Antibody Response to Hypervariable Region 1 Interferes with Broadly Neutralizing Antibodies to Hepatitis C Virus

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ABSTRACT

Hypervariable region 1 (HVR1) (amino acids [aa] 384 to 410) on the E2 glycoprotein of hepatitis C virus contributes to persistent infection by evolving escape mutations that attenuate binding of inhibitory antibodies and by blocking access of broadly neutralizing antibodies to their epitopes. A third proposed mechanism of immune antagonism is that poorly neutralizing antibodies binding to HVR1 interfere with binding of other superior neutralizing antibodies. Epitope mapping of human monoclonal antibodies (HMabs) that bind to an adjacent, conserved domain on E2 encompassing aa 412 to 423 revealed two subsets, designated HC33 HMabs. While both subsets have contact residues within aa 412 to 423, alanine-scanning mutagenesis suggested that one subset, which includes HC33.8, has an additional contact residue within HVR1. To test for interference of anti-HVR1 antibodies with binding of antibodies to aa 412 to 423 and other E2 determinants recognized by broadly neutralizing HMabs, two murine MAbs against HVR1 (H77.16) and aa 412 to 423 (H77.39) were studied. As expected, H77.39 inhibited the binding of all HC33 HMabs. Unexpectedly, H77.16 also inhibited the binding of both subsets of HC33 HMabs. This inhibition also was observed against other broadly neutralizing HMabs to epitopes outside aa 412 to 423. Combination antibody neutralization studies by the median-effect analysis method with H77.16 and broadly reactive HMabs revealed antagonism between these antibodies. Structural studies demonstrated conformational flexibility in this antigenic region, which supports the possibility of anti-HVR1 antibodies hindering the binding of broadly neutralizing MAbs. These findings support the hypothesis that anti-HVR1 antibodies can interfere with a protective humoral response against HCV infection.

IMPORTANCE

HVR1 contributes to persistent infection by evolving mutations that escape from neutralizing antibodies to HVR1 and by shielding broadly neutralizing antibodies from their epitopes. This study provides insight into a novel immune antagonism mechanism by which the binding of antibodies to HVR1 blocks the binding and activity of broadly neutralizing antibodies to HCV. Immunization strategies that avoid the induction of HVR1 antibodies should increase the inhibitory activity of broadly neutralizing anti-HCV antibodies elicited by candidate vaccines.

Up to 170 million people worldwide are infected with hepatitis C virus (HCV), with significant risk for liver failure and hepatocellular carcinoma. The World Health Organization estimates an annual increase in the global burden by two million new infections (1), and in the United States HCV is increasing in young adults from injection drug use (2). Multiple lines of evidence suggest that CD4+ and CD8+ T cell responses are needed to control acute infection but insufficient to prevent long-term persistence (3). Accumulating data indicate that neutralizing antibodies are an important correlate of HCV clearance. In chimpanzee studies, an infectious inoculum obtained during acute infection from a patient who eventually developed chronic HCV hepatitis could be neutralized by in vitro incubation with plasma of the same subject collected at 2 years after the initial infection (4). A neutralizing-antibody response measured against pseudotyped retroviral particles expressing HCV E1E2 glycoproteins (HCVPp) has been associated with control of infection in single-source outbreaks of acute HCV infections (5). In addition, antibodies to HCV E2 prevent infection (6, 7) and clear established infection (8) in a human liver-mouse chimeric model. In spite of the relationship between antibodies and protection against HCV infection, 104 to 106 virions per milliliter of serum can be detected during chronic infection even in the presence of high levels of neutralizing antibodies in serum.

One driver of persistent viremia is a high degree of viral variants, or “quasispecies.” From a viral replication rate of 1012 copies per day with an error-prone NS5B RNA-dependent polymerase, the estimated mutation rate is 2.0 × 10−3 base substitutions per genome per year (9). A major determinant of antibody-mediated neutralization is the first 27 amino acids (aa 384 to 410), i.e., hypervariable region 1 (HVR1), located at the N terminus of HCV E2

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This E2 segment is highly immunogenic, and antibodies against HVR1 can be detected in the majority of HCV-infected individuals (11). However, antibodies to HVR1 over time select for viral variants that escape the existing antibody response (12). The limited nature of the B cell response to this region is shown in studies of HCV evolution from acute to chronic disease (13, 14). Sequential autologous serum antibodies inefficiently neutralize emerging variants, in contrast to their capacity to neutralize earlier quasispecies. Viral escape is associated with mutations within HVR1. Other negative modulators of antibody-mediated neutralization include cell-to-cell transmission, virion-associated lipoproteins, virus envelope protein-associated glycans, and HVR1 itself; HVR1 can mask epitopes or limit access of virus-neutralizing antibodies to their epitopes (15–17). Indeed, virions lacking HVR1 are less susceptible to neutralization by anti-SR-B1 antibodies but are more sensitive to antibody- and soluble CD81-mediated neutralization (18, 19). It has been suggested that HVR1 partly shields CD81 binding sites and conserved epitopes mediating virus neutralization (18).

The concept of interfering antibodies is controversial, but this remains a possible mechanism that contributes to the development of persistent HCV infection in infected subjects (20–23). In this study, we performed more extensive epitope mapping of a panel of human monoclonal antibodies (HMAbs) to a highly conserved region on the E2 glycoprotein encompassing aa 412 to 423, designated HC33-related antibodies (20). Mapping by binding against a library of alanine substitution E2 mutants with mutations from aa 384 to aa 446 revealed two subsets of HC33 HMAbs. Although both subsets have contact residues within aa 412 to 423, one subset included a contact residue within HVR1. This raised the possibility that anti-HVR1 antibodies interfere with the neutralizing activities of antibodies to aa 412 to 423. Two murine MAb were utilized to explore the relationship between anti-HVR1 and antibodies against aa 412 to 423. Surprisingly, the anti-HVR1 antibody inhibited the binding and neutralization of both sets of HC33 HMAbs. This interference of binding and neutralization also was observed against other broadly neutralizing HCV HMAbs that have contact residues outside aa 412 to 423. Thus, immunization strategies that avoid the induction of HVR1 antibodies should increase the inhibitory activity of broadly neutralizing anti-HCV antibodies.

MATERIALS AND METHODS

Cells, viruses, and antibodies. HEK-293T cells were obtained from the ATCC. Hub7.5 cells (generously provided by C. Rice, Rockefeller University) were grown in Dulbecco’s modified minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO) and 2 mM glutamine. The genotype Ia H13-5 recombinant infectious HCV in cell culture (HCVcc) was generously provided by Stanley Lemon (University of North Carolina at Chapel Hill) (24). HMAbs CBH-7, HC-1, HC-11, HC84.20, HC84.20, HC-84.26, HC33.1, HC33.4, HC33.8, and HC33.29 against HCV E2 glycoproteins were produced as described previously (20, 25–28). Mouse MAbs H77.16 and H77.39 and their Fab fragments against HCV E2 glycoprotein were produced as described previously (29).

Binding to E2 glycoprotein. A standard enzyme-linked immunosorbent assay (ELISA) (28) was used to compare HMAB binding to wild-type (wt) and AHVR1 HCV E2 glycoproteins. In some experiments, incubations were performed at a different temperature or in a different sequence when paired with H77.16. Briefly, ELISA plates were coated with Galanthus nivalis lectin (GNA) and blocked with 2.5% nonfat dry milk and 2.5% normal goat serum in phosphate-buffered saline (PBS) supplemented with 0.1% Tween 20. Lysates of cells expressing HCV E2 glycoproteins were captured by GNA onto the microtiter plate, followed by incubation with HMAbs and then washing. Bound HMAb was detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG (Promega; Madison, WI), followed by incubation with p-nitrophenyl phosphate (Sigma) for color development. Absorbance was measured at 405/570 nm. The assay was carried out in triplicate in three independent assays for each HMAb.

Inhibition assay. An ELISA measured the inhibition by mouse MAbs of HMAb binding to GNA-captured E2 glycoproteins (30). Briefly, microtiter plates were coated with GNA and blocked with 2.5% bovine serum albumin (BSA) and 2.5% normal goat serum in 0.1% Tween–PBS. Pretitrated mouse MAb was added to each well at a saturating concentration. After 1 h, HMAb was added at a concentration corresponding to 65% to 75% of the maximal optical density (OD) level, incubated for 30 min at either room temperature (RT) or 4°C, and then washed. Bound HMAb was detected as described above. This assay was carried out in triplicate, a minimum of two times for each HMAb.

Epitope mapping. Epitope mapping was performed using alanine substitution mutants with mutations in a defined E2 region (aa 404 to 425) by ELISA. Alanine substitution mutants were constructed in plasmids carrying the 1a H77C E1E2-coding sequence (GenBank accession no. AF009606), as previously described (31). All mutations were confirmed by DNA sequence analysis (EliBio Pharmaceuticals, Inc. Hayward, CA) for the desired mutation and for exclusion of unexpected residue changes in the full-length E1-E2-encoding sequence. The resulting plasmids were transfected into HEK293T cells for transient protein expression using the calcium phosphate method, as suggested by the manufacturer (Clontech, Mountain View, CA).

Antibody cooperativity for virus neutralization. Synergistic, additive, or antagonistic cooperativity by two antibodies for virus neutralization was evaluated by the median-effect analysis method, as described previously (32, 33), using the CompuSyn software (CompuSyn Inc., Paramus, NJ). The approach takes into account the potency, the shape, and the slope of the dose-dependent neutralization curve of each antibody alone and in combination, at a constant ratio, to calculate a combination index (CI). A CI value of 1 indicates an additive effect, a value of <1 indicates synergism, and a value of >1 indicates antagonism. For each antibody, dose–dependent neutralization was measured initially to determine the concentration that resulted in 50% reduction (50% inhibitory concentration [IC50]). The constant ratio of the combined antibodies was set by the IC50 of the two antibodies. Neutralization of a serial 2-fold dilution of each antibody alone and in combination was then measured in a range of concentrations above and below the IC50. The measured neutralization values were entered in the program as fractional effect (FA) in the range 0.01 < FA < 0.99 for each of the two antibodies alone and in combination. The software determines the linear correlation coefficient, r, of each curve to indicate the fit or conformity of the data with respect to the median-effect method and calculates the CI values in relation to FA values. The 1a HCVcc and each HMAb or combination of HMABs were incubated for 1 h at 37°C and then plated onto Huh7.5 cell monolayers (3×103 cells/well) that were grown in 8-well chamber slides (Nalge Nunc, Rochester, NY) for virus neutralization assay as described previously (20). These assays were carried out in four replicates for each HMAb and combination of HMABs.

Production and purification of recombinant Fab molecules HC33.4 and HC33.8. Synthetic genes that were codon optimized for Drosophila melanogaster encoding heavy and light chains of the Fab regions of each antibody were cloned into a Drosophila S2 Fab expression vector containing a double Strep tag for efficient affinity purification. Drosophila S2 cells were transfected with these plasmids as reported previously (34). For large-scale production, cells were induced with 3 μM CdCl2 at a density of approximately 107 cells/ml for 7 days and pelleted, and Fabs were purified by affinity chromatography from the supernatant using a Strep Tactin
Table 1 Data collection and refinement statistics

| Parameter                      | Valuea for:          |
|--------------------------------|----------------------|
|                                | Fab HC33-4 + peptide| Fab HC33-8 + peptide |
| Data collection                |                      |
| Space group                    | P21                  | P2                   |
| Complexes per AU               | 2                    | 1                    |
| Cell dimensions                |                      |
| \(a, b, c (\text{\AA})\)      | 49.81, 149.43, 67.95 | 49.65, 49.57, 66.39 |
| \(\alpha, \beta, \gamma (^\circ)\) | 90, 90.08, 90        | 90, 98.5, 90         |
| Resolution (\text{\AA})       | 47.25—1.65 (1.75—1.65) | 49.57—1.90 (2.02—1.90) |
| CC(1/2)                        | 99.8 (68.8)          | 99.9 (89.8)          |
| I/\(I_{	ext{free}}\)          | 12.52 (1.63)         | 16.17 (1.21)         |
| Completeness (%)               | 99.7 (98.4)          | 99.6 (97.8)          |
| Redundancy (%)                 | 5.3 (5.2)            | 6.6 (6.4)            |

| Refinement                     |                      |
| Resolution (\text{\AA})       | 24.16—1.65           | 49.57—1.90           |
| No. of reflections             | 118,727              | 33,727               |
| \(R_{\text{work}}/R_{\text{free}}\) | 0.171/0.189         | 0.194/0.236          |
| No. of atoms                   |                      |
| Protein                        | 6,589                | 3,414                |
| Ligand                         | 0                    | 0                    |
| Water                          | 604                  | 116                  |
| Residues per AU                | 862                  | 448                  |
| B factors                      |                      |
| Protein                        | 20.8                 | 38.1                 |
| Ramachandran plot (%)          |                      |
| Favored regions                | 98.00                | 96.80                |
| Allowed regions                 | 2.00                 | 2.70                 |
| Outliers                       | 0                    | 0.50                 |
| Root mean square deviation     |                      |
| Bond length (\text{\AA})      | 0.01                 | 0.01                 |
| Bond angle (\text{\circ})     | 1.09                 | 1.16                 |

a Values in parentheses correspond to the highest-resolution shell.

Superflow column, followed by size exclusion chromatography using a Superdex 200 column. Purified monomeric Fab was concentrated to approximately 25 mg/ml.

Complex formation, crystallization, data collection, structure determination, and refinement. A synthetic peptide comprising residues 406 to 425 (PGAKQNIQLINTNGSWHINST) of the H77 strain was synthesized by GenScript (H9251), and dissolved in water at 10 mg/ml.

Crystal structure analysis. Peptides were aligned using the Match-Maker algorithm implemented in Chimera (H9262) and an iterative alignment process pruning long atom pairs until no pair exceeds 1 Å. Root mean square deviations were calculated using Chimera. Buried solvent-accessible surface areas for the interfaces as well as for individual residues within the peptides were calculated using the PISA server (H11001). Interactions were determined using the protein interactions calculator (PIC) (H9262). Figures were prepared with PyMOL (http://www.pymol.org).

Statistical analysis. Statistical tests were two sided, and \(P\) values (calculated by GraphPad software) below 5% are considered significant.

Protein structure accession numbers. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (www.pdb.org) under accession numbers 5FGB and 5FGC.

RESULTS

HVR1 has different effects on blocking antibody access to epitopes on E2. HVR1 attenuates the activity of broadly neutralizing antibodies to HCV (18, 19). Its ability to decrease the potency of virus-neutralizing antibodies was established with representative HMAbs to clusters of overlapping epitopes, designated antigenic domains B and C (18). To test whether HVR1 acts by shielding access of these and other broadly neutralizing antibodies to their respective epitopes, studies of binding to 1a H77C recombinant E2 protein with and without HVR1 (\(\Delta\)HVR1) were performed (Fig. 1A and B). Representative antigenic domain B antibodies (HC-1 and HC-11 [43]) bound to higher levels to \(\Delta\)HVR1 E2 than wt E2 but with different degrees of increase (86% and 14%; \(P < 0.05\)). Antigenic domain D antibodies (HC84.20, HC84.24, and HC84.26 [28]) also bound more to \(\Delta\)HVR1 E2 than to wt E2 (56 to 75%; \(P < 0.05\)). CBH-7, an antigenic domain C antibody (25), also bound more to \(\Delta\)HVR1 E2 but with only a minimal increase (6%; \(P < 0.05\)).

We next interrogated HMAbs that recognize linear epitopes located in a highly conserved region on E2, encompassing aa 412 to 423, designated antigenic domain E (20, 44), and we observed different patterns. HC33.1 behaved similarly to antigenic domain B and D antibodies, with greater binding to \(\Delta\)HVR1 E2 than wt E2 (56 to 75%; \(P < 0.05\)). Unexpectedly, binding by three other E antibodies, HC33.4, HC33.8, and HC33.9, to \(\Delta\)HVR1 E2 was reduced compared to that with wt E2 protein (65 to 78%; \(P < 0.05\)). The decrease in binding of these MAbs to \(\Delta\)HVR1 E2 suggests that part of their epitopes may fall within HVR1.

Epitope mapping of antibodies to a highly conserved region on E2. Prior mapping of HC33-related HMAb epitopes was limited to aa 411 to 446 by alanine-scanning mutagenesis analysis of H77C E2 (20). That analysis revealed loss-of-binding residues located at L413, G418, and W420. We expanded on these results (36). The crystal structures of the Fab complexes were determined by the molecular replacement method using Phaser (37). The molecular replacement for Fab HC33.4 was performed using separate variable and constant regions of a hypothetical Fab fragment assembled from the light chain of PDB accession code 4JZO (89% amino acid identity) and the heavy chain of PDB accession code 3KDM (91% amino acid identity) as search model. The molecular replacement for Fab HC33.8 was performed using Fab HC33.4 as search model. Model building was performed using Coot (38), and refinement was done using AutoBuster (39). Difference maps calculated after refinement of the Fab molecules revealed an unambiguous side chain density for a tryptophan residue close to the complementarity-determining regions (CDRs), which allowed us to manually build a partial atomic model for the peptide comprising aa 418 to 421 (HC33.4 complex) and aa 415 to 421 (HC33.8).

Crystal structure analysis. Peptides were aligned using the Match-Maker algorithm implemented in Chimera (40) and an iterative alignment process pruning long atom pairs until no pair exceeds 1 Å. Root mean square deviations were calculated using Chimera. Buried solvent-accessible surface areas for the interfaces as well as for individual residues within the peptides were calculated using the PISA server (41). Interactions were determined using the protein interactions calculator (PIC) (42). Figures were prepared with PyMOL (http://www.pymol.org).

Statistical analysis. Statistical tests were two sided, and \(P\) values (calculated by GraphPad software) below 5% are considered significant.

Protein structure accession numbers. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (www.pdb.org) under accession numbers 5FGB and 5FGC.
using alanine-scanning mutagenesis of aa 384 to 446 on E2, which included the entire HVR1 region (aa 384 to 410) (Fig. 1C). The E2 mutants were expressed in 293T cells, and binding by HC33.1, HC33.4, HC33.8, and HC33.29 to cell lysates was measured by ELISA. Expression levels of the mutants were normalized by binding with CBH-17, a nonneutralizing HMAb that recognizes a different linear epitope on HCV E2 (25). A test concentration of 50% maximum binding of each antibody was selected for epitope mapping studies. This was determined by dose-dependent binding of each HC33 HMAb to E2 that showed the test concentration in the linear portion of the binding curve (data not shown). A greater than 80% reduction in binding was observed again for all four HC33-related HMAbs with alanine substitutions at L413A, G418A, and W420A (Fig. 1C), which suggests that they bind to the same or to nearly identical epitopes. Three of these antibodies, HC33.4, HC33.8, and HC33.29, also showed greater than 80% reduction in binding against a K408A mutant E2 protein. Taken together, these mapping studies revealed two subsets of HC33-related antibodies and explain why the deletion of HVR1 led to decreased binding by HC33.4, HC33.8, and HC33.29 but not HC33.1.

Anti-HVR1 antibody blocks the binding of broadly neutralizing antibodies. Epitope mapping of HC33-related antibodies revealed two subsets. One set, HC33.1, has contact residues that are restricted to aa 412 to 423. This is similar to other MAbs to this region, e.g., AP33, HCV1, and H77.39 (29, 45). The other set, HC33.4, HC33.8, and HC33.29, includes a putative contact residue within HVR1 that is located at aa 408. This raises the possibility that at least some anti-HVR1 antibodies can interfere with the functions of antibodies to aa 412 to 423 because of their spatial proximity. To test this hypothesis, blocking studies were performed with two murine MAbs, H77.39 and H77.16 (29). H77.39 binds to an epitope centered at aa 412 to 423, whereas H77.16 recognizes an epitope within HVR1 (29). Epitope mapping by alanine-scanning mutagenesis of H77C E2 from aa 384 to 446 confirmed that H77.39 recognizes residues (K408, D410, and G412) within HVR1 (Fig. 1C).
Mabs was tested for blocking of binding by representative antigenic domain B to E HMAbs (Fig. 2). H77C E2 was first incubated with either H77.39 or H77.16 prior to adding the test HMAb. As expected, H77.39 inhibited the binding to E2 of both subgroups of HC33 HMAbs (67 to 75%; P < 0.05) (Fig. 2A). Inhibition also was observed against representative neutralizing HMAbs to two other epitope clusters, HC-1 and HC-11 (antigenic domain B) were inhibited by H77.39 between 73 and 74% (P < 0.05), and HC84.24 and HC84.26 (antigenic domain D) were inhibited between 64 and 69% (P < 0.05). Against a neutralizing antibody (CBH-7) to a third domain C cluster, essentially no inhibition was observed (P > 0.05). The inhibition of domain D HMAb binding by an antibody to aa 412 to 423 has been observed previously and is unidirectional, which is consistent with proximity but not an overlapping nature of their respective epitopes (20).

Unexpectedly, H77.16, a murine MAb that binds primarily to 1a H77C HVR1 (29), also blocked both subsets of HC33-related HMAbs by 75 to 89% (P < 0.05) (Fig. 2B). Although we anticipated inhibition by H77.16 against HC33.4, HC33.8, and HC33.9 because of the effect of the K408A mutation on their binding, we did not expect a loss of HC33.1 binding (Fig. 1C). H77.16 blocked antigenic domain B antibodies HC-1 and HC-11 by 51% to 53% (P < 0.05) and antigenic domain D antibodies HC84.24 and HC84.26 by 39% to 44% (P < 0.05). In contrast, binding by an antigenic domain C antibody, CBH-7, was not affected by H77.16 (P > 0.05). Contact residues within HVR1 have not been identified for antibodies to antigenic domains B, C, and D in previous studies (25, 28, 43). Thus, the global blocking effect of H77.16 on the binding of these broadly neutralizing antibodies suggests that anti-HVR1 antibodies may interfere with the neutralizing antibodies of antigenic domains B, D, and E.

Anti-HVR1 interferes with broadly neutralizing antibodies. We next examined the neutralization of 1a H77 HCVcc by representative antigenic domain B to E HMAbs in the presence or absence of “interfering” HMAbs. We assessed whether combinations of antibodies were antagonistic, additive, or synergistic using the median-effect analysis method, as described in Materials and Methods (32, 33). A constant ratio between the HVR1 antibody H77.16 and HC33.1 or HC33.4 (domain E), HC-11 (domain B), HC84.26 (domain E), or CBH-7 (domain C) was set by their respective IC_{50}s against the 1a H77 (HJ3-5) HCVcc (24). The IC_{50}s for HC-11 (1.2 μg/ml) and HC84.26 (0.080 μg/ml) were previously established (28, 43). Dose-dependent neutralization was performed for H77.16, HC33.1, HC33.4, and CBH-7, which determined their respective IC_{50}s of 0.5, 3.24, 0.11, and 1.8 μg/ml (data not shown). Dose-dependent neutralization was tested for each antibody alone and in combination in a range of 2-fold dilutions in concentration from 8 × IC_{50} to 1/16 × IC_{50}. A representative set of analyses to determine cooperativity in virus neutralization is shown in Fig. 3A to C. For H77.16 and HC33.4. Dose-dependent neutralization for H77.16, HC33.4, and H77.16 plus HC33.4 from a dose of 8 × IC_{50} to 1/32 × IC_{50} was determined (Fig. 3A); fractional effect (FA) values were plotted in relation to dose (Fig. 3B), and combination index (CI) values were calculated and plotted in relation to FA (Fig. 3C). These studies were performed initially for H77.16 in combination with HC33.1, HC33.4, HC-11, HC84.26, or CBH-7. For each set of analyses, the linear correlation coefficient r was greater than 0.95, indicating a high goodness of fit to the plots (data not shown). The CI values of the paired studies at FA values of the 50% effective dose (ED_{50}), ED_{75}, and ED_{90} were tabulated (Fig. 3D). Aside from CBH-7, the CI values for the remaining antibodies, HC33.1, HC33.4, HC-11, or HC84.26, in combination with H77.16 at the FA of ED_{50} (range, 1.44 to 1.84), ED_{75} (range, 1.31 to 2.76), and ED_{90} (range, 1.18 to 2.32) were all above 1, which indicates antagonism. Moderate (CI ranging between 1.2 and 1.45) antagonism was observed between H77.16 and HC33.1 or HC84.26 (Fig. 4A). Stronger antagonism (ranging between 1.46 and 3.0) was observed between H77.16 and HC33.4 or HC-11. These findings are consistent with the ability of H77.16 to interfere with the binding of representative antigenic domain B (HC-11), D (HC84.26), and E (HC33.1 and HC33.4) HMAbs. Additive cooperativity with CI values near 1.0 (defined as 0.9 to 1.1) was observed between H77.16 and CBH-7, which is consistent with the minimal competition between these two antibodies (Fig. 2B and 4A).

To assess whether the mass of full-length IgG binding to HVR1 decreases access of broadly neutralizing antibodies to their epitopes on E2 by steric hindrance, combination antibody studies were repeated with H77.16 Fab fragments (Fig. 3D). As documented for each pair, the CI values at ED_{50}, ED_{75}, and ED_{90} were

FIG 2 Inhibition of binding by an antibody to HVR1 against broadly neutralizing HMAbs. Results of competition studies with two murine MAs against antigenic domain B to E HMAbs are shown. Lysates of 293T cells expressing recombinant H77C E2 were used. Unlabeled murine MAb H77.39 (A) or H77.16 (B) at 20 μg/ml was first incubated with E2 that had been immobilized on an ELISA plate (28). Test HMAb at 1 μg/ml was then added, and bound HMAb was measured as described previously (28). The percent inhibition was based on test HMAb binding in the absence of the murine MAb. Data are shown as mean percent inhibition ± SD from two experiments performed in triplicate.
substantially lower for H77.16 Fab₂ fragments than for intact H77.16 IgG in combination with HC33.1, HC33.4, HC-11, or HC84.26. The mean CI values for combination studies with H77.16 Fab₂ ranged between 0.93 and 1.07 for H77.16 Fab₂ molecules, which are within the additive range (Fig. 4B). The CI values for H77.16 Fab₂ in combination with CBH-7 remained within the additive range (Fig. 3D and 4B). Because both H77.16 and HC33.4 have at least one critical residue within HVR1 as determined by alanine substitution studies (Fig. 1C) and H77.16 Fab₂ interferes less than full-length H77.16 against HC33.4 neutralization, inhibition-of-binding studies were performed. The findings showed that full-length H77.16 inhibited HC33.4 binding to E2 more than H77.16 Fab₂, \( P < 0.05 \) (Fig. 4C). Thus, the mass of an intact IgG binding to an epitope in HVR1 decreases the access of broadly neutralizing antibodies to their respective epitopes.

Temperature and time of addition alter anti-HVR1 interference of binding by broadly neutralizing antibodies. Previous studies with HCV (46) and other flaviviruses (47) demonstrated that higher incubation temperatures lead to greater antibody binding to cryptic epitopes because of enhanced viral protein motion. We hypothesized that at higher temperatures, binding by broadly neutralizing antibodies would be increased due to greater exposure of their epitopes and decreased steric hindrance by anti-HVR1 MAbs. Two sets of H77C E2 were first exposed to H77.16 (20 μg/ml) at room temperature (RT). One (test) set was incubated at 40°C prior to addition of antigenic domain E, B, and D HMAbs; the other (control) set remained at RT prior to addition of the HMAbs. After 30 min at either RT or 40°C, detection of test HMAb binding was determined by ELISA. Indeed, greater binding by each antigenic domain E, B, and D HMAb was observed at 40°C than at RT, and this was associated with significantly less inhibition by H77.16 at 40°C than at RT, \( P < 0.05 \), Fig. 5A). Furthermore, we tested whether preincubation with either broadly neutralizing antibodies or H77.16 affected the ability of H77.16 to interfere with the binding of E, B, and D HMAbs. When E2 was first incubated with each E, B, or D HMAb and then with H77.16, virtually no inhibition of binding by H77.16 was observed, as expected (Fig. 5B). When E2 was first incubated by H77.16 and then with each E, B, or D HMAb, the magnitudes of inhibition by H77.16 against the domain E, B, or E HMAbs were similar to the observed inhibition, as shown in Fig. 5A at RT. Binding of the control antibody, CBH-7, to E2 was not affected by H77.16 under either test conditions (Fig. 5B). These observations support the hypothesis of steric hin-
Determination of binding of broadly neutralizing MAbs by anti-HVR1 MAbs.

**Determination of the structure of Fab-peptide complexes.**

Because the HC33.4, HC33.8, and HC33.29 epitopes appear to overlap between HVR1 and aa 412 to 423, structural analysis was performed. We expressed the Fab fragments derived from HC33.4 and HC33.8 and performed cocrystallization trials of complexes containing the Fab and a peptide comprising residues 406 to 425 (PGAKQNIQLINTNGSWHINST) of the genotype 1a strain H77. This peptide was chosen to include all putative contacts revealed by the alanine-scanning mutagenesis, i.e., residues K408, L413, G418, and W420. We obtained crystals diffracting to 1.65-Å resolution (Fab HC33.4) and 1.9-Å resolution (Fab HC33.8), respectively (Table 1). The structures of both complexes were determined by the molecular replacement method (see Materials and Methods for more details). Since comparison of peptides from both complexes revealed an identical amino acid backbone conformation and the peptide in the HC33.8 complex structure is more complete, we concentrated our further analysis on this complex.

**Molecular determinants of Fab HC33.8 interaction with its peptide epitope.**

The conformation of the peptide in complex with Fab HC33.8 resembles the recently described conformation of a similar peptide in complex with Fab HC33.1 (48). The interaction between the peptide and Fab HC33.8 is dominated by the side chain of W420 that protrudes and is deeply immersed into a cavity formed by the long complementarity-determining region 3 loop of the heavy chain (CDR-H3), the framework residues around CDR-H2, and the CDR-L3 loop (Fig. 6B and C). This pattern agrees with the results of the alanine-scanning mutagenesis, with W420 being a primary determinant of antibody binding. The second residue that is suggested by the alanine-scanning mutagenesis as crucial for an antibody–E2 interaction is G418. This glycine residue makes three hydrogen bonds involving main-chain atoms; however, this cannot explain the amino acid specificity suggested by alanine-scanning mutagenesis. It is more likely that the extensive flexibility of the glycine residue is required to facilitate the protrusion of the W420 side chain into the described cavity in the paratope. In contrast to the case for the HC33.1 complex, in which electron density was observed for residues 412 to 423, peptide residues 412 to 414 of the HC33.8 complex are likely to be disordered, and no evidence for further direct interactions between antibody and peptide were observed.

The peptide in the HC33.8 complex is exposed to the solvent, and particularly N415 (i.e., the region directly downstream of the disordered part of the peptide) is located in a solvent channel (Fig. 6A), suggesting that crystal packing does not prevent a direct interaction between K408 and the antibody. However, the N-terminal peptide parts of the HC33.1 and HC33.8 complexes likely differ due to a short stretch at the end of β-strand F in the heavy-chain variable region of HC33.8 (101VFTDS105, versus 101VSSDI105 in HC33.1). The distances between superposed Ca atoms from the corresponding segments of HC33.1 and HC33.8 amount to 0.2 Å and 0.6 Å at residues 101 and 105, respectively, and to 4.3 Å in the middle, with the HC33.8 segment bulging out. Fab HC33.1 tightly interacts with I414 in the N-terminal part of the peptide via two main-chain hydrogen bonds from SH103, presumably stabilizing the peptide conformation in the HC33.1 complex. The bulge observed in the HC33.8 complex prohibits this interaction, suggesting major differences in the N-terminal part of the interface. The complex structure can therefore neither confirm nor rule out a direct interaction between antibody and the N-terminal part of the peptide.

**Conformation of the E2 peptide from aa 415 to 423 in complex with Fab HC33.8.**

The peptide in the HC33.8 complex adopts an extended conformation similar to the conformation observed in complex with the related HC33.1 Fab (48). However, this binding mode contrasts with the β-hairpin formed in complex with
three independent neutralizing antibodies (49, 50) or with the extended conformation observed in complex with MAb 3/11 (51) (Fig. 7). All three backbone conformations have been observed in complex with broadly neutralizing antibodies, underlining the structural flexibility at the surface of infectious virus particles. Our data suggest that different conformations of the antigenic region aa 412 to 423 are in equilibrium and that independent antibodies recognize this site following the principle of induced fit or conformational selection. The demonstrated conformational flexibility in this antigenic region supports the hypothesis of a temperature-dependent steric hindrance of binding of broadly neutralizing MAbs by anti-HVR1 MAbs. An intact IgG that dangles upstream of the flexible antigenic domain E (i.e., one that binds the C terminus of HVR1) sterically blocks binding of broadly neutralizing antibodies to adjacent epitopes.

**FIG 5** Inhibition of broadly neutralizing antibody binding is altered by changes in temperature of incubation and by timing of exposure. Cell lysates of 293T cells expressing recombinant H77C E2 were captured with GNA-precoated wells in duplicate sets. (A) Unlabeled murine MAb H77.16 at 20 μg/ml was added to both sets of ELISA plates and left for 30 min at RT (28). One set was washed with buffer at RT and the other (test) set washed with buffer at 40°C. The test set was placed in a 40°C incubator for 10 min, and the control set remained at RT. Each antigenic domain E (H33.1 and H33.4), B (HC-1 and HC-11), or D (HC84.24 and HC84.26) HMAb at 1 μg/ml was then added, and after 30 min at either RT or 40°C, bound HMAb was measured at RT as described previously (28). The percent inhibition at either RT or 40°C was based on each HMAb binding in the absence of the murine MAb. (B) H77.16 (labeled H77.16 1st) or E, B, or D HMAb (labeled H77.16 2nd) was added to E2 and left for 30 min at RT. After washing, each E, B, or D HMAb was added to E2 preincubated with H77.16, or H77.16 was added to E2 preincubated with E, B, or D HMAb, and left for an additional 30 min. Control antibody was CBH-7, a domain C HMAb. Detection and calculation of percent inhibition were the same as for panel A. Data are shown as mean percent inhibition from two experiments performed in triplicate.
Diminished access against antibodies to antigenic domains B (HC-1 and HC-11) and D (HC-33.20, HC-33.24, and HC-33.26) also was observed. The major binding regions for domains B and D have been mapped to aa 429 to 440 and aa 440 to 446, respectively (28, 43). The effect of anti-HVR1 on neutralization by antibodies recognizing antigenic domain B, D, and E antibodies was apparent in the antagonism studies by use of the median-effect analysis method. When the mass of the anti-HVR1 antibody was determined by alanine substitution studies. A possible explanation is that structural studies with both antibodies are nearly identical, which means that HC33.4 does not include a true contact residue at aa 408 (48). Taken together, these findings suggest that anti-HVR1 Abs interfere with the binding of broadly neutralizing antibodies by steric hindrance. Finally, the interference by H77.16 against domain E antibodies (HC33-related HMAbs) appears to be greater than that by H77.39, although H77.39 binds to the same region as antigenic domain E antibodies (Fig. 2). The difference is probably due to the different mechanisms of interference. H77.39 directly competes for the same binding sites as the HC33-related antibodies, which can be affected by the relative binding affinities of the two antibodies. In contrast, H77.16 binds to an adjacent site in which the bulk of the antibody molecule is preventing the binding of HC33-related antibodies to their respective epitopes. The extent of blockade by steric hindrance is less dependent on differences in the affinities of the competing antibodies, which could be more effective with the tested antibodies. The concept of antibody-mediated interference of HCV is not new. It has been proposed that epitopes within the E2 segment encompassing aa 434 to 446 elicit nonneutralizing antibodies and that these antibodies interfere with neutralizing antibodies directed at an adjacent E2 segment (aa 412 to 426) (22, 23). However, other studies employing similar approaches of isolating polyclonal antibodies to synthetic peptides encompassing aa 412 to 426 and aa 434 to 446 showed no interference in virus neutralization (21). The lack of interference by antibodies to aa 434 to 446 was assessed by combination studies of HMAbs that bind to aa 412 to 426 or aa 434 to 446 (20, 28). The median-effect analysis method was applied, and it was determined that the effect was additive and not antagonistic (20).

We performed structural studies to further understand how the binding of anti-HVR1 MAbs interferes with broadly neutralizing antibodies. The antigenic region aa 412 to 423 is positioned downstream of HVR1, which is a structurally flexible region at the N terminus of E2. HVR1 was reported to interfere with binding of neutralizing antibodies by shielding conserved epitopes (18, 19). The different structures observed for peptides comprising aa 412 to 423 bound to multiple neutralizing antibodies suggest that this region also has considerable structural flexibility in the HCV particle. These observations indicate that there is a long, highly flexible region at the N terminus of E2, which extends beyond HVR1 and includes conserved residues strongly implicated in CD81 binding. Since the majority of neutralizing epitopes within E2 are located in close proximity to this flexible region, MAbs targeting epitopes within this region likely can interfere, even in the absence of direct competition for binding residues, with binding of neutralizing antibodies to other antigenic domains within E2 by steric hindrance.

Overall, our findings indicate that in addition to a direct shielding role of HVR1, an indirect shielding role is plausible and could be exerted by antibodies binding to the extended structurally flexible region at the N terminus of E2 (including aa 412 to 423). Thus, immunization strategies that avoid the generation of HR antibodies may be needed to enable broadly neutralizing antibodies to function optimally and prevent new infections or control established infections.

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**REFERENCES**

1. Shepard CW, Finelli L, Alter MJ. 2005. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 5:558–567. http://dx.doi.org/10.1016/S1473-3099(05)70216-4.
2. Klevens RM, Hu DJ, Jiles R, Holmberg SD. 2012. Evolving epidemiology of hepatitis C virus in the United States. Clin Infect Dis 55(Suppl 1):S3–S9. http://dx.doi.org/10.1093/cid/cis393.
3. Bowen DG, Walker CM. 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature 436:946–952. http://dx.doi.org/10.1038/nature04079.

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**FIG 7 Conformations of the antigenic region from aa 412 to 423. A comparison of peptide conformations observed for aa 412 to 423 is shown. The peptides in complex with Fabs HC33.8 (A), 3/11 (B) (PDB 4WHT) and, HCV-1 (C) (PDB 4DGV) (49) as an example for the β-hairpin conformation are shown as cartoons with the side chains shown as sticks and colored by atom type (red and blue for oxygen and nitrogen, respectively, and carbon atoms colored orange [HC33.8], gray [3/11], or green [HCV-1]) to illustrate the differences in the backbone conformation observed for the three structures.**
Anti-HVR1 Hinders Broadly Neutralizing Antibodies

4. Farci P, Alter HJ, Wong DC, Miller RH, Govindarajan S, Englert R, Shapiro M, Purcell RH. 1994. Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated in vitro neutralization. Proc Natl Acad Sci U S A 91:7792–7796. http://dx.doi.org/10.1073/pnas.91.16.7792.

5. Pestka JM, Zeisel MB, Blaser E, Schurrmann P, Bartosch B, Cossut FL, Pattni NM, Meisel H, Baumert T, Viazov S, Rispeker K, Blum HE, Roggendorf M, Baumert TF. 2007. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. Proc Natl Acad Sci U S A 104:6025–6030. http://dx.doi.org/10.1073/pnas.0607026104.

6. Law M, Maruyama T, Lewis J, Giang E, Tarr AW, Stamatakis Z, Gasta-minza P, Chisari FV, Jones IM, Fox RI, Ball JK, Mecking J, Knete ...

10. Shimizu Zibert A, Schreier E, Roggendorf M. 2016. March 2016 Volume 90 Number 6 jvi.asm.org

11. Dowd Prentoe J, Jensen TB, Meuleman P, Serre SB, Scheel TK, Leroux-Roels G, Gottwein JM, Bukh J. 2011. Hypervariable region 1 differences impacts viability of hepatitis C virus strains of genotypes 1 to 6 and impair virus neutralization. J Virol 85:2224–2234. http://dx.doi.org/10.1128/JVI.01594-10.

12. Keck Z, Wang W, Wang Y, Lau P, Carlsen TH, Prentoe J, Xia J, Patel AH, Bukh J, Founge SK. 2013. Cooperation in virus neutralization by human monoclonal antibodies to two adjacent regions located at the amino terminus of hepatitis C virus E2 glycoprotein. J Virol 87:53–57. http://dx.doi.org/10.1128/JVI.01941-12.

13. Tarr AW, Urbanowicz RA, Jayaraj D, Brown RJ, McKeating JA, Irving WL, Ball JK. 2012. Naturally occurring antibodies that recognize linear epitopes in the amino terminus of the hepatitis C virus e2 protein confer noninterfering, additive neutralization. J Virol 86:2739–2749. http://dx.doi.org/10.1128/JVI.04692-11.

14. Zhang P, Zhong L, Struble EB, Watanabe H, Kachko A, Mihalkik L, Virata-Theimer ML, Yu MY, Alter HJ, Feinestone SM. 2007. Hepatitis C virus epitope-specific neutralizing antibodies in Igs prepared from human plasma. Proc Natl Acad Sci U S A 104:8449–8454. http://dx.doi.org/10.1073/pnas.0703039104.

15. Zhang P, Zhong L, Struble EB, Watanabe H, Kachko A, Mihalkik L, Virata-Theimer ML, Alter HJ, Feinestone SM. 2009. Depletion of interfering antibodies in chronic hepatitis C patients and vaccinated chimpanzees reveals broad cross-genotype neutralizing activity. Proc Natl Acad Sci U S A 106:7537–7541. http://dx.doi.org/10.1073/pnas.0902794106.

16. Yi M, Villanueva RA, Thomas DL, Wakti T, Lemon SM. 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc Natl Acad Sci U S A 103:2310–2315. http://dx.doi.org/10.1073/pnas.0510271103.

17. Hadlock KG, Landorf RE, Perkins S, Rowe J, Yang Q, Levy S, Pileri P, Ball JK, Maruyama T, Bukh J, Founge SK. 2007. Human monoclonal antibodies that inhibit binding of hepatitis C virus E2 protein to CD81 and recognize conserved conformational epitopes. J Virol 74:10407–10416. http://dx.doi.org/10.1128/JVI.07714-10.10416-2009.

18. Keck ZY, Sung VM, Perkins S, Rowe J, Paul S, Liang T, Lai MM, Founge SK. 2004. Human monoclonal antibody to hepatitis C virus E1 glycoprotein that blocks virus attachment and viral infectivity. J Virol 78:7257–7263. http://dx.doi.org/10.1128/JVI.78.17.7257-7263.2004.

19. Keck ZY, Xia J, Cai Z, Li TK, Owiesanka AM, Patel AH, Luo G, Founge SK. 2007. Immunogenic and functional organization of hepatitis C virus (HCV) glycoprotein E2 on infectious HCV virions. J Virol 81:1043–1047. http://dx.doi.org/10.1128/JVI.01710-06.

20. Keck ZY, Xia J, Wang Y, Wang W, Krey T, Prentoe J, Carlsen T, Li AY, Patel AH, Lemon SM, Bukh J, Rey FA, Founge SK. 2012. Human monoclonal antibodies to a novel cluster of conformational epitopes on HCV E2 with resistance to neutralization escape in a genotype 2a isolate. PLoS Pathog 8:e1002653. http://dx.doi.org/10.1371/journal.ppat.1002653.

21. Sabo MC, Luca VC, Prentoe J, Hopcraft SE, Blight KJ, Yi M, Lemon SM, Ball JK, Bukh J, Evans MJ, Fremont DH, Diamond MS. 2011. Broadly neutralizing monoclonal antibodies against hepatitis C virus E2 protein bind discontinuous epitopes and inhibit infection at a postattachment step. J Virol 85:7005–7019. http://dx.doi.org/10.1128/JVI.00086-11.

22. Keck ZY, Op De Beeck A, Hadlock KG, Xia J, Li TK, Dubuisson J, Founge SK. 2004. Hepatitis C virus E2 has three immunogenic domains containing conformational epitopes with distinct properties and biological functions. J Virol 78:9224–9232. http://dx.doi.org/10.1128/JVI.78.17.9224-9232.2004.

23. Keck ZY, Saha A, Xia J, Wang Y, Lau P, Krey T, Rey FA, Founge SK. 2011. Mapping a region of hepatitis C virus E2 that is responsible for escape from neutralizing antibodies and a core CD81-binding region that does not tolerate neutralization escape mutations. J Virol 85:10451–10463. http://dx.doi.org/10.1128/JVI.05239-11.

24. Chou TC. 2010. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 70:440–446. http://dx.doi.org/10.1158/0008-5472.CAN-09-1947.

25. Chou TC, Talalay P. 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–38. http://dx.doi.org/10.1016/0065-2714(84)90007-4.

26. Johansson DX, Krey T, Andersson O. 2012. Production of recombinant antibodies in Drosophila melanogaster S2 cells. Methods Mol Biol 907:359–370. http://dx.doi.org/10.1007/978-1-61779-974-7_21.

27. Kabsch W. 1988. Automatic indexing of rotation diffraction patterns. J Appl Crystallogr 21:67–71. http://dx.doi.org/10.1107/S0021889887009373.

28. Collaborative Computational Project. 1994. The CCP4 suite: programs for protein crystallography. Acta Crystallogr D Biol Crystallogr 50:760–763. http://dx.doi.org/10.1107/S0909444094003112.

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March 2016 Volume 90 Number 6 Journal of Virology jvi.asm.org 3121

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37. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. 2007. Phaser crystallographic software. J Appl Crystallogr 40: 658–674. http://dx.doi.org/10.1107/S0021889807021206.

38. Emsley P, Lohkamp B, Scott WG, Cowtan K. 2010. Features and development of Coot. Acta Crystallogr D Biol Crystallogr 66:486–501. http://dx.doi.org/10.1107/S0907444910007493.

39. Bricogne G, Blanc E, Brandl M, Flensburg C, Keller P, et al. 2010. BUSTER version 2.9. Global Phasing Ltd., Cambridge, United Kingdom.

40. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. 2004. UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 25:1605–1612. http://dx.doi.org/10.1002/jcc.20084.

41. Krissinel E, Henrick K. 2007. Inference of macromolecular assemblies from crystalline state. J Mol Biol 372:774–797. http://dx.doi.org/10.1016/j.jmb.2007.05.022.

42. Tina KG, Bhadra R, Srinivasan N. 2007. PIC: Protein Interactions Calculator. Nucleic Acids Res 35:W473–W476. http://dx.doi.org/10.1093/nar/gkm423.

43. Keck ZY, Li TK, Xia J, Gal-Tanamy M, Olson O, Li SH, Patel AH, Ball JK, Lemon SM, Foung SK. 2008. Definition of a conserved immunodominant domain on hepatitis C virus E2 glycoprotein by neutralizing human monoclonal antibodies. J Virol 82:6061–6066. http://dx.doi.org/10.1128/JVI.02475-07.

44. Keck ZY, Angua AG, Wang W, Lau P, Wang Y, Gatherer D, Patel AH, Foung SK. 2014. Non-random escape pathways from a broadly neutralizing human monoclonal antibody map to a highly conserved region on the hepatitis C virus E2 glycoprotein encompassing amino acids 412-423. PLoS Pathog 10:e1004297. http://dx.doi.org/10.1371/journal.ppat.1004297.

45. Owsianka A, Tarr AW, Jutla VS, Lavillette D, Bartosch B, Cosset FL, Ball JK, Patel AH. 2005. Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. J Virol 79:11095–11104. http://dx.doi.org/10.1128/JVI.79.17.11095-11104.2005.

46. Sabo MC, Luca VC, Ray S, Bukh J, Fremont DH, diamond, MS. 2012. Hepatitis C virus epitope exposure and neutralization by antibodies is affected by time and temperature. Virology 422:174–184. http://dx.doi.org/10.1016/j.virology.2011.10.023.

47. Dowd KA, Jost CA, Durbin AP, Whitehead SS, Pierson TC. 2011. A dynamic landscape for antibody binding modulates antibody-mediated neutralization of West Nile virus. PLoS Pathog 7:e1002111. http://dx.doi.org/10.1371/journal.ppat.1002111.

48. Li Y, Pierce BG, Wang Q, Keck ZY, Fuerst TR, Foung SK, Mariuzza RA. 2015. Structural basis for penetration of the glycan shield of hepatitis C virus E2 glycoprotein by a broadly neutralizing human antibody. J Biol Chem 290:10117–10125. http://dx.doi.org/10.1074/jbc.M115.643528.

49. Kong L, Giang E, Robbins JB, Stanfield RL, Burton DR, Wilson IA, Law M. 2012. Structural basis of hepatitis C virus neutralization by broadly neutralizing antibody HCV1. Proc Natl Acad Sci USA 109:9499–9504. http://dx.doi.org/10.1073/pnas.120294109.

50. Pantua H, Diao J, Ullsch M, Hazen M, Mathieu M, McCutcheon K, Takeda K, Date S, Cheung TK, Phung Q, Hass P, Arnott D, Hongo JA, Matthews DJ, Brown A, Patel AH, Kelley RF, Eigenbrot C, Kapadia SB. 2013. Glycan shifting on hepatitis C virus (HCV) E2 glycoprotein is a mechanism for escape from broadly neutralizing antibodies. J Mol Biol 425:1899–1914. http://dx.doi.org/10.1016/j.jmb.2013.02.025.

51. Meola A, Tarr AW, England P, Meredith LW, McClure CP, Foung SK, McKeating JA, Ball JK, Rey FA, Krey T. 2015. Structural flexibility of a conserved antigenic region in hepatitis C virus glycoprotein E2 recognized by broadly neutralizing antibodies. J Virol 89:2170–2181. http://dx.doi.org/10.1128/JVI.02190-14.