Cooperativity in Virus Neutralization by Human Monoclonal Antibodies to Two Adjacent Regions Located at the Amino Terminus of Hepatitis C Virus E2 Glycoprotein

Zhenyong Keck, Wenyan Wang, Yong Wang, Patrick Lau, Thomas H. R. Carlse, Jannick Prentoe, Jinming Xia, Arvind H. Patel, Jens Buhk, Steven K. H. Foung

Department of Pathology, Stanford University School of Medicine, Stanford, California, USA; Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases and Clinical Research Centre, Copenhagen University Hospital Hvidovre, and Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark; MRC-University of Glasgow Centre for Virus Research, University of Glasgow, Glasgow, United Kingdom

A challenge for hepatitis C virus (HCV) vaccine development is defining conserved epitopes that induce protective antibodies against this highly diverse virus. An envelope glycoprotein (E2) segment located at amino acids (aa) 412 to 423 contains highly conserved neutralizing epitopes. While polyclonal antibodies to aa 412 to 423 from HCV-infected individuals confirmed broad neutralization, conflicting findings have been reported on polyclonal antibodies to an adjacent region, aa 434 to 446, that may or may not interfere with neutralization by antibodies to aa 412 to 423. To define the interplay between these antibodies, we isolated human monoclonal antibodies (HMAbs) to aa 412 to 423, designated HC33-related HMAbs (HC33 HMAbs), and characterized their interactions with other HMAbs to aa 434 to 446. A subset of the HC33 HMAbs neutralized genotype 1 to 6 infectious cell culture-derived HCV virions (HCVcc) with various activities. Although nonneutralizing HC33 HMAbs were isolated, they had lower binding affinities than neutralizing HC33 HMAbs. These antibodies could be converted to neutralizing antibodies by affinity maturation. Unidirectional competition for binding to E2 was observed between HC33 HMAbs and HMAbs to aa 434 to 446. When HMAbs to aa 434 to 446, which mediated neutralization, were combined with neutralizing HC33 HMAbs, biphasic patterns in neutralization were observed. A modest degree of antagonism was observed at lower concentrations, and a modest degree of synergism was observed at higher concentrations. However, the overall effect was additive neutralization. A similar pattern was observed when these antibodies were combined to block E2 binding to the HCV coreceptor, CD81. These findings demonstrate that both of these E2 regions participate in epitopes mediating virus neutralization and that the antibodies to aa 412 to 423 and aa 434 to 446 do not hinder their respective virus-neutralizing activities.

Infection with hepatitis C virus (HCV) leads to chronic hepatitis in the majority of infected individuals, many of whom are at significant risk for developing cirrhosis, liver failure, and hepatocellular carcinoma. The World Health Organization has estimated an annual increase in the global HCV burden of 3 to 4 million new infections (1). A critical first step in a rational vaccine design for HCV is to identify the relevant mechanisms of immune protection. While CD4+ and CD8+ T cell responses appear to be necessary for controlling acute HCV infection, they are inadequate for preventing long-term persistence in most infected individuals (2). Nonetheless, a phase I study of the first T cell-based HCV vaccine for humans was recently reported (3). The adenoviral-based delivery showed a good safety profile and induced both CD4+ and CD8+ T cell responses, with some evidence of cross-genotype immunity. Although this is an encouraging development, the challenge is to overcome the huge diversity of the virus and its potential to escape host immune responses. Virus-neutralizing antibodies are increasingly recognized to contribute to HCV clearance (4–10), but the virus envelope glycoproteins E1 and E2, the targets of neutralizing antibodies, display some of the highest levels of genetic diversity found in HCV. Hypervariable region 1 (HVR1), found at the N terminus of E2, is highly immunogenic but is a major determinant for isolate-specific neutralizing antibody responses associated with viral escape (11–13). Thus, a significant challenge for vaccine development is defining conserved epitopes in the envelope proteins that are capable of eliciting protective antibodies against this highly diverse virus. An E2 segment that is adjacent to HVR1, encompassing amino acids (aa) 412 to 423, is recognized as containing highly conserved neutralizing epitopes. Initially, the mouse monoclonal antibody AP33 defined a mostly linear epitope in this region, which has contact residues within aa 412 to 423 (14,15). This antibody displayed broad neutralizing activities against HCV retroviral pseudotype particles (HCVpp) expressing E1E2 that represented the major HCV genotypes 1 through 6 (15), which is consistent with this epitope being highly conserved. Other monoclonal antibodies, both murine and human, have been reported to bind to epitopes located within aa 412 to 423 and to display broad neutralizing activities (16–18). Epitope mapping revealed that W420 is a critical residue for virus binding to the HCV coreceptor, CD81 (19). Another murine monoclonal antibody, H77.39, has
been found to bind to an epitope containing contact residues within aa 412 to 423 at N415 and N417 (18). This antibody appears to mediate virus neutralization by inhibiting E2 binding to both CD81 and another HCV coreceptor, the scavenger class B type 1 receptor (SR-B1). Collectively, these findings show that the E2 region encompassing aa 412 to 423 encodes highly conserved epitopes that elicit broadly neutralizing antibodies and consequently should be highly relevant in HCV vaccine development.

In addition to the genetic mutation strategy of HCV for escape from neutralization, exemplified by antibodies to HVR1, the virus possesses other evasion strategies for negatively modulating the neutralizing antibody response. Glycosylation of the virus envelope, nonneutralizing antibodies, or virion-associated lipoproteins may interfere with antibody-mediated neutralization by masking neutralizing epitopes or otherwise limiting access of neutralizing antibodies to their cognate epitopes (20–22). Studies with HCVpp and infectious cell culture-derived HCV virions (HCVcc) have indicated that N-linked glycans at conserved Asn residues of the E2 protein hinder neutralizing activities of HCV-specific polyclonal sera as well as neutralizing human monoclonal antibodies (HMABs) (20, 21, 23). Circulating HCV virions in the blood of patients are associated with host lipoproteins and immunoglobulins in the form of lipo-viro particles (LVP) and are heterogeneous in density and infectivity (24). The extent to which the association of HCV with lipoproteins impedes the access of neutralizing antibodies to the virion surface is not well known. Infectious HCVcc particles can be readily precipitated with antibody to apolipoprotein E (ApoE) but are nonetheless neutralized with virus-specific HMABs (25). In the presence of high-density lipoprotein (HDL) or human serum, the neutralization of HCVpp and HCVcc by anti-E2 neutralizing antibodies could be attenuated (26).

Interestingly, it has been proposed that an epitope located in a segment of E2 encompassing aa 434 to 446, named epitope II, elicits nonneutralizing antibodies and that these antibodies interfere with the neutralizing activities of antibodies directed against an adjacent E2 segment encompassing aa 412 to 426, named epitope I (27, 28). Synthetic peptides of epitopes I and II were used to elute polyclonal antibodies specific for each peptide from a gamma-globulin preparation produced from the plasma of HCV-seropositive individuals. Neutralization activity was observed with epitope I-specific antibodies but not with epitope II-specific antibodies. More importantly, the presence of epitope II-specific antibodies interfered with the neutralization activity of epitope I-specific antibodies. However, conflicting studies that employed similar approaches of isolating polyclonal antibodies to synthetic peptides encompassing aa 412 to 423 and aa 434 to 446 also have been reported (29). Eluted polyclonal antibodies from both peptides neutralized HCV, and when combined, they showed additive neutralizing activities. A limitation of these studies, however, was the use of polyclonal antibodies to establish the relationship between the antibodies elicited by these two regions on E2 during infection. Although the eluted antibodies were sequence specific to either aa 412 to 423 or aa 434 to 446, multiple antibodies against different epitopes were present in each IgG preparation. The relative ratios of different antibodies with different affinities are unknown, and it is not possible to finely map their respective epitopes. Any measurement of their functional properties is a composite of effects by different antibodies.

Because of the potential importance of conserved epitopes within aa 412 to 423 in vaccine development, we isolated and characterized a panel of HMABs to this region, designated HC33-related human antibodies (HC33 HMABs). Furthermore, we assessed their relationships with other HCV HMABs that are directed at two distinct clusters of overlapping epitopes, designated antigenic domains B and D (30–34). Antibodies to antigenic domain B share two contact residues located within aa 434 to 446, e.g., an epitope identified by an antibody designated HC-11 (35, 37). Antigenic domain D has been identified by the use of a panel of antibodies, designated HC84-related HMABs, and they bind to epitopes having contact residues located within aa 434 to 446. A subset of these antibodies binds to a synthetic peptide comprising aa 434 to 446 (aa-434-to-446 synthetic peptide) (33). These HMABs to antigenic domains B and D exhibited broad virus neutralization against a panel of HCVcc of different major genotypes. Some of the HC33 HMABs mediated neutralization. Competition studies revealed that the HC84-related HMABs and HC-11 had little to no interference with the binding of labeled neutralizing HC33-related HMABs to E2. However, HC33-related HMABs moderately inhibited the binding of HC84-related HMABs to E2. When neutralizing HC33-related HMABs were combined with either HC84-related HMABs or HC-11 for virus neutralization, antagonistic effects were observed at lower antibody concentrations and synergistic effects were observed at higher antibody concentrations. A similar pattern occurred when blocking the E2 glycoprotein from binding to the HCV coreceptor CD81 by combined HC33- and HC84-related HMABs. Affinity maturation of selected nonneutralizing HC33 HMABs led to clones that mediated neutralization.

MATERIALS AND METHODS

Cells and viruses. HEK 293T cells were obtained from the ATCC. Huh7.5 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 (FCS) (Sigma-Aldrich Co., St. Louis, MO) and 2 mM glutamine. Saccharomyces cerevisiae strain EBY-100 (GAL1-AGA1:URA3 uri3-52 trpl1 leu2Δ1 his3Δ200 pep::HIS3 prb1Δ1.6r can1 GAL) (Invitrogen, Carlsbad, CA) was maintained in yeast-extract-peptone-dextrose (YPD) broth (Difco). The JFH1 (genotype 2a) recombinant isolate was generously provided by T. Wakita (National Institute of Infectious Diseases, Japan) (38). Chimeric JFH1 HCVcc bearing C, E1, E2, p7, and NS2 from six major genotypes are designated genotype 1a (H77C/JFH1), 2a (J6/JFH1), 3a (S52/JFH1), 4a (ED43/JFH1), 5a (SA13/JFH1), and 6a (HK64/JFH1) (39–43); all of these isolates except the 2a virus contained adaptive mutations, but only in the case of HK64/JFH1 were they located in the envelope genes.

Antibodies and reagents. HMABs CBH-4D, CBH-4G, CBH-7, HC-1, HC-11, HCV84, 20, and HC-84.26 against HCV E2 glycoprotein were produced as described previously (32, 33, 44, 45). HCV MAb against the NS3 protein was generously provided by G. Luo (University of Kentucky). MAb AP33 to aa 412 to 423 was used as a control antibody (14). Mabs against the human CD81 (clone JS-81) and the V-5 tag were purchased from BD Bioscience (San Jose, CA) and Invitrogen (Carlsbad, CA), respectively. The detection MAb used in fluorescence-activated cell sorting (FACS), namely, phycocerythrin (PE)-labeled donkey anti-human IgG (Fcγ specific), fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Fcγ specific), and allopoxycyanin (APC)-conjugated donkey anti-human IgG (Fcγ specific), were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). A molecular clone encoding the
CD81 large extracellular loop fused to glutathione S-transferase was generously provided by S. Levy (Stanford University) and affinity purified over a GSTrap FF affinity column according to the manufacturer’s instructions (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The yeast display vector pYD2 was kindly provided by J. D. Marks (UCSF) (46). IgG1-Avec for full-length IgG1 expression was kindly provided by P. Wilson (University of Chicago). Biotinylated peptides (1410NIQLINTNGSWHINST245) were synthesized using a C-terminal biotin residue with a Gly-Ser-Gly linker (American Peptide, Sunnyvale, CA).

Isolation of HMABs targeting HCV E2 glycoprotein within region 412 to 423. The methods for generation of the yeast single-chain variable fragment antibody (scFv)-displayed library, selection of HCV E2-specific scFv, and production of scFvs and IgG1 HMABs have been described in detail previously (33). Briefly, the yeast library was grown in synthetic dextrose Casamino acids (SD-CAA) with glucose replaced by galactose (SG-CAA) for 48 h at 18°C. We first selected 2 × 10⁸ yeast cells by two rounds of magnetically activated cell sorting (MACS) using biotinylated peptides (1410NIQLINTNGSWHINST245), followed by two rounds of FACS with 1 × 10⁵ MACS output cells. Between each round of selection, the collected cells were grown in SD-CAA and induced in SG-CAA medium. Selection was performed using a BD Bioscience FACS Vantage sorter. The collected cells were plated on SD-CAA plates. Individual clones reactive to E2 by flow analysis were analyzed by fingerprint with BstNI digestion and followed by sequencing based on different digestion patterns. The primers used to amplify the representative scFv inserts for BstNI digestion and followed by sequencing based on different digestion constructions were PYDF (5′-AGT AAC GTT TGT CAA TGG C-3′) and PYDR (5′-GTC GAT TTT GAT ACA TCT ACA C-3′). The PCR products were sequenced with the primer GAP5 (5′-TAA AGC TCT TGC AGG CTA GTG-3′) (Eli Lilly Biopharmaceuticals, Inc., Hayward, CA).Soluble scFv and full-length IgG1 were produced and purified as previously described (33).

Neutralization assay. Neutralization against different HCVcc isolates was evaluated as described previously (33). Against 2a JFH1 HCVcc, antibody at 20 μg/ml was mixed with virus inoculum (containing 50 focus-forming units [FFU]), and the mixture was incubated for 1 h at 37°C. The virus-HMAB mixture was then added to Huh7.5 cell monolayers (3.2 × 10⁶ cells/well) that were grown in 8-well chamber slides (Nalg Nunc, Rochester, NY). Cells were fixed and stained for NS3 protein expression at 72 h postinfection. The percent neutralization was calculated as the percent reduction in FFU compared with that with virus incubated with an irrelevant control antibody. These assays were carried out in duplicate in three independent assays for each HMAB. For neutralization experiments performed with the HCVcc panel for JFH1-based genotypes 1 to 6, a virus inoculum (~100 FFU) was incubated for 1 h at 37°C with 50 μg/ml specific HMABs prior to 3 h of incubation with 6 × 10⁵ Huh7.5 cells/well in poly-γ-lysine-coated 96-well plates (Nunc). Cells were fixed and stained for NS5A expression at 48 h postinfection (39–41, 43). For each test, neutralization was done in four replicates with six control wells containing the virus only. The percent neutralization was calculated in relation to the mean of virus-only controls. Titration studies to calculate the 50% inhibitory concentration (IC₅₀) against HCVcc in genotypes 1 to 6 were performed in a similar fashion with selected antibodies. Data transformation and four-parameter nonlinear regression analysis were performed using GraphPad Prism software.

Antibody cooperativity for virus neutralization. Synergistic, additive, and antagonistic cooperativity by two antibodies for virus neutralization was evaluated by the median effect analysis method, as described by Chou and Talalay (47, 48), using the CompuSyn software (CompuSyn Inc., Paramus, NJ). This 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, <1 indicates synergism, and >1 indicates antagonism. For each antibody, dose-dependent neutralization was measured initially to determine the concentration that resulted in a 50% reduction in FFU (IC₅₀). The constant ratio of the combined antibodies was set by the IC₅₀ of the two antibodies. Neutralization values of serial 2-fold dilutions of each antibody alone and in combination were then measured in a range of concentrations above and below the IC₅₀. The measured neutralization values were entered into the program as fractional effects (FA) in the range of 0.01 < FA < 0.99 for each of the two antibodies and for both 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 JFH1 HCVcc and each HMAB or combination of HMABs were incubated for 1 h at 37°C and then plated onto Huh7.5 cell monolayers (3.2 × 10⁶ cells/well) that were grown in 8-well chamber slides (Nalg Nunc, Rochester, NY) for assay, as described above for virus neutralization. These assays were carried out in four replicates for each HMAB and combination of HMABs.

Measurement of scFv affinity. scFv binding kinetics were measured using surface plasmon resonance in a BIAcore 3000 (Pharmacia Biosensor) and used to calculate the equilibrium dissociation constant (KD) as described previously (33). Approximately 135,000 response units (RU) of CBH-4D, a nonneutralizing HMAB to a conformational epitope on E2 (32, 44, 49), was coupled to a CM5 sensor chip by using N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Approximately 250 RU of purified secreted E2 (sE2) in HBS-EP buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.005% [vol/vol] surfactant P20) (BIAcore BR-1001-88; GE Healthcare) was captured by CBH-4D onto the surface of the chip. Another flow cell without sE2 capture was set as a reference. The purified HC33 scFv at concentrations ranging from 1,000 nM to 31.25 nM (with 2-fold serial dilutions) was injected for 2 min using a flow rate of 30 μl/min. Dissociation of bound HC33 scFv in HBS-EP buffer flow was measured for 3 min. The surfaces (E2 and HC33 scFv) were regenerated after each cycle using regeneration solution (10 mM glycine-HCl [pH 2.5]). All sensorgrams were double referenced before data analysis. First, the response from the reference flow cell (without E2) was subtracted. Second, the response from an average of two blank injections (0 nM E2) of HBS-EP buffer was subtracted. The sensorgrams (duplicates for each concentration) were globally fit with the parameters KD (association rate constant) and Kdoff (dissociation rate constant) using Scrubber 2.0 (Center for Biomolecular Interaction Analysis, University of Utah, UT). Kd was calculated as Koff/Kd.

Binding to native and denatured E2 glycoprotein. A standard enzyme-linked immunosorbent assay (ELISA) was used (33) to compare HMAB binding to native and denatured HCV E1E2 glycoproteins. Briefly, ELISA plates were coated with Galanthus nivalis agglutinin (GNA) and blocked with 2.5% nonfat dry milk and 2.5% normal goat serum in 0.1% Tween-phosphate-buffered saline (PBS). Lysates of cells expressing HCV E1E2 or denatured E1E2 glycoproteins were captured by GNA onto a microtiter plate, followed by incubation with HMABs at various concentrations ranging from 0.02 to 5 μg/ml, and then washed. E1E2 was denatured by boiling for 5 min in Tris-buffered saline (TBS)-10% FCS containing 1.0% sodium dodecyl sulfate and 50 mM dithiothreitol. 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.

Competition assay. Purified HMABs were biotinylated using an EZ-Link NHS-PEO solid-phase biotinylation kit (Thermo Scientific, Rockford, IL). The competition between unlabeled and biotinylated HMABs for binding to GNA-captured E2 glycoproteins was measured by ELISA (31). 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-20PBS. Pretrititated unlabeled competing HMAB was added to each well at a saturating concentration. After 1 h, biotinylated antibody was added at a concentration corresponding to 65% to 75% of the maximal binding.
RESULTS

Isolation of human monoclonal antibodies to an HCV E2 region encompassing aa 412 to 423. Serum samples from HCV-infected blood donors were tested for reactivity to a synthetic peptide composed of a genotype 1a H77C sequence, aa 410 to 425, to identify individuals having antibodies against aa 412 to 423 on the HCV E2. Thirteen of 88 (15%) serum samples reacted to this peptide as measured by ELISA, which is a percentage that was significantly higher than those previously reported (2 to 3%) (29, 50). The peripheral B cells from a donor infected with HCV genotype 2b who had a high serum antibody binding titer to aa 410 to 425 and >1:10,000 neutralizing titer against genotype 2a JFH1 HCVcc were employed to construct a yeast display scFv antibody library, as described previously (33). Fifty clones that bound to aa 410 to 425 were selected for further analysis. Sequencing analysis of the heavy and light chains of these scFvs in CDR regions 1, 2, and 3 resulted in 13 unique variable region (VH/VL) clones but with seven unique V\(_{\text{H}}\)\(_{\text{D}}\)\(_{\text{S}}\). These scFvs were converted to full-length IgG1 and designated HC33.1, HC33.2, HC33.3, HC33.4, HC33.5, HC33.6, HC33.7, HC33.8, HC33.9, HC33.10, HC33.11, HC33.29, and HC33.32.

HC33 HMAbs mediate virus neutralization and inhibit E2-CD81 interaction. Previously identified murine and human monoclonal antibodies that bound to aa 412 to 423 were generated by immunizing mice or transgenic mice with HCV glycoproteins. These antibodies broadly neutralized different HCV genotype and subtype isolates (14-18). To test whether the HC33 HMAbs elicited as part of the antibody response during the course of a natural HCV infection also neutralized the virus, an initial screen was performed by blocking HuH7.5 cell infection by 2a JFH1 HCVcc. Each HC33 HMAb was tested at 20 µg/ml, and neutralization activity was determined using a cutoff value of 40%. The results divided the antibodies into two groups (Fig. 1A). Group I HMAbs, HC33.1, HC33.4, HC33.8, HC33.29, and HC33.32, neutralized JFH1 2a HCVcc by 50% to >90%. Group II HMAbs, HC33.2, HC33.3, HC33.5, HC33.6, HC33.7, HC33.9, HC33.10, and HC33.11, had neutralization values of <40%. HC-11, a neutralizing HCV HMAb, served as a positive control (35), and R04, an isotype-matched HMAb to HCMV, served as a negative control (44). Since the E2 region, aa 412 to 423, is highly conserved among all HCV genotypes and subtypes, group I antibodies were further tested at 50 µg/ml for their breadth of neutralization against a panel of chimeric 2a JFH1 HCVcc bearing C, E1, E2, p7, and NS2 from genotypes 1a (strain H77C), 2a (J6), 3a (S52), 4a (ED43), 5a (SA13), and 6a (HK6a) (Fig. 1B) (39-43). In contrast to the mouse-derived antibodies, the group I HC33 HMAbs displayed various degrees of neutralization against different HCV genotypes. HC33.4, HC33.8, and HC33.29 neutralized five of six HCVcc genotypes by >40%; they failed to neutralize the genotype 6a, 2a (J6), and 3a HCVcc. HC33.1 neutralized three HCVcc isolates (genotypes 1a, 4a, and 5a), and HC33.32 neutralized two isolates (genotypes 4a and 5a). These findings suggest that the five antibodies at 50 µg/ml were different in their neutralizing potencies toward the epitope as presented in the different genotypes. The 50% inhibitory antibody concentration, IC\(_{50}\), was determined for HC33.1 and HC33.4, and these values ranged from 0.1 µg/ml to >50 µg/ml against the six genotype isolates, which included two 2a isolates (Table 1). IC\(_{50}\) were obtained for HC33.8 and HC33.29 against genotypes 1, 2, and 4. The most striking observation was the neutralization activities against the 2a JFH1 HCVcc by all five antibodies at 20 µg/ml (Fig. 1A) compared to their low neutralizing activities (<10% to 50%) against the 2a J6/JFH1 HCVcc (Fig. 1B). This suggested that contact residues that are outside aa 410 to 425 possibly participate in the HC33 epitopes, or that sequence differences outside the aa 412 to 423 region have a structural impact on this region, which affects the accessibility of the HC33-related epitopes on J6/JFH1.

To investigate the mechanism of virus neutralization, soluble CD81-LEL binding to the 1a H77C E2 protein was measured in the presence of each of the five neutralizing HC33 HMAbs by ELISA. HC-11, known to inhibit E2 binding to CD81, served as the positive control, and R04 served as the negative control (Fig. 1C). Compared to a no-antibody control or R04, 80 to 100% inhibition of E2 binding to CD81 was observed in the presence of HC33.1, HC33.4, HC33.8, or HC33.29, and there was approximately 55% inhibition by HC33.32. These findings roughly parallel the relative neutralizing activities of these antibodies (Fig. 1A). When genotype 1a was used, HC33.1, HC33.4, HC33.8, and HC33.29 inhibited E2-CD81 interaction as effectively as HC-11.

Binding to linear epitopes, measurements of antibody affinities, and mapping of epitopes. Binding of the five neutralizing
FIG 1  Antibody Cooperativity in Virus Neutralization

HC33 HMAbs to native and denatured E1E2 antigens was measured to determine the extent to which these antibodies are directed at linear epitopes (Fig. 2A). Denaturation of E1E2 led to a 3 to 12% reduction in antibody binding compared to native E1E2 at an antibody concentration of 5 μg/ml, indicating that these HC33 HMAbs are directed at primarily linear epitopes on the E2 glycoprotein. AP33 showed a similar degree of 9% reduction. This is consistent with the results of Western blot analyses that showed these antibodies detecting denatured E2 proteins (data not shown). Binding of HC-11, an antibody to a conformational epitope, was reduced by 99% against denatured E1E2. CBH-17, an antibody to an unrelated linear epitope on E2, showed an increase of 34%, which indicates that this epitope is less accessible in the native antigen. The binding affinities of HC33.1, HC33.4, HC33.8, HC33.29, and HC33.32, were next analyzed by employing purified scFv of each antibody, as determined by surface plasmon resonance (SPR) in a Biacore 3000 (Fig. 2B and C). Purified secreted 1a H77C E2 was first captured by CBH-4D, an antigenic domain A HMAb to a conformational epitope, which was coupled onto a sensor chip to ensure that native E2 was employed in these measurements (33, 51). A representative trace of overlay plots of association and dissociation curves to obtain the $K_{on}$, $K_{off}$, and $K_D$ values for HMAb HC-33.29 is shown in Fig. 2B. The $K_{on}$, $K_{off}$, and $K_D$ values were calculated and are presented in ranked order from the highest (HC-33.8) to the lowest (HC33.32) affinity (Fig. 2C). The $K_{on}$ values for HC33.8, HC33.4, and HC33.29 were similar, moderately higher than that for HC33.1, and a log higher than that for HC33.32. These binding affinities to H77C E2 correlated closely with neutralization potencies against the 1a H77/JFH1 HCVcc (Table 1).

The precise contact residues of HC33 HMAbs within aa 412 to 423 were determined by alanine mutagenesis analysis of H77C E2 (Fig. 2D). Mapping was also performed in the adjacent sequence aa 434 to 446, in which the alanine at aa 439 was substituted with glycine. The E2 mutants were expressed in HEK 293T cells, and binding by neutralizing HC33 HMAbs was measured by ELISA. Expression levels of the mutants were normalized by binding with CBH-17, a nonneutralizing HMAb to a different linear epitope on HCV E2 (44). A test concentration of 0.2 μg/ml was selected for the epitope-mapping studies. This concentration was determined by dose-dependent binding of each HC33 HMAb to E2, which showed the test concentration in the linear portion of the binding curve (data not shown). To confirm that the native E2 structure was not affected by each alanine substitution, we also measured binding by representative antibodies to mostly nonoverlapping clusters of conformational epitopes on E2, CBH-4D (a nonneutralizing HMAb in a cluster designated antigenic domain A), and CBH-7 (a neutralizing HMAb in a cluster designated antigenic domain C) (44). None of the alanine replacements resulted in reduced binding by CBH-4D or CBH-7; thus, these substitutions did not have a global effect on E2 conformation. A >80% reduction in binding was observed for all five HC33-related neutralizing antibodies, with alanine substitutions at L413A, G418A, and test HMAb at 10 μg/ml. The antibody-antigen complex was then added onto CD81-LEL precoated microtiter wells. Detection of E2 bound to CD81-LEL was measured with biotinylated CBH-4D (44) and secondary streptavidin. HC-11 was used as a positive control. The experiments were performed twice, each in triplicate. The error bars indicate 1 standard deviation from the mean.
W420A (Fig. 2D), indicating that they bind to the same or to nearly identical epitopes. The exception was retention of 40% binding to G418A by HC33.4. Since the HC33-related antibodies have similar binding affinities and displayed different patterns of neutralization against genotype 1 to 6 HCVcc, it remains possible that other residues are involved and can differentiate the HC33-related epitopes (Fig. 1B). An alternative possibility is that differences in sequences at distant regions in different genotypes could have structural effects on the aa 412 to 423 region. Taken together, the characteristics of these antibodies show that, at the molecular level, they interact differently with nearly identical epitopes.

**Unidirectional competition between HCV HMAbs to aa 412 to 423 and aa 434 to 446 for binding to E2.** To assess the interplay of antibodies directed at aa 412 to 423 and aa 434 to 446, we first investigated the extent to which previously isolated HMAbs to epitopes having contact residues within aa 434 to 446 affected the binding of HC33 HMAbs to the E2 glycoprotein. In these and subsequent studies, HC33.32 was not included due to its low binding affinity to E2. We evaluated three “interfering” antibodies that have contact residues within aa 434 to 446, HC84.20, HC84.26, and HC-11 (Fig. 3A) (33, 37). None of these antibodies contained contact residues within aa 412 to 423, and none bound to the aa-410-to-425 synthetic peptide (data not shown). While all three of these antibodies are directed to conformational epitopes, they have two binding patterns: HC84.26 bound to the aa-434-to-446 synthetic peptide, and HC84.20 and HC-11 did not bind (33). The HC-11 epitope also contains contact residues just outside aa 412 to 423 and within aa 434 to 446 (37). GNA-captured H77C E2 was first incubated with 50 μg/ml of unlabeled interfering HMAb prior to adding 2 μg/ml of biotinylated HC33 HMAb. The data showed that the two HC84 antibodies and the HC-11 antibody decreased the binding by each labeled HC33 HMAb by ≤20%, except for HC84.20, which inhibited HC33.8 by 25% (Fig. 3B). The negative controls (R04) showed 0 to ≤10% inhibition. Positive controls consisted of each HC33 HMAb inhibiting itself by 80 to 95% (Fig. 3B, Self). Simultaneous coinoculation of blocking antibodies with labeled HC33 HMAbs showed similar findings of approximately ≤20% inhibition of binding (data not shown). Reciprocal inhibition of binding was tested to determine whether HC33 HMAbs inhibited the binding of biotinylated HC84 HMAbs. As shown in Fig. 3C, each HC33 HMAb inhibited the binding of labeled HC84.20 (at 1 μg/ml) to E2 by 66 to 86% and HC84.26 (at 1 μg/ml) by 69 to 81%. Positive controls consisted of each HC84 HMAb inhibiting itself by 61 to 82%. The unidirectional competition by HC33 and HC84 HMAbs is consistent with proximity but not overlap of their respective clusters of epitopes.

**Additive neutralization by HMAbs to aa 412 to 423 and aa 434 to 446.** We next examined the neutralization of 2a JFH1 HCVcc by HC33 HMAbs in the presence and absence of interfering HMAbs. Whether the combined antibodies were antagonistic, additive, or synergistic was assessed by the median effect analysis method, as described in Materials and Methods (47, 48). A constant ratio between each HC33 HMAb and either HC84.20, HC84.26, or HC-11 was set by their respective IC50 concentrations, as noted in Table 1 for the HC33 HMAbs against 2a JFH1 HCVcc. The IC50s for HC84.20 (0.003 μg/ml), HC84.26 (0.010 μg/ml), and HC-11 (0.04 μg/ml) were established previously (33, 35). Dose-dependent neutralization was tested for each antibody and antibodies in combination in a range of 2-fold dilutions in concentrations from 8 times the IC50 to 1/16 of the IC50. A representative set of analyses to determine cooperativity in virus neutralization is shown in Fig. 4A, B, and C for HC33.1 and HC84.20. Dose-dependent neutralization for HC33.1 and HC84.20 separately and in combination from a dose of 8 times the IC50 to 1/16 of the IC50 were determined (Fig. 4A). On the x axis in Fig. 4B, the doses are expressed as fractions or multiples of their respective IC50 antibody concentrations. Neutralization percentages were entered into the CompSyn program as fractional effect (FA) values from 0.01 to 0.99 and were plotted as the fractional effect in relation to dose. The combination index (CI) values were calculated and plotted in relation to the FA (Fig. 4C). On a logarithmic CI scale, values above 0 are indicative of antagonism, and values below 0 are indicative of synergism. Studies to determine these values were performed for HC33.1, HC33.4, HC33.8, and HC33.29 in combination with HC84.20, HC84.26, or HC-11. 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 of each of the HC33 HMAbs at FA values of 50%, 75%, and 90% effective dose (ED50, ED75, and ED90) are shown in Fig. 4D. The majority of CI values for the FA at ED50 were slightly above 1.0 (range, 0.98 to 1.30), at ED75 were near 1.0 (range, 0.86 to 1.07), and at ED90 were slightly below 1.0 (range, 0.78 to 1.27). These findings suggest a modest degree of antagonism or a partially additive effect at lower antibody concentrations and a modest degree of synergism at higher antibody concentrations. Similar patterns were observed for each HC33 HMAb in combination with HC-11.

**HMAbs to aa 412 to 423 and aa 434 to 446 additively inhibit E2 binding to CD81.** Both sets of HMAbs to aa 412 to 423 and aa 434 to 446 are virus-neutralizing antibodies that function by blocking virus binding to the HCV coreceptor, CD81. This made it possible for us to perform analyses to determine whether HC84.20 or HC84.26 adversely affected the ability of HC33 antibodies to inhibit E2 binding to CD81. These analyses were performed in a manner analogous to the median effect analysis method used in combination studies for virus neutralization (47, 48). We initially tested dose-dependent blocking of E2 binding to CD81 by HC33.1, HC33.4, HC33.8, HC33.29, HC84.20, HC84.26,
and HC-11 to determine their respective IC50s (2.2, 0.5, 5.2, 0.75, 0.15, 0.15, and 0.6 μg/ml) (data not shown). Dose-dependent blocking of binding was tested for each antibody and in combination in a range of 2-fold dilutions in concentrations from 8 times the IC50 to 1/16 of the IC50. Each antibody combination was tested at a constant ratio, as established by its IC50. A representative set of analyses to determine cooperativity in blocking of binding is shown in Fig. 5A, B, and C for HC33.1 and HC84.20. Dose-dependent inhibition for HC33.1, HC84.20, and both in combination was measured (Fig. 5A). On the x axis, doses are expressed as fractions or multiples of the respective IC50 antibody concentrations. The percentages of the blocking of E2 binding to CD81 were entered in the CompuSyn program as FA values and were plotted versus dosage (Fig. 5B). The program also provided a plot of CI values in relation to FA values (Fig. 5C). On a logarithmic CI scale, values above 0 are indicative of antagonism, and values below 0 are indicative of synergism. These studies were performed for HC33.1, HC33.4, HC33.8, and HC33.29 in combination with
HC84.20, HC84.26, or HC-11. For each set of analyses, the linear correlation coefficient $r$ was greater than 0.95 (data not shown). Tabulations of the CI values of the paired studies of each HC33 HMAb at FA values of the 50%, 75%, and 90% effective doses (ED$_{50}$, ED$_{75}$, and ED$_{90}$) are shown in Fig. 5D. CI values were mainly above 1.0 (range, 0.99 to 1.40) at ED$_{50}$, near 1.0 (range, 0.94 to 1.21) at ED$_{75}$, and below 1.0 (range, 0.74 to 1.05) at ED$_{90}$. Analogous to the neutralization patterns, modest degrees of antagonism were observed at lower concentrations, and modest degrees of synergism were observed at higher concentrations.

To define the overall cooperativity in virus neutralization and blocking of E2 binding to CD81 by each pair of antibodies, we plotted the averages of the CI values at FA values of 0.5, 0.75, and 0.90 for both sets of data (Fig. 6). For virus neutralization, the CI averages showed essentially additive neutralization between HC33 and HC84 HMAbs (Fig. 6A). In addition, additive neutralization was observed with each HC33 HMAb and HC-11. Similarly, the average CI values for blocking E2 binding to CD81 (plotted in Fig. 6B) showed mainly additive effects between HC33 and HC84 HMAbs. Taken together, these studies indicated no overall interference by antibodies to aa 412 to 423 and aa 434 to 446 in their respective ability to block virus entry.

**Binding properties of nonneutralizing HC33-related HMAbs and affinity maturation.** Eight of 13 HC33 HMAbs had $<40\%$...
virus-neutralizing activities against JFH1 HCVcc (Fig. 1A). This result initially suggested the possibilities that these antibodies do not share the same epitope or that they are of lower antibody binding affinities because of different light-chain pairing during yeast library selection with the aa-410-to-425 synthetic peptide. We epitope mapped nonneutralizing HC33 HMAbs by site-directed alanine mutagenesis (Fig. 7) under the same conditions as the mapping of neutralizing HC33 HMAbs (Fig. 2D). As a cluster, the HC-33 epitopes not associated with virus neutralization mapped with contact residues at L413, G418, and W420, as defined by 20% retention of binding by their respective antibodies. These are the same contact residues as observed for neutralizing HC33 HMAbs (Fig. 2D). However, the majority of nonneutralizing epitopes included other potential contact residues between L413 and W420 (as shown for HC33.5, HC33.6, HC33.7, HC33.10, and HC33.11) and two residues at H421 and/or H422 (as shown for HC33.5, HC33.10, and HC33.11). Epitope maps for HC33.2, HC33.3, and HC33.9 were similar to those for neutralizing HC33 HMAbs, with contact residues restricted to L413, G418, and W420. Binding affinities for representative nonneutralizing HC33 HMAbs were measured by SPR using purified scFvs, and the $K_{on}$, $K_{off}$, and $K_D$ values were calculated (Table 2). Indeed, the binding affinities of nonneutralizing HC33 HMAbs were nearly 100-fold less than those for neutralizing HC33 HMAbs (Fig. 2C).

To confirm that antibody binding affinity has a direct role in determining virus neutralization, we tested whether affinity maturation by V kappa ($V_K$) light-chain swap can increase binding affinity and neutralizing activity (52). Nonneutralizing HC33.2, HC33.3, and HC33.6 were selected based on the ability of their unique $V_K$ to be paired with three different light chains from neutralizing HC33.1, HC33.4, and HC33.8 (as $V_K$ light-chain donors) (Fig. 8A). The resulting nine scFvs were converted to IgG1 and tested for 2a JFH1 HCVcc neutralization. Among the nine antibodies, HC33.2, HC33.3, or HC33.6 V_H, when paired with $V_K$ from HC33.4 or HC33.8 (indicated as HC33.2, HC33.4, and HC33.8), displayed 80 to 90% neutralization (Fig. 8B). $V_K$ provided by HC33.1 showed high (85%), moderate (50%), or no neutralization when paired with HC33.2 (indicated as HC33.2.1), HC33.3 (HC33.3.1), and HC33.6 (HC33.6.1), respectively. Thus, light-chain shuffle resulted in the conversion of nonneutralizing antibodies to neutralizing antibodies. The HC33.6 affinity-matured antibodies (HC33.6.1, HC33.6.4, and HC33.6.8) were chosen for binding affinity studies since light-chain shuffle led to high (HC33.6.4 and HC33.6.8) or no change (HC33.6.1) in virus-neutralizing activities. To evaluate binding kinetics of the antibodies with the E2 antigen, we produced purified scFvs of parental HC-33.6 and affinity-matured clones for SPR analysis (Table 3). The $K_D$ for HC33.6.1 was 1 log less than that for parental HC33.6, which was due mainly to a 1-log drop in the on rate, $K_{on}$, as a result of mismatched H and L chains. This is consistent with the 40% neutralization by HC33.6.1, as observed with the parental HC33.6. The other two affinity-matured clones, HC33.6.4 and HC33.6.8, achieved 267- and 172-fold improvements, respectively, in $K_D$ values that were the results of both higher on rates and lower off rates. The mechanism of virus neutralization by the affinity-matured clones was assessed by inhibition of E2 binding to CD81 (Fig. 8C). In contrast to no inhibition by parental HC33.6 or clone 33.6.1, clones HC33.6.4 and HC33.6.8 inhibited E2 binding to CD81 in a
dose-dependent manner. At the higher concentration, they achieved the same degree of inhibition as the positive-control HMAb, HC-11. R04 served as a negative control along with a no-antibody control. Collectively, these results demonstrate a direct correlation between antibody binding affinity to E2, virus neutralization, and inhibition of E2 binding to CD81.

Epitope mapping of a series of affinity-matured HC33 HMAbs. We next explored whether improved binding affinity leads to a change in the contact points between antibody and antigen. Epitope mapping was performed with the three light-chain-swapped HC33.6 clones by site-directed alanine mutagenesis within aa 410 to 423 of H77C E1E2 (Fig. 8D). The epitopes for
HC33.6-related antibodies consistently showed >80% reduction in binding with E2 mutants at L413A, G418A, and W420A. In addition, binding by parental HC33.6 was reduced to similar degrees with I414A, N415A, T416A, N417A, H421A, and N423A. For the two neutralizing clones, HC33.6.4 and HC33.6.8, binding reduction was not observed at six of these nine mutations (I414A, N415A, T416A, N417A, H421A, and N423A). The epitope pattern for HC33.6.1 was similar to that for parental HC33.6, in which the same nine alanine substitution E2 mutants led to significantly reduced binding, as defined by <40% binding (Fig. 8D). Interestingly, we observed that improved binding affinity led to a change in the epitope specificity. It is possible that the bindings of lower-affinity antibodies to the same epitope are more easily dislodged by structural changes associated with mutations at residues that are located close to true contact residues.

**DISCUSSION**

The antibody responses to two adjacent regions, aa 412 to 423 and aa 434 to 446, located at the N terminus on the HCV E2 glycoprotein, have drawn much research interest. Although some studies have suggested that the aa region from 434 to 446 is associated with nonneutralizing and interfering antibodies (27, 28), we and others have isolated monoclonal antibodies to both of these regions (14, 16–18, 33, 53, 54) and showed that these antibodies mediate broad neutralization against diverse HCV genotypes and subtypes. Hence, both regions are relevant in a rational design expectation was that the heavy chain would be found to play a more significant role in epitope specificity. It is possible that the bindings of lower-affinity antibodies to the same epitope are more easily dislodged by structural changes associated with mutations at residues that are located close to true contact residues.

**TABLE 2**

| scFv | $K_{\text{in}}$ ($M^{-1} s^{-1}$) | $k_{\text{off}}$ ($s^{-1}$) | $K_{\text{D}}$ ($M$) |
|------|----------------|----------------|----------------|
| HC33.3 | 3.06e-03 | 7.39e-04 | 2.42e-07 |
| HC33.6 | 3.05e-03 | 1.72e-03 | 5.64e-07 |
| HC33.7 | 3.31e-03 | 3.82e-04 | 1.15e-07 |
| HC33.10 | 1.65e-03 | 7.48e-04 | 4.54e-07 |

Antibody Cooperativity in Virus Neutralization

FIG 7  Epitope mapping of nonneutralizing HC33 HMAbs. E2 mutant proteins encompassing aa 412 to 423 and aa 434 to 446 were expressed in HEK 293T cells, and cell lysates were analyzed by ELISA. Identification of the contact residue for each antibody was the same as the epitope mapping described for Fig. 2D for the neutralizing HC33 HMAbs.
Epitope mapping revealed that the key contact residues within aa 412 to 423 for neutralizing HC33 HMAbs are L413, G418, and W420. The initial expectation was that these antibodies would exhibit broad neutralization, as has been observed for AP33 and HCV1 (14,16). In fact, none of the five HC33 HMAbs neutralized all six genotypes of HCVcc. Although the sequence from aa 412 to 423 is the same for 2a JFH1 and J6/JFH1, all of these antibodies had significant reduction (>60%) in their neutralization potency against the J6/JFH1 HCVcc isolate (Fig. 1B; Table 1). While it remains possible that there are other contact residues outside aa 412 to 423 participating in the HC33 epitopes, the nearly equal binding to native and denatured E2 proteins indicates that HC33 HMAbs are linear epitopes without a conformational component. A possible interpretation of these findings is that the accessibility of the aa-412-to-423 epitope is presented unequally to the HC33 HMAbs among the different HCVcc isolates. The structural presentation of aa 412 to 423 could be influenced by other regions of the E2 glycoprotein in spite of sequence conservation. Other studies employing human polyclonal antibodies to aa 412 to 423 have revealed variable reactivity between genotypes 1a and 2a in HCVcc isolates (29).

To answer the question of whether antibodies to aa 434 to 446 mediate virus neutralization and whether they interfere with antibodies to aa 412 to 423, two sets of monoclonal antibodies were studied: an antigenic domain B HMAb, HC-11, and two antigenic domain D HMAbs, HC84.20 and HC84.26 (33,35, 37). These antibodies react to residues within aa 434 to 446 in three patterns: (i) binding to an aa-434-to-446 synthetic peptide (HC84.26), (ii) binding to epitopes having contact residues within aa 434 to 446 and aa 613 to 616 but not to the synthetic peptide (HC84.20), or (iii) binding to residues within aa 434 to 446, in the segment between aa 412 to 423 and aa 434 to 446, and aa 613 to 616 but not to the synthetic peptide (HC84.20), or (iii) binding to residues within aa 434 to 446, in the segment between aa 412 to 423 and aa 434 to 446, and aa 613 to 616 but not to the synthetic peptide (HC84.20), or (iii) binding to residues within aa 434 to 446, in the segment between aa 412 to 423 and aa 434 to 446, and aa 613 to 616 but not to the synthetic peptide (HC84.20), or (iii) binding to residues within aa 434 to 446, in the segment between aa 412 to 423 and aa 434 to 446, and aa 613 to 616 but not to the synthetic peptide (HC84.20), or (iii) binding to residues within aa 434 to 446, in the segment between aa 412 to 423 and aa 434 to 446, and aa 613 to 616 but not to the synthetic peptide (HC84.20).

Epitope mapping of the affinity-matured antibodies in the E2 region of aa 412 to 423. The assay was performed as described for Fig. 2D.
bodies to epitopes that include 8 of 12 residues within aa 434 to 446 and at least one residue, HC84.26, that binds to an aa-434-to-446 synthetic peptide. Moreover, all of these antibodies are neutralizing, which definitively proves that antibodies to aa 434 to 446 mediate virus neutralization. Yet, Zhang and colleagues reported that polyclonal antibodies to this peptide are nonneutralizing (27). A limitation of polyclonal antibodies is that any measurement of their functional properties is a composite of effects of different antibodies, and the concentrations of specific antibodies are unknown. Our prior studies with HC84 HMAbs that bind to aa 434 to 446 found that their respective FcγRIIa against different HCVcc isolates are vastly different from antibody to antibody. For example, the 2a JFH1 HCVcc was 3 to 4 logs more sensitive to virus neutralization by the HC84-related HMAbs than the 2a J6/JFH1 HCVcc (33). It is possible that the polyclonal preparation of antibodies to aa 434 to 446 in the Zhang et al. study contained weaker and/or lower amounts of tier-neutralizing antibodies against the J6/JFH1 HCVcc isolate. Tarr and colleagues used the more sensitive 2a JFH1 isolate in a study in which they demonstrated that polyclonal antibodies to this region mediated virus neutralization (29).

Having established that both regions induce neutralizing antibodies, we performed combined studies to assess antibody cooperativity. Whether the antibodies induced by these regions are additive, synergistic, or perhaps antagonistic is relevant for vaccine design. We first investigated the influence of antibodies to aa 434 to 446 on neutralizing antibodies to aa 412 to 423. Unidirectional competition was observed between antibodies to these two regions for binding to E2, which is consistent with their epitopes being in proximity rather than overlapping on E2 (31, 59). Furthermore, HC84 or HC-11 HMAbs, when combined with each of the HC33 HMAbs at low concentrations, had CI values modestly above 1 for virus neutralization, which is consistent with some degree of antagonism or partially additive neutralization. Similar findings were observed when HC33 and HC84 HMAbs were combined at lower concentrations, with some degree of antagonism in blocking E2 binding to CD81. Taken together, the data suggest that there is a moderate degree of steric hindrance in the binding of these two sets of antibodies. This is consistent with a proposed model of the tertiary organization of HCV E2, in which four β strands in domain I form the tertiary structure that interacts with CD81 (60). The regions aa 412 to 423 and aa 434 to 446 are located on the two adjacent β strands, designated C0 and D0, forming part of the β sheet that contains most of the contact residues that bind to CD81. The proximity of C0 and D0 explains the observed steric hindrance when antibodies bind to these β strands. However, at higher antibody concentrations, the steric hindrance is overcome, and in fact, at FA values of ED90 some degree of synergy was detected in both virus neutralization and blocking of E2 binding to CD81 when HC33 and HC84 HMAbs were combined. The average CI values over a range of antibody concentrations suggest that the overall relationship between antibodies to these two adjacent regions on E2 is mostly additive for virus neutralization.

Screening the yeast-displayed library with aa 412 to 423 identified eight HC33 HMAbs with low or no neutralizing activities. The striking similarity of the two groups is that all of their epitopes included the three key residues located at aa 413, 418, and 420 on E2. This raised the question of whether both neutralizing and nonneutralizing antibodies are elicited by this region. If that is the case, then nonneutralizing HC33 HMAbs could interfere with neutralizing HC33 HMAbs. Although this possibility cannot be ruled out, the nonneutralizing HMAb binding affinities were 2 logs less than those of the neutralizing HC33 HMAbs. Affinity maturation by light-chain swap increased the affinities of the three selected nonneutralizing antibodies and led to virus neutralization. Interestingly, in the case of HC-33.6, epitope mapping revealed a more restricted number of contact residues with the affinity-matured clones that were more analogous to the neutralizing HC33 HMAbs. These findings suggest that light chains can influence epitope specificity. Lower-affinity antibodies are more easily dislodged from binding to their epitopes by substitutions at residues that are near their true contact residues. However, it is also possible that epitope mapping by alanine substitution is less reliable with lower-affinity antibodies. More importantly, the HMAbs targeting aa 412 to 423 showed a direct correlation between Kd values, virus neutralization, and the ability to block E2 binding to CD81. In conclusion, the two adjacent regions of aa 412 to 423 and aa 434 to 446 are relevant for vaccine design in that they contain epitopes associated with broadly neutralizing antibodies. Moreover, the induction of both sets of these antibodies will be generally equal to the predicted effect of their respective neutralizing activities.

ACKNOWLEDGMENTS

We thank J. Lu and A. Saha for technical support.

This work is supported in part by a Ph.D. stipend from the Faculty of Health Science, University of Copenhagen (to T.H.R.C.), an individual postdoctoral stipend from the Danish Council for Independent Research, Medical Sciences (to J.P.), research grants from the Lundbeck Foundation, the Danish Cancer Society, and the Novo Nordisk Foundation (to J.B.), and PHS grant AI081903 (to S.K.H.F.).

REFERENCES

1. World Health Organization. 2012. Hepatitis C fact sheet no. 164. http://www.who.int/mediacentre/factsheets/fs164/en/index.html.
2. Bowen DG, Walker CM. 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature 436:946–952.
3. Barnes E, Folgori A, Capone S, Swadling L, Aston S, Kurioka A, Meyer J, Huddart R, Smith K, Townsend R, Brown A, Antrobus R, Ammendola V, Naddoe M, O’Hara G, Willberg C, Harrison A, Grazzini F, Esposito ML, Siani L, Traboni C, Oo Y, Adams D, Hill A, Colloca S, Nicosa A, Cortese R, Kleinerman P. 2012. Novel adenovirus-based vaccines induce broad and sustained T cell responses to HCV in humans. Sci. Transl. Med. 4:115ra111. doi:10.1126/scitranslmed.3003155.
4. Bartosch T, Bukh J, Meunier JC, Granier C, Engle RE, Blackwelder WC, Emerson SU, Cosset FL, Purcell RH. 2003. In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. Proc. Natl. Acad. Sci. U. S. A. 100:14199–14204.
5. Dorner M, Horwitz JA, Robbins JB, Barry WT, Feng Q, Mu K, Jones CT, Schoggins JW, Catanese MT, Burton DR, Law M, Rice CM, Ploss A. 2011. A genetically humanized mouse model for hepatitis C virus infection. Nature 474:208–211.
6. Lavillette D, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, Sakellariou G, Intrator I, Bartosch T, Pawlotsky JM, Cosset FL. 2005. Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. J. Virol. 79:6023–6034.
7. Law M, Maruyama T, Lewis J, Giang E, Tarr AW, Stamataki Z, Gastañiza P, Chiari FV, Jones JM, Fox R, Ball JK, McKeating J, Kneteman N, Burton DR. 2008. Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. Nat. Med. 14:25–27.
8. Meuleman P, Bukh J, Verhoye L, Farhoudi A, Vanwolleghem T, Wang RY, Desombre I, Alter H, Purcell RH, Leroux-Roels G. 2011. In vivo evaluation of the cross-genotype neutralizing activity of polyclonal antibodies against hepatitis C virus. Hepatology 53:755–762.
9. Pestka JM, Zeisel MB, Blaser E, Schurmann P, Bartosch T, Cosset FL,

January 2013 Volume 87 Number 1 jvi.asm.org
Patel AH, Meisel H, Baumert J, Viazov S, Rispeker K, Blum HE, Roggemann D, 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.

10. Vanwolleghem T, Buhk J, Meuleman P, Desombre I, Meunier JC, Altendorf K, Purcell R, Rodats G. 2008. Polynuclear immunoglobulins from a chronic hepatitis C virus patient protect human liver-chimeric mice from infection with a homologous hepatitis C virus strain. Hepatology 47:1846–1855.

11. Farci P, Alter HJ, Wong DC, Miller RH, Govindarajan S, Engle 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.

12. Shimizu YK, Hijiakata M, Iwamoto A, Alter HJ, Purcell RH, Yoshikura H. 1994. Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. J. Virol. 68:1494–1500.

13. von Hahn T, Yoon JC, Alter H, Rice CM, Rehermann B, Balfe P, McKeating JA. 2006. Characterization of the hepatitis C virus E2 epitope defined by the broadly neutralizing monoclonal antibody AP33. Hepatology 43:592–601.

14. Broering TJ, Garrity KA, Boatright NK, Sloan SE, Sandor F, Thomas WD, Jr, Szabo G, Finberg RW, Ambrosino DM, Babcock GJ. 2009. Identification and characterization of broadly neutralizing human monoclonal antibodies directed against the E2 envelope glycoprotein of hepatitis C virus. J. Virol. 83:12473–12482.

15. Helle F, Vieyres G, Elkrief L, Popescu CI, Wychowski C, Descamps V, Andre P, Komurian-Pradel F, Deforges S, Perret M, Berland JL, Sodoyer M, Pol S, Brechot C, Paranhos-Baccala G, Lotteau V. 2002. N-linked glycans in the functions of hepatitis C virus envelope proteins. J. Virol. 76:623–6242.

16. Thomsen R, Bonk S, Propele C, Heermann KH, Kochel HG, Uy A. 1992. Association of hepatitis C virus in human sera with beta-lipoprotein. Med. Microbiol. Immunol. 181:293–300.

17. Helle F, Vieyres G, Elkhrief L, Popescu CI, Wychowski C, Descamps V, Castelain S, Roingeard P, Brechot C, Paranhos-Baccala G, Lotteau V. 2000. Characterization of a low- and very-low-density hepatitis C virus RNA-containing particles. J. Virol. 74:1959–1969.

18. Chang KS, Jiang J, Cai Z, Luo G. 2007. Human apolipoprotein E is required for infectivity and production of hepatitis C virus in cell culture. J. Virol. 81:13783–13793.

19. Dreux M, Pietschmann T, Granier C, Vioisset C, Ricard-Blum S, Mangeot PE, Keck Z, Foung S, Vu-Dac N, Dubuisson J, Bartenschlager R, Lavillette D, Cosset FL. 2006. High density lipoprotein inhibits hepatitis C virus-neutralizing antibodies by stimulating cell entry via activation of the scavenger receptor BI. J. Biol. Chem. 281:18285–18295.

20. Zhang P, Wu CG, Mihalik K, Virata-Theimer ML, Yu MY, Alter HJ, Feinstone SM. 2007. Hepatitis C virus epitope-specific neutralizing antibodies in Lgs prepared from human plasma. Proc. Natl. Acad. Sci. U. S. A. 104:8449–8454.
45. Keck ZY, Sung VM, Perkins S, Rowe J, Paul S, Liang TJ, Lai MM, Foug SK. 2004. Human monoclonal antibody to hepatitis C virus E1 glycoprotein that blocks virus attachment and viral infectivity. J. Virol. 78:7257–7263.

46. Razai A, Garcia-Rodriguez C, Lou J, Geren IN, Forsyth CM, Robles Y, Tsai R, Smith TJ, Smith LA, Siegel RW, Feldhaus M, Marks JD. 2005. Molecular evolution of antibody affinity for sensitive detection of botulinum neurotoxin type A. J. Mol. Biol. 351:158–169.

47. Chou TC. 2010. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 70:440–446.

48. Chou TC, Talalay P. 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22:27–55.

49. Keck ZY, Li TK, Xia J, Bartosch B, Cosset FL, Dubuisson J, Foung SK. 2005. Analysis of a highly flexible conformational immunogenic domain A in hepatitis C virus E2. J. Virol. 79:13199–13208.

50. Tarr AW, Owsianka AM, Jayaraj D, Brown RJ, Hickling TP, Irving WL, Patel AH, Ball JK. 2007. Determination of the human antibody response to the epitope defined by the hepatitis C virus-neutralizing monoclonal antibody AP33. J. Gen. Virol. 88:2991–3001.

51. Flint M, Dubuisson J, Maidens C, Harrop R, Guile GR, Borrow P, McKeating JA. 2000. Functional characterization of intracellular and secreted forms of a truncated hepatitis C virus E2 glycoprotein. J. Virol. 74:702–709.

52. Wang Y, Keck ZY, Saha A, Xia J, Eckart M, Lou J, Eckart M, Marks JD, Foug SK. 2011. Affinity maturation to improve human monoclonal antibody neutralization potency and breadth against hepatitis C virus. J. Biol. Chem. 286:44218–44233.

53. Owsianka A, Clayton RF, Loomis-Price LD, McKeating JA, Patel AH. 2001. Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. J. Gen. Virol. 82:1877–1883.

54. Perotti M, Mancini N, Diotti RA, Tarr AW, Ball JK, Owsianka A, Adair R, Patel AH, Clementi M, Burioni R. 2008. Identification of a broadly cross-reacting and neutralizing human monoclonal antibody directed against the hepatitis C virus E2 protein. J. Virol. 82:1047–1052.

55. Nainan OV, Alter MJ, Kruszon-Moran D, Gao FX, Xia G, McQuillan G, Margolis HS. 2006. Hepatitis C virus genotypes and viral concentrations in participants of a general population survey in the United States. Gastroenterology 131:478–484.

56. Helle F, Duverlie G, Dubuisson J. 2011. The hepatitis C virus glycan shield and evasion of the humoral immune response. Viruses 3:1909–1932.

57. Burioni R, Bugli F, Mancini N, Rosa D, Di Campli C, Moroncini G, Manzin A, Abranches S, Varaldo PE, Clementi M, Fadda G. 2001. Nonneutralizing human antibody fragments against hepatitis C virus E2 glycoprotein modulate neutralization of binding activity of human recombinant Fabs. Virology 288:29–35.

58. Burioni R, Mancini N, Carletti S, Perotti M, Grecco A, Canducci F, Varaldo PE, Clementi M. 2004. Cross-reactive pseudovirus-neutralizing anti-envelope antibodies coexist with antibodies devoid of such activity in persistent hepatitis C virus infection. Virology 327:242–248.

59. Moore J, Sodroski J. 1996. Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein. J. Virol. 70:1863–1872.

60. Krey T, d’Alayer J, Kikut CM, Saulnier A, Damier-Piolle L, Petitpas I, Johansson DX, Tawar RG, Baron B, Robert B, England P, Persson MA, Martin A, Rey FA. 2010. The disulfide bonds in glycoprotein E2 of hepatitis C virus reveal the tertiary organization of the molecule. PLoS Pathog. 6:e1000762. doi:10.1371/journal.ppat.1000762.

61. Gottwein JM, Scheel TK, Callendret B, Li YP, Eccleston HB, Engle RE, Govindarajan S, Satterfield W, Purcell RH, Walker CM, Bukh J. 2010. Novel infectious cDNA clones of hepatitis C virus genotype 3a (strain S52) and 4a (strain ED43): genetic analyses and in vivo pathogenesis studies. J. Virol. 84:5277–5293.

62. Prentoe J, Jensen TB, Meuleman P, Serre SB, Scheel TK, Leroux-Roels G, Gottwein JM, Bukh J. 2011. Hypervariable region 1 differentially impacts viability of hepatitis C virus strains of genotypes 1 to 6 and impairs virus neutralization. J. Virol. 85:2224–2234.