing antibodies directed against hepatitis C virus (HCV) are present in Igs made from anti-HCV-positive plasma. However, these HCV-specific Igs are largely ineffective *in vivo*. The mechanism for the poor effectiveness is currently unknown. We hypothesize that the presence of nonneutralizing antibodies in HCV-specific Igs interferes with the function of neutralizing antibodies, resulting in the reduction or blockage of their effect. In the present study, we identified at least two epitopes at amino acid residues 412–419 (epitope I) and 434–446 (epitope II), located downstream of the hypervariable region I within the HCV E2 protein. We demonstrated that epitope I, but not epitope II, was implicated in HCV neutralization and that binding of a nonneutralizing antibody to epitope II completely disrupted virus neutralization mediated by antibody binding at epitope I. The dynamic interaction between nonneutralizing and neutralizing antibodies may thus play a key role in determining the outcomes of HCV infection. Further exploration of this interplay should lead to a better understanding of the mechanisms of neutralization and immune escape and may indicate pathways for the manufacture of an effective HCV-specific Ig product for immune prophylaxis of HCV infection.

anti-hepatitis C virus-positive plasma | Ig intravenous

More than 70% of the estimated 170 million people worldwide who are infected with hepatitis C virus (HCV) develop chronic infection. Chronic HCV infections can lead to chronic liver disease, cirrhosis, and hepatocellular carcinoma. Chronic HCV infection is the leading indication for liver transplantation in the United States (1). Antiviral treatment of HCV is now successful in about half of the cases, but it is expensive, requires long-term treatment, and is associated with serious side effects. There is no vaccine currently available for the prevention of HCV infection.

HCV is a positive-sense RNA virus belonging to the Flaviviridae family. It encodes a single polyprotein of ≈3,000 aa. Through the action of a combination of host and viral proteases, the polyprotein is cleaved into structural proteins (core, E1, E2, and p7) and nonstructural proteins (NS2–NS5B). The two envelope glycoproteins, E1 and E2, are believed to form heterodimers/oligomers on the surface of HCV particles that participate in the process of cell entry (2).

The mechanisms that govern the clinical outcomes of HCV infection are not well understood. Whereas cellular immune responses have been considered essential for controlling viral infection (3), the role of the humoral immune response remains to be defined. Increasing evidence demonstrates that neutralizing antibodies are present in patients with chronic hepatitis C (4, 5), and that epitopes located within the E2 protein are important for HCV neutralization (6–14). Our recent data indicated that an experimental Ig preparation made from anti-HCV-positive plasma (HCIGIV) prevented HCV infection when mixed with a virus inoculum *ex vivo* before infusion into chimpanzees (15). Unfortunately, the *in vivo* efficacy of HCIGIV in both chim-

panzees and humans has been disappointing. Two clinical studies failed to show that anti-HCV Ig preparations could decrease HCV RNA levels or prevent recurrent infections after liver transplantation (16, 17). One current hypothesis is that insufficient amounts of the HCV-specific Ig preparations were administered (reviewed in ref. 18). However, before undertaking a new clinical trial or merely increasing dosage, it is important to understand more fully the mechanisms underlying HCV neutralization. In the present study, we found that HCV epitope-specific neutralizing antibody can be recovered from HCIGIV, but that a preexisting network consisting of both nonneutralization and neutralization epitopes affects the dynamic of antibody binding and subsequent neutralization. We suggest that simply increasing the frequency of administration or elevating the dose of HCIGIV would not be adequate to inactivate circulating infectious virus. We propose that enrichment of HCIGIV with antibodies directed specifically against neutralization epitopes, as described herein, may provide an approach to the improvement of current anti-HCV Ig products.

Results

Presence of HCV-Specific Antibodies in HCIGIV. Previous studies indicated that the HCV E2 protein contained neutralization epitopes that were recognizable by a number of monoclonal antibodies (6–14). These epitopes formed a cluster within a short peptide between hypervariable regions I and II. To determine whether any epitope within this segment could be recognized by human Igs, we tested HCIGIV for its ability to bind a 36-aa-long peptide (peptide A; amino acids 412–447) derived from the E2 protein (Fig. 1). As shown in Fig. 2A, HCIGIV reacted with peptide A in a dose-dependent manner and remained positive up to a dilution of 1:3,200. Negative controls, albumin and a commercial Ig intravenous (IGIV) made from anti-HCV-negative plasma, did not react with peptide A. In the second experiment, HCIGIV at different dilutions was added to the control IGIV. Binding of these Ig mixtures to peptide A could be observed at dilutions up through 1:3,200 (Fig. 2B). These results indicate that HCIGIV contains antibodies specific to epitope(s) within the HCV E2 protein between residues 412 and 447.

Dynamic Interactions Between Epitope-Specific Antibodies. Because each peptide was biotinylated at the C terminus (Fig. 1), streptavidin-coated plates were used to immobilize the peptide.
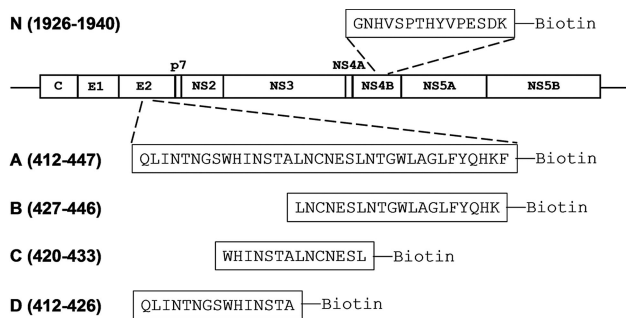
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Fig. 1. Schematic representation of HCV polyprotein and biotin-linked peptides (A, B, C, D, and N) used in this study. All peptides were synthesized based on the sequence of strain H77. The numbers indicate the position of these peptides in HCV polyprotein, and single letters are used to represent amino acids.

After affinity binding of HCIGIV, eluted antibodies specific for each peptide (peptide A, B, C, D, or N) were collected; these eluates were designated A_E, B_E, C_E, D_E, and N_E, respectively. Experiments were carried out to examine the specific binding of each eluate to individual peptides. As shown in Figs. 3 and 4A, A_E, as predicted, reacted with peptide A. It also reacted with peptide B, but not with peptide C, D, or N. Similarly, B_E reacted only with peptide A and peptide B. Under the experimental conditions used, C_E exhibited no detectable binding activity for any of these peptides, suggesting that there was no epitope within peptide C that was recognizable by C_E. D_E bound equally to peptide A and peptide D, although its overall binding activity for peptide A was only about one-fourth that of A_E. Although HCIGIV, as a positive control, could recognize all of the peptides tested, the binding was much stronger for peptide A, B,
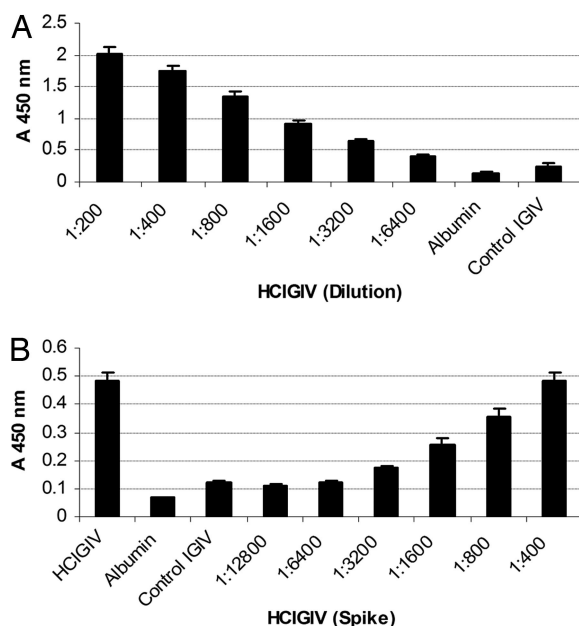

Fig. 2. Presence of HCV E2 peptide-specific antibodies in HCIGIV. (*A*) Determination of HCIGIV antibody titer to peptide A. The *x* axis indicates the dilution of HCIGIV, and the *y* axis indicates absorbance at 450 nm in ELISA. Albumin (5%) and a control IGIV (5%) at 1:400 dilution in PBS were used as controls. (*B*) Spiking of HCIGIV into the control IGIV. The *x* axis indicates dilution of HCIGIV in the control IGIV. HCIGIV at 1:400 dilution alone was used as the positive control. Albumin (5%) and the control IGIV (5%) at 1:400 dilution were used as negative controls. The *y* axis indicates absorbance at 450 nm in ELISA.

D, or N than for peptide C. As negative controls, albumin and control IGIV did not bind to any of these peptides. These data suggest that this region of the E2 protein contains two epitopes: epitope I at amino acids 412–419 and epitope II at amino acids 434–446, respectively (Fig. 4B).

B_E reacted with peptide A more strongly than it did with peptide B (Figs. 3 and 4A). This difference suggested that deletion of the N-terminal sequence 412–426 and amino acid residue 447 reduced antibody binding to epitope II, implying that amino acids 412–426 and/or 447 could enhance antibody binding to epitope II at amino acids 434–446. This conclusion, in turn, suggested a conformational nature of epitope II.

Surprisingly, A_E did not exhibit detectable binding activity for peptide D, which had been previously shown to include epitope I. This observation suggested that most, if not all, of the activity in A_E was directed against epitope II. By contrast, D_E reacted equally with both peptide A and peptide D (Figs. 3 and 4A). These results indicated that deletion of C-terminal residues 427–447 or depletion of epitope II-binding antibodies from HCIGIV permitted antibody binding to epitope I. Thus, antibody binding to epitope II might concurrently disrupt antibody recognition of epitope I. In control experiments, N_E reacted only with peptide N (Figs. 3 and 4A); the control IGIV and albumin contained no detectable antibody-binding activity to any of the peptides tested (Fig. 3).

To characterize further the nature of epitope II, A_E was used to screen a random peptide phage display library. Two major clusters of phage were recognized by antibodies in A_E (Fig. 5A). The phage-displayed peptides had a significant sequence homology with peptide A. [441]LFY[443] appeared to constitute the key residues for antibody binding. These observations prompted us to examine epitope II further by an analysis of sequence alignment of the six major HCV genotypes (10, 19). In contrast to epitope I, which had only four variant amino acids among genotypes (at residues 412, 413, 414, and 416), epitope II showed multiple variations among these genotypes, particularly in residues 444–446 (Fig. 5B). However, the skein [441]LFY[443] appeared to be conserved. We then asked whether introduction of a mutation within epitope II (including residues [441]LFY[443]) would disrupt antibody binding. The peptide sequence [439]AGLFYQH[445] was replaced by an epitope (NAPATV) from the severe acute respiratory syndrome virus in the context of peptide B (Fig. 6A). As a consequence of this substitution, the binding activity of B_E for peptide B mutant was eliminated (Fig. 6B). Similarly, the substitution also resulted in significant loss of antibody binding to epitope II by HCIGIV. In a control experiment, the mutant could be recognized by 341C, a monoclonal antibody specific for the severe acute respiratory syndrome epitope. These data confirm that there are at least two epitopes in the HCV envelope protein, one at amino acids 412–419 and the other at amino acids 434–446.

Neutralization of HCV by HCIGIV Eluates. We investigated the capacity of each HCIGIV eluate to block virus entry in a cell culture model. In this study, the virus stock was generated based on a chimera of genotype 2a. A_E, B_E, C_E, and N_E did not cause any significant reduction of virus entry (Fig. 7A). By contrast, D_E at 1:40 dilution neutralized HCV ($P < 0.05$).

These data suggested that the binding of neutralizing antibodies to epitope I was likely blocked by the presence of nonneutralizing antibodies specific to epitope II. To confirm this hypothesis, the neutralizing activity of D_E was tested in the presence of A_E (Fig. 7B). When A_E was mixed with D_E at a ratio of 1:1 or 2:1, neutralizing activity, which had been previously observed with D_E, was no longer detectable ($P < 0.05$).
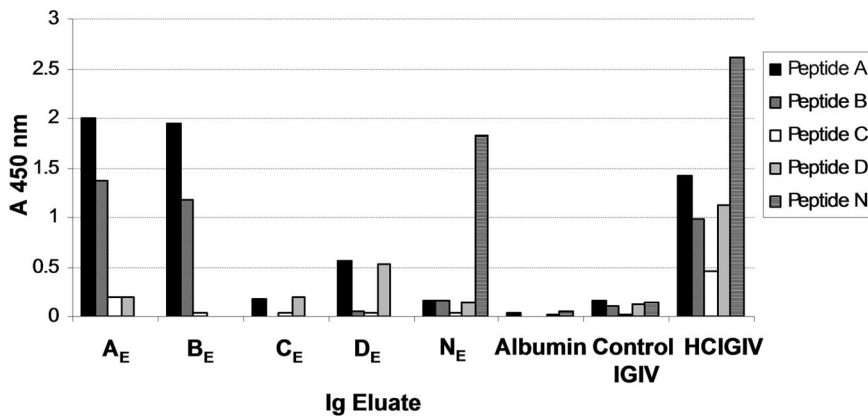
Fig. 3. Determination of HCV epitope-specific antibodies in HCIGIV. The *x* axis indicates Ig eluates (A$_E$, B$_E$, C$_E$, D$_E$, or N$_E$) collected after affinity binding and elution of HCIGIV by using a given peptide (peptide A, B, C, D, or N). HCIGIV at 1:400 dilution alone was used as the positive control. Albumin (5%) and the control IGIV (5%) at 1:400 dilution were used as negative controls. The *y* axis indicates absorbance at 450 nm in ELISA, representing specific binding of a given Ig eluate to each individual peptide.

Discussion

HCV-specific Ig preparations have not been effective in preventing HCV recurrence in patients who have undergone liver transplantation (reviewed in ref. 18). This behavior is in contrast to that of the hepatitis B virus-specific Ig product, which has been shown to be highly effective in hepatitis B virus-infected patients undergoing similar procedures (20). The poor outcome in HCV infection has been attributed primarily to insufficient quantity or frequency of the administered dose of HCV-specific antibodies. However, attributing poor outcome to usage/dosage alone may overlook the particular mechanism by which antibody neutralizes HCV, and also the possible role of competing and/or interfering antibodies in the preparations. Therefore, exploring the nature of antigen–antibody interactions in HCV infection may lead to better understanding of the mechanism(s) contributing to the poor performance of anti-HCV specific Igs and, in turn, may pave the way for the manufacture of more effective HCV-specific Ig products for immune prophylaxis of HCV infection.

In this study, we found that HCV epitope-specific neutralizing antibodies could be recovered from an HCIGIV by using affinity binding and elution. We precisely mapped two epitopes within a short segment of E2: epitope I, at amino acids 412–419, and epitope II, at amino acids 434–446. We demonstrated that

epitope I, but not epitope II, was involved in virus neutralization under our experimental conditions, which involved HCV cell culture with a genotype 2a chimera virus stock. This finding was somewhat unexpected because the region encompassing amino acids 432–447 can be recognized by at least three monoclonal antibodies (2/69a, 7/16b, 11/20). These monoclonal antibodies have been shown to be involved in neutralization, as demonstrated in an HCV pseudoparticle assay (8). The fact that the assay system and virus genotypes used in our study differ from those used in earlier studies may in part explain the observed difference between these results. Knowing that genotypes 1, 4, 5, and 6 are serologically more closely related to each other than to genotypes 2 and 3 (5), we cannot exclude the possibility that epitope II in other genotypes is associated with neutralization. In addition, it is of interest that two monoclonal antibodies (3/11 and AP33) recognize amino acids 412–426, which encompass epitope I (amino acids 412–419), but have neutralizing abilities that differ markedly *in vitro* (8, 10, 11), and their corresponding epitope(s) remain to be dissected.

The significance of identifying neutralization epitope I lies in its potential for facilitating the production of new HCIGIV
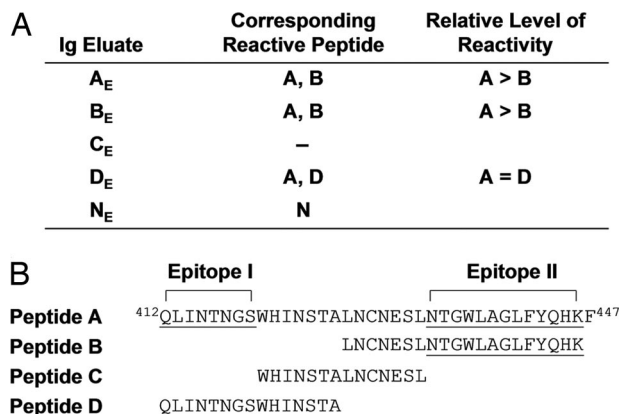

A

Ig Eluate	Corresponding Reactive Peptide	Relative Level of Reactivity
A$_E$	A, B	A > B
B$_E$	A, B	A > B
C$_E$	–	
D$_E$	A, D	A = D
N$_E$	N	

B

	Epitope I	Epitope II
Peptide A	^{412}QLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKF^{447}	
Peptide B		LNCNESLNTGWLAGLFYQHK
Peptide C	WHINSTALNCNESL	
Peptide D	QLINTNGSWHINSTA	

Fig. 4. Summary of antibody binding and location of epitopes. (*A*) Antibody-binding activity in Ig eluates for individual peptides. The data from Fig. 3 are summarized. >, stronger than; =, equal to; –, no detectable peptide binding. (*B*) Identification of epitopes within HCV E2 protein. The sequences of the identified epitopes are underlined.
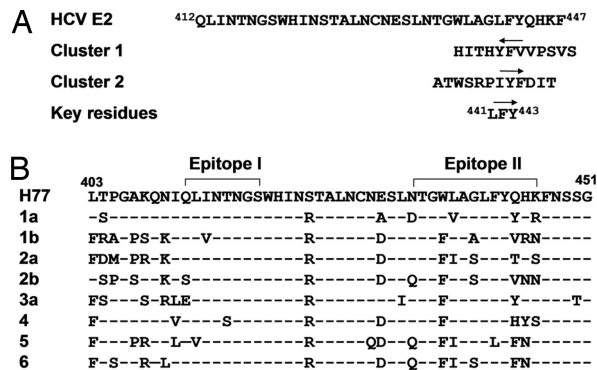

A

HCV E2 ^{412}QLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKF^{447}

Cluster 1 HITHYFVVPSVS

Cluster 2 ATWSRPIYFDIT

Key residues ^{441}LFY^{443}

B

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                Epitope I                    Epitope II
      403                                                      451
H77   LTPGAKQNIQLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKFNSSG
1a    -S-------------------------R------A--D---V-----Y-R-----
1b    FRA-PS-K---V---------------R------D-----F--A---VRN-----
2a    FDM-PR-K-------------------R------FI-S---T-S-----
2b    -SP-S--K-S-----------------R------D--Q--F--S---VNN-----
3a    FS---S-RLE-----------------R--------I---F------Y-----T-
4     F-------V----S-------------R------D-----F------HYS-----
5     F---PR--L-V---------------R------QD--Q--FI---L-FN-----
6     F-S---R-L-----------------R------D--Q--FI-S---FN------
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Fig. 5. Epitope mapping. (*A*) HCV epitope mapping by screening a random peptide phage-display library. Amino acid sequences of two phage clusters identified by screening a phage-display library (PhD-12) with A$_E$ as a source of antibodies are indicated. The direction of the arrow indicates the arrangement of the residues in the peptide critical for antibody binding. The key residues for the epitope are numbered based on the amino acid sequence of strain H77. (*B*) Alignment of amino acid sequences of the E2 regions among various HCV genotypes (10, 22). Epitopes I and II identified in this study are shown. A hyphen indicates an amino acid residue identical to that of the H77 sequence.
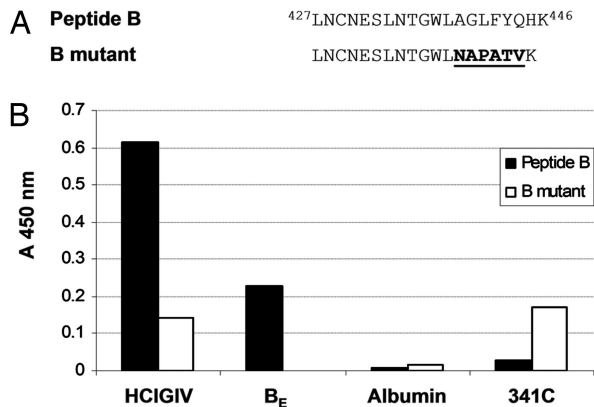
A **Peptide B** ^{427}LNCNESLNTGWLAGLFYQHK^{446}

B mutant LNCNESLNTGWL**NAPATV**K

B

Fig. 6. Identification of HCV epitope by mutation analysis. (*A*) Mutation of epitope II. Amino acid sequences for peptide B and its mutation (B mutant) are presented. The mutation site is underlined. (*B*) Detection of antibody binding by ELISA. A total of 100 ng of biotin-conjugated peptide B and its mutant (B mutant) were added to streptavidin-coated 96-well plates in an ELISA. The *x* axis indicates antibodies that were used in this assay. HCIGIV at 1:800 dilution was used as the positive control, and albumin (5%) at 1:800 dilution was used as the negative control. 341C, a monoclonal antibody that recognizes the sequence NAPATV, was used at 1:200 dilution. The *y* axis indicates absorbance at 450 nm, representing specific binding of a given antibody to each individual peptide.

preparations. In principle, epitope I can be used to achieve selective enrichment of neutralizing antibodies from current HCIGIV preparations. On the other hand, the finding of neutralization (epitope I) and nonneutralization (epitope II) epitopes within a short E2 peptide could assist the establishment of a specific "epitope-based" neutralization assay for monitoring the neutralizing antibody titer in patients' plasma and HCIGIV products. Currently, the level of anti-HCV antibody is measured by using recombinant HCV envelope proteins (16, 17). However, binding of antibody to nonneutralization epitope(s) can lead to an overestimation of the actual level of neutralizing antibodies in HCIGIV preparations, as well as in patients' plasma. Therefore,
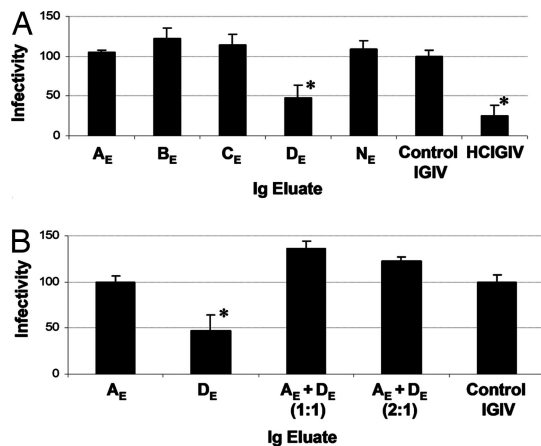

Fig. 7. HCV neutralization in cell culture. (*A*) HCV neutralization by Ig eluates. The *x* axis indicates Ig eluates that were used in this assay at 1:40 dilution. HCIGIV at 1:100 dilution was used as the positive control, and an IGIV (5%) at 1:100 dilution was used as the negative control. The *y* axis indicates infectivity (percentage of negative control). The asterisk indicates statistical significance ($P < 0.05$). (*B*) Blocking neutralizing activity of D_E by nonneutralizing Ig in A_E. The *x* axis indicates Ig A_E or D_E alone at 1:40 dilution or a mixture of A_E and D_E ($A_E + D_E$) at 1:1 or 2:1 ratio. An IGIV (5%) at 1:100 dilution was used as the negative control. The *y* axis indicates infectivity (percentage of negative control). The asterisk indicates statistical significance ($P < 0.05$).

the epitopes identified in the present study may provide the basis for the design of potency assays more reflective of the neutralization capacities of HCIGIV preparations.

Our studies demonstrated that epitopes I and II are not presented independently and equally to the antibodies. Epitope I shares a sequence (amino acids 412–426) with an element that enhances antibody binding to epitope II (amino acids 434–446), via a yet to be identified mechanism. However, once epitope II is bound by an antibody, the site of epitope I (amino acids 412–419) becomes masked. Epitope I could thus no longer be recognized by the specific antibodies (namely, D_E) directed against this epitope. Consistent with these findings, mixing nonneutralizing antibody (A_E) with neutralizing antibody (D_E) diminished the neutralizing activity of D_E. These observations immediately suggest a self-protective mechanism by which HCV can escape from antibody responses. If such a mechanism operates *in vivo*, one would anticipate that a preexisting network consisting of both neutralization and nonneutralization epitopes affects the dynamic of antibody binding, thus influencing the course of HCV infection. Furthermore, the *in vivo* efficacy of such enriched HCIGIV preparations may also depend on the binding affinity of nonneutralizing antibodies in the recipient and their capacity to interfere with the function of the selected neutralizing antibodies. Therefore, the depletion of interfering antibodies could enhance HCV neutralization.

According to this model, simply increasing the frequency of administration or elevating the dose of current HCIGIV products would not be adequate to achieve complete inactivation of circulating infectious virus. Instead, success in the enrichment of neutralization epitope-specific antibodies, accompanied by depletion of certain specific competing and/or interfering antibodies from current HCIGIV preparations, may permit systematic evaluation of the roles played by an array of neutralizing antibodies in immune prophylaxis of HCV infection.

Materials and Methods

Igs and Monoclonal Antibody. HCIGIV, an experimental 5% IGIV made from anti-HCV-positive plasma, was kindly provided by Nabi Biopharmaceuticals (Boca Raton, FL). It was made from the pooled plasma of 198 anti-HCV (EIA-2)-positive donors who otherwise met the requirements for normal plasma donations, i.e., negative for both anti-HIV and hepatitis B surface antigen and without elevated levels of alanine aminotransferase. This HCIGIV preparation had been treated by a solvent–detergent process to inactivate potential contaminating viruses. It was previously shown to neutralize HCV in both a pseudoparticle system and a chimpanzee model (15). A commercial 5% IGIV solution, which was manufactured from anti-HCV (EIA-2)-negative plasma donations, was used as a negative control. This IGIV preparation was also virally inactivated by a solvent–detergent treatment. Albumin was a commercial 25% albumin (human) that had been virally inactivated by heating at 60°C for 10 h. It was diluted to 5% with PBS before use as a control. A murine monoclonal antibody (341C), specific for peptide NAPATV (P.Z., unpublished data), was used as a control (21).

Peptide Synthesis. All peptides were synthesized by the Core Laboratory of the Center for Biologics Evaluation and Research, Food and Drug Administration, with an Applied Biosystems (Foster City, CA) Model 433A Peptide Synthesizer by using standard FastMoc chemistry (22). Synthesis of biotinylated peptides was carried out with Fmoc-Lys (Biotin-LC)-Wang resin (AnaSpec, San Jose, CA). The crude peptides were precipitated, washed with butyl methyl ether, dried under vacuum, purified by RP-HPLC by using a DeltaPak C-18 reversed-phase column (Waters, Milford, MA), and analyzed by MALDI-TOF MS on a Voyager DE-RP MALDI-TOF mass spectrometer (Applied Biosystems).

Affinity Binding and Elution. Biotinylated peptides (100 ng) were incubated for 1 h at room temperature in each well of 96-well plates precoated with streptavidin in PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T). After blocking with blocking buffer (Blocker BSA; Pierce, Rockford, IL), an appropriately diluted antibody was added to the well and incubated for 1 h. After 10 washes with PBS-T, the bound antibody was eluted with 0.2 M glycine-HCl buffer (pH 2.2) for 10 min at room temperature and neutralized immediately with 1 M Tris·HCl (pH 9.1).

ELISA. Streptavidin-coated 96-well plates were used for ELISA according to the manufacturer's instructions (Pierce). Briefly, biotinylated peptides (100 ng in 100 μl) were added to streptavidin-coated wells and blocked with Blocker BSA for 1 h. After washings with PBS-T, antibodies were added to the wells and incubated for 1 h at room temperature. After removal of unbound antibodies by washing with PBS-T, a goat anti-human peroxidase-conjugated IgG (Sigma–Aldrich, St. Louis, MO) at 1:3,000 dilution was added to the wells. After washings, the plates were kept in darkness for 10 min with 100 μl of a solution containing a tablet of orthophenylene diamine dihydrochloride (Sigma–Aldrich) diluted to 0.4 mg/ml in 0.05 M phosphate/citrate buffer (pH 5.0) containing 0.03% sodium perborate (Sigma–Aldrich). The reaction was stopped by adding 50 μl of 1 M H_2SO_4. The absorbance of each well was measured at 450 nm with a microtiter plate reader (Optimax; Molecular Devices, Palo Alto, CA).

Phage Display. Selection of peptides from a random peptide display-phage library (New England Biolabs, Beverly, MA; PhD-12) was described previously (23). Briefly, $\approx 10^{10}$ phages were incubated with individual Ig eluate/protein A mixtures for 20 min at room temperature. After eight washings with 0.05 M Tris·HCl buffer (pH 7.5) containing 0.15 M NaCl and 0.05% Tween 20, the phages were eluted from the complex with 0.1 M HCl for 8 min at room temperature. The eluted phages were then amplified in the host strain ER2738. Amplified phages were subjected to three additional rounds of selection with antibody. After selection, collected phages were grown on LB-agar plates. DNA from each single-phage plaque was sequenced, and the corresponding peptide sequence was then deduced from the DNA sequence. The sequence homology of phage-displayed peptides with different HCV genotypes (11, 19) was determined.

Neutralization Assay. FL-J6/JFH1 virus was a gift from Charles Rice at the Rockefeller University (New York, NY). Virus stock was prepared by infecting Huh 7.5 cells according to the procedures described previously (24–26). For the neutralization assay, Huh 7.5 cells were seeded at a density of 4–5×10^3 cells per well in 96-well plates to obtain 50–60% confluence after 24 h. The virus stock was titrated in Huh 7.5 cells, and a dilution was selected that would give ≈ 50 immunostained foci per 50 μl. This dilution was chosen because, in the final incubation of the test for neutralization capacity (see below), each well contained 50 μl of the virus stock and an equal volume of an appropriate dilution of the antibody preparation being tested. As a control, a dilution series was prepared from the virus stock to check the input virus titer. To test neutralization capacity, 200 μl of test antibodies appropriately diluted in DMEM containing 10% FCS, in parallel with a positive (HCIGIV) and a negative control IGIV, was mixed with 200 μl of virus stock and incubated at 37°C for 1 h. The virus/antibody mixture in aliquots of 100 μl was then added to each of four wells of the microtiter plate containing the Huh 7.5 cells. After 3 days in culture, the cells were washed, fixed with cold methanol, and then probed with a mouse monoclonal antibody directed against the HCV core antigen (kindly provided by Harry Greenberg, Stanford University School of Medicine, Stanford, CA), followed by washing and probing with a horseradish peroxidase-conjugated anti-mouse IgG (H and L) (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Stained foci were developed by using HistoMark TrueBlue (Kirkegaard & Perry Laboratories). Stained foci were counted in quadruplicate wells, and the mean number of foci per well was calculated. Infectivity was expressed as a percentage of the mean number of foci per well in the negative control group. Thus, neutralizing activity was equivalent to the decrease, if any, from 100%.

Statistical Analysis. JMP, version 5.0, software (SAS Institute, Cary, NC) was used for analyzing data. Pairwise comparisons of the means between two Ig eluates at a time were performed by using Student's t test. For an overall comparison of means, the Tukey–Kramer honestly significant difference test was used. Statistical significance was set at $\alpha = 0.05$. A positive test value generated between two means is indicative of a significant difference.

Note. While this paper was being reviewed, we were made aware of a comprehensive study of clinical samples collected after a single-source outbreak of hepatitis C, suggesting that neutralizing antibodies produced early in the infection play an important role in the clearance of HCV, i.e., in avoiding chronicity (27). An E2 epitope encompassing amino acid residues 412–423 appeared to be involved in the entry of virus particles into cells. This epitope is highly similar to, if not identical with, epitope I identified in the present study.

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