Affinity Maturation to Improve Human Monoclonal Antibody Neutralization Potency and Breadth against Hepatitis C Virus*§

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Background: A neutralizing antibody to a conserved HCV epitope could be useful for immunotherapy.

Results: Affinity maturation of an antibody, HC-1, by yeast-displayed scFv improved binding affinity. Some of these antibodies neutralized a viral isolate not neutralized by WT HC-1.

Conclusion: Affinity maturation can improve both the potency and the breadth of antibody reactivity.

Significance: Affinity-matured HC-1 antibodies are excellent candidates for therapeutic development.

A potent neutralizing antibody to a conserved hepatitis C virus (HCV) epitope might overcome its extreme variability, allowing immunotherapy. The human monoclonal antibody HC-1 recognizes a conformational epitope on the HCV E2 glycoprotein. Previous studies showed that HC-1 neutralizes most HCV genotypes but has modest potency. To improve neutralization, we affinity-matured HC-1 by constructing a library of yeast-displayed HC-1 single chain Fv (scFv) mutants, using for selection an E2 antigen from one of the poorly neutralized HCVpp. We developed an approach by parallel mutagenesis of the heavy chain variable (VH) and κ-chain variable (Vk) genes separately, then combining the optimized VH and Vk mutants. This resulted in the generation of HC-1-related scFv variants exhibiting improved affinities. The best scFv variant had a 92-fold improved affinity. After conversion to IgG1, some of the antibodies exhibited a 30-fold improvement in neutralization activity. Both surface plasmon resonance and solution kinetic exclusion analysis showed that the increase in affinity was largely due to a lowering of the dissociation rate constant, $K_{\text{off}}$. Neutralization against a panel of HCV pseudoparticles and infectious 2a HCV virus improved with the affinity-matured IgG1 antibodies. Interestingly, some of these antibodies neutralized a viral isolate that was not neutralized by wild-type HC-1. Moreover, propagating 2a HCVcc under the selective pressure of WT HC-1 or affinity-matured HC-1 antibodies yielded no viral escape mutants and, with the affinity-matured IgG1, needed 100-fold less antibody to achieve complete virus elimination. Taken together, these findings suggest that affinity-matured HC-1 antibodies are excellent candidates for therapeutic development.

End-stage liver disease caused by hepatitis C virus (HCV)² is a leading indication of liver transplantation. However, reinfection with HCV occurs universally, and allograft failure due to reinfection is the most common cause of retransplantation and death among HCV-infected liver transplant recipients. More than one-third of liver transplant recipients develop cirrhosis and need retransplantation by the fifth postoperative year (1–3). Combined treatment with pegylated interferon and ribavirin has poor tolerability and efficacy in liver transplant recipients, with only ~30% sustained virological clearance in treated patients (4–6). Recent advances in in vitro and in vivo HCV infection systems and an increased understanding of HCV virology have led to the development of many HCV-specific small molecules with antiviral activity. Completion of phase III studies with several protease inhibitors was recently presented with promising results (7). However, the potential for HCV mutants to escape from these recently FDA-approved protease inhibitors, the proviral effects of post-liver transplant immunosuppression on HCV biology, the diminished tolerability of interferon and ribavirin in the post-transplant setting, and the potential for interactions of new antivirals with immunosuppressive agents are likely to limit the utility of new antiviral therapies in liver transplant recipients at least in the medium term. A model for HCV is available in liver transplantation for hepatitis B (HBV). Although nucleotide and nucleoside analogs are well tolerated and effective for suppression of HBV, hepatitis B immunoglobulin is required and is a standard of care for prevention of post-liver transplant HBV infection. Hepatitis B

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² The abbreviations used are: HCV, hepatitis C virus; HBV, hepatitis B virus; hmAb, human monoclonal antibody(ies); HCVpp, HCV retroviral pseudotype particle; HCVcc, cell culture-infectious hepatitis C virus; scFv, single chain Fv; VH, heavy chain variable region; Vk, κ-chain variable region; CDR, complementarity-determining region; $K_{\text{on}}$, association rate constant; $K_{\text{off}}$, dissociation rate constant, off rate; $K_{\text{eq}}$, equilibrium dissociation constant; $K_{\text{ass}}$, equilibrium association constant; MACS, magnetic-activated cell sorting; Ni-NTA, nickel-nitrilotriacetic acid; ABC, active binding site concentration; GNA, Galanthus nivalis agglutinin; SPR, surface plasmon resonance.
Ig has moved HBV-infected patients from the ranks of the not transplantable to ideal candidates for liver transplantation. Thus, an effective hepatitis C immunoglobulin is a possible cornerstone for prevention of post-liver transplant HCV infection, even if more efficacious and well tolerated oral anti-HCV therapies are developed.

HCV can be classified into seven genetically distinct genotypes and further subdivided into a large number of subtypes, of which the seven major genotypes differ by ~30%, and the subtypes differ by 20 to 25%, at the nucleotide level (8, 9). A significant challenge for immunotherapeutic development is the identification of protective epitopes conserved in the majority of viral genotypes and subtypes. This problem is compounded by the fact that the envelope E1E2 glycoproteins, the natural targets for the neutralizing response, are two of the most variable proteins (10). The error-prone nature of the RNA-dependent RNA polymerase, together with the high HCV replicative rate in vivo (11), results in the production of viral quasispecies (10, 12). Selected antibodies ideally should be broadly reactive to different HCV genotypes, each inhibiting at different steps of virus entry, and be synergistic in their ability to control virus infection. A major determinant of virus neutralization is the highly immunogenic hypervariable region located at the N terminus of HCV E2 (HVR-1) (13, 14). Neutralization by antibodies to HVR-1 is felt to be mediated by inhibiting E2 binding to the scavenger receptor class B type 1 (SR-B1) (15, 16). However, antibodies to this region are of limited utility because the B cell response leads to rapid viral escape associated with mutations within HVR-1, as shown in a study of sequential HCV isolates from one patient over a 26-year period (17).

We have described the isolation of human monoclonal antibodies (hmAbs) to conformational epitopes on HCV E2 glycoprotein employing peripheral B cells from individuals with chronic HCV infection (18–20). Cross-competition analyses delineate at least three immunogenic clusters of overlapping epitopes with distinct properties. Non-neutralizing hmAb fall into one cluster, which we designated domain A, whereas neutralizing hmAb segregated into two clusters, designated domains B and C. Antibodies within domains B and C mediate neutralization by inhibiting E2 binding to the required co-receptor CD81 (18, 19). Domain B antibodies mediate varying degrees of neutralization against HCV pseudotype particles (HCVpp) containing E1E2 glycoproteins of HCV genotypes 1 to 6, with some hmAb neutralizing all genotypes. Alanine-scanning mutagenesis revealed that two conserved E2 residues (Gly-523 and Asp-535) are required for binding of all domain B hmAbs, whereas Gly-523 or Trp-529 is required for some but not all of these antibodies (20). Gly-523, Trp-529, Gly-530 and Asp-535 are also contact residues for HCV attachment to CD81 (21). Thus, these findings suggest that domain B hmAbs exert potent and potentially broad neutralizing effects on HCV by competing with CD81 for binding to conserved residues on E2 that are important for viral entry. Consistent with this, broadly neutralizing hmAb isolated from combinatorial libraries derived from B cells from individuals with chronic hepatitis C also recognize conformational epitopes containing these contact residues (22–24). However, not all domain B antibodies are equal in their properties. Some can lead to virus escape without compromising virus fitness when infectious cell culture virions, HCVcc, are propagated under increasing antibody concentration of the selecting antibody (25). Under the selective pressure of other domain B antibodies, virus escape was associated with a loss of viral fitness, or at a critical antibody concentration, complete viral replication suppression was observed.

HC-1, an IgG2a HCV-neutralizing domain B hmAb, recognizes a conformational epitope on HCV E2 and at a critical antibody concentration is able to completely suppress viral replication (20, 25). In addition, this antibody binds broadly with known contact residues that are absolutely conserved. However, the neutralization potency is modest to moderate against some isolates, which limits its therapeutic applications. This report describes our effort to improve HC-1 antibody binding and neutralization by affinity maturation. In vitro antibody displays, both phage and yeast displays, have been widely employed to increase antibody affinity (26). The advantages of yeast over phage display include avoiding expression, purification, and characterization of a large number of different phage display scFv, the ability to measure $K_D$ directly on the yeast surface, and the ability to enrich for higher affinity clones with decreasing antigen concentration by flow cytometry (27–30). Employing this approach, we have isolated a series of affinity-matured HC-1 clones that have improved binding and neutralization activities against HCV isolates that were neutralized poorly by wild-type (WT) HC-1 hmAb. Interestingly, an HCV isolate not neutralized by WT HC-1 was neutralized by affinity-matured HC-1 IgG clones.

**EXPERIMENTAL PROCEDURES**

**Cells, Virus, Strains, Media, and Antibodies—HEK293 T cells were obtained from the ATCC. Huh7.5 cells (generously provided by Dr. C. Rice, Rockefeller University) and were grown in Dulbecco’s modified minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (Sigma-Aldrich) and 2 mM glutamine. JFH-1 2a cell culture infectious virus (HCVcc) was generously provided by Dr. T. Wakita (National Institute of Infectious Diseases, Japan). The yeast strain EBY100 (GAL1-AGA1:URA3 ura3–52 trp1 leu2Δ1 his3Δ200 pep4:His2 pro1Δ1.6R can1 GAL) was maintained in YPD medium (31). EBY100 transformed with expression vector pYD2 (27) was selected on SD-CAA medium (31). ScFv yeast surface display was induced by transferring yeast cultures from SD-CAA to SG-CAA medium (identical to SD-CAA medium except the glucose was replaced by galactose) supplemented with 12.5 μg/ml tetracycline and 50 μg/ml kanamycin and grown at 18 °C for 48 h as described (31). The bacterial strain Escherichia coli DH5α, (K12, Δ(lac-pro), supE, thi, hisD55F’ traD36, proA+B+, lacI, lacZΔM15) was used for cloning and preparation of plasmid DNA. HCV hmAb CBH-17, HC-1, CBH-4D, and CBH-4G were used (18–20). For IgG detection by flow cytometry (FACS), phycoerythrin-labeled donkey anti-human IgG (Fcγ-specific, catalogue number 709-116-098), fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Fcγ-specific, catalogue number 115-095-071), or allophycocyanin-conjugated donkey anti-human IgG (Fcγ-specific, catalogue number 709-136-098) was employed (Jackson ImmunoResearch)
Construction and Expression of Mutant scFv Display Libraries—The gene encoding the WT HC-1 scFv was randomized using a Stratagene GeneMorph® II random mutagenesis kit to give a low to high mutagenesis rate. The template used for library construction was pYD2.HC-1. Primers pYDFor and pYDRev were designed to PCR-amplify the HC-1 gene from pYD2.HC-1 (PYDFor, 5'-AGT AAC GTT TGT CAT TCG GTA TTC and pYDRev, 5'-GTC GAT TTT GTT ACA TCT ACA C-3'). The mutated HC-1 scFv gene was gel-purified using gel extraction kit (Qiagen). For the HC-1 VH-mutant (Mut) library, HC-1 VH gene fragments were PCR-re-amplified with the primers Gap3 and HulHJR (Gap 5, 5'-AGT AAC GTT TGT CAT TCG GTA TTC and HulHJR, 5'-GCC ACC TCC GCC AGG ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC 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buffer and incubated with certain dilutions of HCV E2 secretion medium. After incubation with E2, yeasts were washed and resuspended in anti-V5 (1:5000) and CBH-4G (10 μg/ml) for 1 h at 4 °C. The cells were washed and incubated with a 1:200 dilution of FITC-labeled anti-mouse and phycoerythrin-labeled anti-human IgG (Fcγ-specific) for 30 min at 4 °C. The labeled cells were washed and sorted. Typically the highest 0.2–2% of the E2-binding population was gated in the sorting. Collected cells were grown in SD-CAA medium and used for the next round of sorting after induction in SG-CAA as described above. After the final round of sorting, 96 individual clones were picked, induced, and stained with E2 containing medium, as detected by CBH-4G and anti-V5 antibody to identify the best candidates in terms of E2 binding.

Measurement of Yeast-displayed scFv Affinity for HCV E2—Quantitative equilibrium binding was determined using yeast-displayed scFv and flow cytometry as described (29, 30). In general, eight concentrations in a 5-fold dilution from 1 mg/ml to 0.013 μg/ml of purified HCV E2 (based on A280 measurement) were used. Incubation volumes and the number of yeast cells stained were chosen to keep the number of antigen molecules in 10-fold excess above the number of scFv, assuming 5.0 × 10^5 scFv/yeast. Binding of E2 to yeast-displayed scFv was detected using CBH-4G and anti-V5 antibody. Typically the highest 0.2–2% of the E2-binding population was gated in the sorting. Collected cells were grown in SD-CAA medium and used for quantitative equilibrium binding. HC-1 and its affinity-matured mutants were captured onto the surface. The purified E2 (384–661) at concentrations ranging from 14.2 nM to 4.49 μM was injected for 2 min using a flow rate of 30 μl/min. Dissociation of bound antigen in Hepes-buffered saline buffer flow was followed for 5 min. The surfaces (HC-1 antibody and E2) were regenerated after each cycle using regeneration solution (3 mM MgCl2). The association rate constant (K_on) was determined from a plot of (ln(dR/dt))/t versus concentration. The dissociation rate constant (K_off) was determined from the dissociation part of the sensogram at the highest concentration of E2. K_D was calculated as K_off/K_on.

Affinity Maturation of HCV-neutralizing Antibody

Measurement of IgG Affinity for HC-1 and Its Affinity-matured Mutants—IgG binding kinetics were measured using surface plasmon resonance in a BIAcore 3000 (Pharmacia Biocisor) and used to calculate the K_D. The HC-1 and its mutants were affinity captured using a human antibody capture kit (GE Healthcare, BIAcore BR-1008-39). Approximately 7000 response units of mouse anti-human antibody were coupled to a CM5 sensor chip by using N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Approximately 100 response units of purified IgG in Hepes-buffered saline (pH 7.4) were captured onto the surface. The purified E2 (384–661) at concentrations ranging from 14.2 nM to 4.49 μM was injected for 2 min using a flow rate of 30 μl/min. Dissociation of bound antigen in Hepes-buffered saline buffer flow was followed for 5 min. The surfaces (HC-1 antibody and E2) were regenerated after each cycle using regeneration solution (3 mM MgCl2). The association rate constant (K_on) was determined from a plot of (ln(dR/dt))/t versus concentration. The dissociation rate constant (K_off) was determined from the dissociation part of the sensogram at the highest concentration of E2. K_D was calculated as K_off/K_on.

Binding affinity was also measured using a KinExA 3000 flow fluorimeter to quantify the free antigen at equilibrium in 1.5-ml solutions of constant antigen concentration mixed with varying concentrations of antibody. Studies of reaction mixtures were performed in PBS (pH 7.4) with 1 mg/ml BSA with 0.02% (w/v) sodium azide added as a preservative. Antibody solution was diluted serially into a constant concentration of E2 solution sufficient to produce a reasonable signal, and the antibody concentration was varied from less than 0.1 to greater than 10-fold above the value of the apparent K_D. The E2 concentrations were limited to below 10-fold above the K_D to ensure a K_D-controlled experiment. Samples were allowed to reach equilibrium, and then each of five dilutions was passed over a flow cell packed with a 4-mm column of N-hydroxysuccinimide-activated Sepharose 4 Fast Flow beads (GE Healthcare) covalently coated with VH Vk3.67 antibody, which shares its E2 epitope with all the measured clones, so that only free antigen was captured from the mixture. An Alexa 647-labeled hamAb binding to an alternate epitope on E2 (CBH-4D) was passed over the beads to detect the free antigen by binding and producing a signal relative to the amount of free E2 bound to the beads. The equilibrium titration and kinetics data were fit to a 1:1 reversible binding model using KinExA Pro software (version 3.0.6; Sapidyne Instruments) to determine the K_D. The binding kinetics of the antibody/antigen interaction were also measured using a KinExA 3000 to quantify the decrease in free antigen as mixtures of IgG and antigen came to equilibrium. A single mixture of antibody solution and E2 solution was prepared for each clone and measured on the instrument by being passed in 0.5-ml volumes over a fresh antigen-binding bead pack at intervals of ~550 s and detected with Alexa 647-labeled CBH-4D as described above. The exponential decrease in the concentration of free antigen as a function of time was fit to a standard bimolecular rate equation using the KinExA Pro software to determine the K_on × K_D. An experiment was also performed with an E2 concentration over 200-fold above the measured K_D to produce a receptor-controlled data curve that would accurately measure the E2 capture by E2 concentration. The b1b4 E2 input concentration was 50 nm. HC-1.Vk1.9 was used for this study; its concentration was serially diluted 1:10, from 50 nm to 5 pm, in a
constant E2 concentration. The error curve for the active binding site concentration (ABC; active antigen in the experimental samples) was generated by the software by varying the ABC value for the optimized $K_D$ curve at each point while holding other parameters constant and then plotting the “best fit.” The low point of the curve then reflects the most likely ABC value, as it reflects the least error, and the 95% confidence interval is determined similarly.

HCV Retroviral Pseudoparticle Production and Neutralization—A panel of different HCVpp genotypes and subtypes was generously provided by Dr. Jonathan Ball (University of Nottingham, United Kingdom) (UKN genotypes 1A20.8, 1B5.23, 2A1.2, 2B2.8, 3A1.9, 4.11.1, and 5.15.7; with GenBankTM accession numbers EU155192, AY734976, AY349777, AY734983, AY734985, AY734986, and EF427672). HCVpp were produced as described previously (34, 35) by co-transfection of 293T cells with plasmids containing the env-deficient proviral genome and an expression plasmid encoding the HCV glycoproteins. The virus-containing extracellular medium was collected 48 h after transfection and separated from cell debris by passage through a 0.45-μm filter. For the neutralization assay, the virus-containing medium was incubated with each hmAb at various concentrations, or with phosphate-buffered saline instead of the antibodies as an infectivity control, plus 4 μg/ml Polybrene at 37 °C for 60 min. The HCVpp-antibody mixture was transferred to Huh7.5 cells (8 × 10³ cells/well) preseeded in 96-well plates, and infections were centrifuged at 730 × g for 2 h at room temperature. After incubation at 37 °C in the presence of 5% CO₂ for 15 h, the unbound virus was replaced with fresh complete medium followed by an additional incubation for a total of 72 h. For detection of HCVpp entry, 100 ml of reconstituted Bright-Glo (Promega, Madison, WI) was added to each well followed by 2 min of mixing and light detection in a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). The neutralizing activity of an antibody was calculated as the percent reduction of luciferase activity compared with an inoculum containing PBS. For HCVpp infectivity studies, the virus-containing extracellular medium was normalized for HIV p24 expression using a QuickTiter lentivirus titer kit (Cell Biolabs, San Diego).

GNA Capture ELISA—An ELISA to detect hmAb binding to E2 was performed as described previously (36). In brief, HEK293T cells were transfected with plasmids encoding the E1E2 glycoproteins from different HCV genotypes. Clarified lysates from transfected cells were captured onto GNA-coated microtiter plates. Antibody titration studies were performed at concentrations of 0.01 to 200 μg/ml hmAb. The bound hmAb was incubated with alkaline phosphatase-conjugated goat anti-human IgG (Promega) followed by incubation with p-nitrophenyl phosphate disodium hexahydrate for color development. Absorbance was measured at 405 nm with an FL600 plate reader from Bio-Tek Instruments (Winooski, VT). The data were analyzed by nonlinear regression to measure the antibody disassociation constant ($K_D$) using Prism software (GraphPad).

HCV Neutralization Assay—The plasmid pHF-1, containing the full-length cDNA of JFH-1, was used to generate HCVcc as described previously (37, 38). Briefly, the pHF-1 plasmid was linearized by XbaI digestion and used as a template for in vitro transcription with the MEGAscript kit (Ambion, Austin, TX). In vitro transcribed RNA was delivered to Huh7.5 cells by electroporation, and viral stocks were obtained by harvesting cell culture supernatants at 3–4 days after transfection. A virus inoculum (containing 50 focus-forming units) was incubated with serial dilutions of antibodies for 1 h at 37 °C before inoculation onto Huh7.5 cells (8 × 10³ cells/well) seeded 24 h previously into 96-well plate. After 3 h of incubation at 37 °C in the presence of 5% CO₂, the inoculum was replaced with 100 μl of fresh complete medium followed by incubation for a total of 72 h. The medium was taken out, and infected cells were lysed and examined for HCV E2 protein expression with CBH-5 by GNA capture ELISA as described above. The percent neutralization was calculated as the percent reduction in $A_{405nm}$ absorbance compared with virus incubated with an irrelevant control antibody (R04). The antibody concentrations (μg/ml) causing 50 or 90% inhibition in ELISA were determined by linear regression analysis.

Selection of Antibody-resistant Neutralization Escape Mutants—Huh7.5 cells (3.2 × 10⁴/ml) seeded 24 h previously in a 24-well plate were infected with 2a HCVcc (1 × 10⁴ focus-forming units). The initial concentration of the neutralizing antibody employed to isolate escape HCVcc mutants was adjusted to the 50% inhibitory concentration (IC₅₀) of the antibody against the 2a HCVcc. The infectious virus was first incubated with the selection antibody for 1 h at 37 °C prior to inoculation onto naive Huh7.5 cells. This was followed by a second incubation for 3 h at 37 °C before the medium was replaced with fresh medium containing the same antibody concentration. The cultures were maintained for 3 days in the presence of WT HC-1, affinity-matured HC-1, or R04 (as mock human IgG selection), and the extracellular virus was harvested for the next round of selection. The entire process constituted one passage of infectious virus under antibody selection. At each antibody concentration, the virus was repeatedly passaged until the virus titer reached 1 × 10⁴ focus-forming units prior to subjecting the virus to the next higher antibody concentration. The number of rounds of amplification required for this purpose varied from antibody to antibody. Starting at IC₅₀, the antibody concentration was progressively increased (0.05, 0.1, 0.5, 1, 5, 10, 20, 40, 60, 80, and 100 μg/ml). Viral growth and the emergence of escape variants were monitored weekly by two-color confocal immunofluorescence microscopy staining with the respective neutralizing antibody and an anti-NS3 antibody. Viral supernatant and cells were collected weekly and stored at −80 °C for sequence analysis. Viral supernatants were used for neutralization studies against escape mutants and as a source of virus stock.

Confocal Immunofluorescence Microscopy—Cells from mock- or virus-infected cultures were harvested by trypsinization, counted, resuspended in DMEM, and plated onto a 24-spot Teflon-coated slide. The cells were air-dried and fixed with 100% acetone. Slides were incubated with a mixture of mouse anti-NS3 (1:100) and HC-1 (10 μg/ml) as primary antibodies for 30 min followed by FITC-labeled anti-human IgG (Jackson ImmunoResearch) or goat anti-mouse IgG conjugated to Alexa 594 (Invitrogen) as secondary antibodies for 30 min at
37 °C. Slides were washed, counterstained with Hoechst 33342 and mounted in mounting medium (Vector Laboratories, Burlingame, CA), sealed, and then examined with a Leica DM2500 laser-scanning confocal microscope. The captured images were superimposed using LaserSharp software (Bio-Rad Laboratories). To verify the binding specificity of HC-1 affinity-matured clones, 293T cells either expressing HCVpp 1aH77c E1E2 proteins or not were immunostained by using HC-1 WT and affinity-matured IgG clones (10 g/ml). R04 antibody was used as a negative control. Detection with anti-human IgG, counterstaining with Hoechst 33342, and scanning confocal microscopy were as described above.

RESULTS

**Determination of HC-1 Binding and Neutralization against a Panel of HCV 1a and 1b Isolates**—Our previous study had indicated that HC-1 is a broadly neutralizing antibody against the different HCV genotypes and subtypes (20). Patients who eventually require liver transplantation are infected, for the most part, with either HCV genotype 1a or 1b. To evaluate the potency of HC-1 broad reactivity to HCV 1a and 1b subtypes, we performed dose-dependent binding and neutralization assays against a panel of HCVpp-expressing E1E2 glycoproteins of different genotype 1a and 1b isolates as shown and listed in Fig. 1. HC-1 binding to E1E2 in viral lysates of different isolates was measured by GNA capture ELISA, and the equilibrium dissociation constants (K_D) were calculated (Fig. 1B). The neutralization capability was determined by calculating the IC_{50}. Fig. 1B summarizes the differential neutralization conferred by HC-1 against HCVpp 1a and 1b subtypes together with the K_D determined by GNA capture ELISA. HC-1 neutralized both 1a and 1b isolates. However, significant variation in the neutralization potency was observed. The IC_{50} values differed by almost 300 orders of magnitude, ranging from 0.16 to 47.78 μg/ml HC-1 IgG. Importantly, HC-1 failed to neutralize the 1A20.8 HCVpp isolate. The maximum neutralization efficiency was only 9% in the presence of 100 μg/ml HC-1 IgG. In comparing the binding data with the neutralization potency, we noted that the isolates more sensitive to neutralization, such as the one designated as HCV 1aH77c (Fig. 1B), showed the best binding with the lowest K_D value, whereas the poorly neutralized HCV 1b34 and UKN 1B5.23 bound with moderate K_D values, and the non-neutralized HCV 1A20.8 with the highest K_D (Fig. 1, A and B). These results suggest that affinity to E1E2 is a major determinant of neutralization by HC-1, which indicates that performing affinity maturation might be a plausible way to improve neutralization.
Construction and Sorting of HC-1 VH and Vk Random Mutagenesis Yeast Libraries—A yeast surface display approach was employed to achieve affinity maturation. First, a plasmid for yeast-displayed HC-1 scFv, pYD2.HC-1, was generated in the pYD2 vector as described elsewhere (27). To facilitate cloning, we introduced a SalI site at the 5' end and a BspEI site at the 3' end of the (Gly4-Ser)3 linker, which joined the HC-1 variable fragment of the heavy chain (VH) and the light chain (Vk) together, as shown in the top line of Fig. 2A. HC-1 affinity was increased sequentially by creating three different mutation libraries following the strategy shown in Figs. 2A and C, and 3A. To evaluate the contribution of VH or Vk to affinity and
neutralization, we mutated VH and Vk separately in two different libraries. To achieve this, the HC-1 VH fragment gene was randomly mutated using error-prone PCR. The resulting repertoire was directly cloned into S. cerevisiae strain EBY100 by gap repair into pYD2-HC-1-Vk (which was obtained by digesting pYD2-HC-1 plasmid with NcoI and SalI and recovering the vector fragment). This resulted in a HC-1 VH-Mut (mutant) library containing $2.5 \times 10^7$ transformants. An HC-1 Vk-Mut library with $2.9 \times 10^7$ transformants was generated by a similar method, except the larger vector fragment was obtained by digesting pYD2-HC-1 plasmid with BspEI and NotI (Fig. 2A).

Higher affinity HC-1 mutants were enriched sequentially by MACS and FACS. We chose a weakly bound antigen on the premise that it would be a better candidate for affinity improvement, thus selecting HCV 1b34 as the antigen for these procedures. Between the E1E2 lysate and secreted E2, we preferred the latter, mainly based on the relative ease of obtaining purified E2 and better tolerance of yeast cells to this antigen. HCV-secreted E2 containing amino acids 384–661 with a His$_x$ tag for Ni-NTA purification was expressed in 293T cells as described and used elsewhere (39–41). The C terminus of E2 was not included in this construct because it has been reported that this region is highly hydrophobic and can anchor E2 to the endoplasmic reticulum, preventing secretion of the protein (42). The purified 1b34 E2 (383–661) protein was characterized by SDS-PAGE under reducing and nonreducing conditions (supplemental Fig. S1A) and by immunoblotting with HC33.1.53, an antibody to a linear epitope on E2 (supplemental Fig. S1B). Under reducing condition, the E2 was mainly a broad band with molecular mass ranging from 55 to 100 kDa, which reflects different degrees of glycosylation as observed previously (40). However, reportedly only a fraction of the total E2 is functionally active because of the intrinsic deficiency of secreted E2 produced by overexpression in mammalian cells, which includes misfolding, aggregation, and different degrees of glycosylation (40). Under nonreducing condition, the disulfide-linked dimeric (D) and multimeric (A) bands were observed (supplemental Fig. S1A), with the monomeric E2 fraction representing 36.3% of the total protein as calculated by ImageJ software. Immuno blotting under nonreducing conditions showed that the disulfide-linked dimeric and multimeric bands were less reactive to anti-E2, suggesting that the anti-E2 binding epitope was hindered or masked in the disulfide-linked aggregation (supplemental Fig. S1B). Although the functionally active E2 is located in the monomeric E2 fraction, not all of the monomeric E2 proteins were expected to be active, as indicated previously by the difference between the intracellular and secreted forms of monomeric E2 in their respective reactions with CD81, an HCV co-receptor molecule (40). To verify the antigenicity of the secreted HCV E2 (384–661), glycoproteins of 1b34, 1aH77c, and 1bSF (hereafter referred to as 1b34 E2, 1aH77c E2, and 1bSF E2) were compared with the corresponding HCV E1E2 lysate for binding of HC-1. The data showed that HC-1 bound nearly equally to both E2 and E1E2 (data not shown).

The secreted E2 was selected over the cell lysate because of better tolerance of yeast cells to the purified secreted E2.

Two rounds of enrichment were carried out on magnetic bead columns using an equilibrium method with the secreted E2 as the medium base (43). For the first round, we used secreted 1b34 E2 in a 1:5 dilution, with biotin-CBH-4G (an hmAb to a non-neutralizing epitope on HCV E2) (44) as the detection antibody and streptavidin-magnetic microbeads to capture the E2-bound yeast. In the subsequent round of MACS, we lowered the concentration of 1b34 E2 to a 1:20 dilution and altered the microbeads to anti-biotin magnetic microbeads. The output was subjected to one more round of sorting on FACS by gating the highest 0.5% binders against secreted 1b34 E2 in a 1:25 dilution. Fig. 2B shows the flow cytometry analysis of the output. After two rounds of MACS (VH-M2 or Vk-M2) plus one round of FACS enrichment (VH-M2F1 or Vk-M2F1), both libraries showed improvement of binding compared with the WT HC-1. The sorted output was plated to allow for characterization of individual HC-1 yeast-displayed mutants. Ninety-six colonies from each library were picked randomly and screened for binding to 1b34 E2. The eight clones with the highest mean fluorescence intensity were picked and sequenced. The sequence alignments indicated that the G100R mutation in the VH-CDR2 region appeared in all eight VH mutants, T56I in VH-CDR2 appeared in six of the eight (supplemental Table S1), and the S31R mutation in the Vk-CDR1 region occurred in all eight Vk mutants (supplemental Table S2). Although two more rounds of FACS selection with secreted 1b34 E2 in 1:50 or 1:100 dilutions were performed, no higher affinity clone was identified. Analysis indicated that VH1.48 and Vk1.9 were the best clones in the VH-Mut and Vk-Mut libraries, and these were chosen for further study.

**Generation and Sorting of HC-1 VH/Vk Mutant Sublibraries—**To further increase E2 binding affinity, a third HC-1 mutation yeast library was constructed following the steps indicated in Fig. 2C. The output of the HC-1 VH-Mut and HC-1 Vk-Mut libraries generated after the first round of FACS enrichment provided mutants with abundant diversity and the most improved affinity. The output plasmid pools were extracted from yeast and transfected into E. coli; these were named HC-1 VH-Mut sublibrary and HC-1 Vk-Mut sublibrary, respectively. Each of the sublibraries yielded 1.5 × $10^6$ transformants. The Vk gene mutants were PCR-amplified from the HC-1 Vk-Mut sublibrary template and gap-repaired into the BspEI- and NotI-digested HC-1 VH-Mut sublibrary. Thus, the beneficial HC-1 VH mutants were randomly paired with beneficial HC-1 Vk mutants, producing a HC-1 VH/Vk-Mut library. This library was subjected to three rounds of FACS selection using decreasing concentrations of 1b34 E2 in dilutions varying from 1:100, to 1:200, to 1:500. From the output of the last round of FACS enrichment, we identified three more clones, VHvk3.42, VHvk3.64, and VHvk3.67 (Fig. 3A), which showed the highest 1b34 E2-binding values in monoclonal screening. Compared with the previously isolated HC-1 VH1.48 and Vkl.9, these clones showed higher mean fluorescence intensity values. As indicated by Fig. 2D, in the presence of 1b34 E2 in 1:500 dilution, no binding signal could be detected for WT HC-1 and VH1.48, whereas further improved binding was shown for VHvk3.42 and VHvk3.64.

**Sequence Characterization Analysis of WT HC-1 and Affinity-matured Mutants—**Six unique affinity-matured mutants were isolated for further characterization. Fig. 3A lists the
library and selection methods by which these individual mutants were derived. In the case of VH1.48-Vk1.9, the VH gene of VH1.48 and the Vk gene of Vk1.9 were combined to assess whether the combination produced some advantage. The alignment of the amino acid sequences of the affinity-matured clone with the parental WT HC-1 is presented in Table 1, which also lists the locations at which the mutations were introduced. There was a total of 10 mutated residues (28 times) that differed from the wild type, among which only one mutation occurred in the VH framework region 3 (VH-FR3); the others occurred in the complementarity-determining region (CDR). The alignment revealed two absolute consensus mutations, a threonine-to-isoleucine change at residue 56 (T56I) in VH-CDR2 and a serine-to-arginine at residue 31 (S31R) in Vk-CDR1, which appeared in all of the affinity-improved clones. The $K_D$ improvement of each affinity-matured clone was measured by flow cytometry with the purified HCV 1b34 E2 and compared with WT HC-1. The data show that the $K_D$ of all of the affinity-matured clones was higher than that of the WT HC-1 scFv (Fig. 3B). The best clone was VHVK3.42, which showed a 92-fold increase in binding. In the case of VH1.48-Vk1.9, the combination resulted in an additive effect on affinity, which showed a 58-fold improvement compared with WT HC-1, whereas VH1.48 and Vk1.9 exhibited, respectively, a 16- and 22-fold improvement (Fig. 3C).

Conversion of scFv to IgG and Measurement of IgG Affinity
Using Both SPR and KinExA—For functional characterization, we converted these scFv to full-length IgG with the described protocol (33). Briefly, we separately cloned the VH or Vk gene of WT HC-1 or its mutants into two mammalian expression vectors designated specifically for VH or Vk. Co-transfection of these two vectors into a 293T cell line transiently produced the IgG. To evaluate the impact of conversion of yeast-displayed scFv to IgG on affinity, we used surface plasmon resonance in BIAcore. In addition, this kinetic analysis allowed us to address which parameter, the association rate constant ($K_{on}$) or the dissociation rate constant ($K_{off}$), contributes more to affinity improvement. As shown in
Fig. 4B, the affinity of IgG measured by SPR was comparable with the affinity of yeast-displayed scFv (Fig. 3C). The trend was maintained, but some variation was observed. Compared with the WT HC-1, there was improvement in both $K_{\text{on}}$ and $K_{\text{off}}$; however, the $K_{\text{off}}$ improvement was more dramatic. The highest affinity clone was VH1.48-Vk1.9, which displayed 3.17-fold improvement in $K_{\text{on}}$ and 12.7-fold improvement in $K_{\text{off}}$, with a total 40.5-fold improvement in $K_D$.

To confirm the affinity measures, another solution affinity method, KinExA, was used to analyze the affinity and kinetic. KinExA may be used to accurately determine the affinity of an interaction, the concentration of actively binding antigen, or both depending on the ratio of antigen concentration to measured $K_D$, by measuring the decrease in free antigen as mixtures of IgG and antigen came to equilibrium. The exponential decrease in the concentration of free antigen as a function of time was fit to a standard bimolecular rate equation using KinExA Pro software to determine $K_{\text{on}}$. The $K_{\text{off}}$ value was calculated from the product of $K_{\text{on}} \times K_D$. $K_{\text{on}}$ is the rate constant of association, $K_{\text{off}}$ the rate constant of dissociation, $K_D$ the equilibrium dissociation constant, and $K_a$ the equilibrium association constant.

![Fig. 4. Measurement of WT HC-1 and affinity-matured mutants IgG affinity to HCV 1a E2 on SPR. A, overlay plot of association and dissociation curves obtained for HCV1b 34E2 antigen at 4.49 μM concentration against immobilized HC-1 IgG. The HC-1 affinity-matured mutants show significant slower dropping of dissociation curves comparing to WT HC-1. B, binding kinetics and affinities of IgG constructed from heavy and light chain variable regions of HC-1 wild type and affinity-matured scFv as measured by BIAcore 3000 with the HCV1b34E2 antigen. C, binding curves for HC-1 WT and affinity-matured IgG as determined by flow fluorimetry in a KinExA 3000. Percentage of free E2 as a function of antibody concentration was measured by KinExA. Binding was determined in duplicate for each IgG and the data plotted. D, binding kinetics and affinities of HC-1 WT and affinity-matured IgG as measured by the KinExA 3000 to quantify the decrease in free antigen as mixtures of IgG and antigen came to equilibrium. The exponential decrease in the concentration of free antigen as a function of time was fit to a standard bimolecular rate equation using KinExA Pro software to determine $K_{\text{on}}$. The $K_{\text{off}}$ value was calculated from the product of $K_{\text{on}} \times K_D$. $K_{\text{on}}$ is the rate constant of association, $K_{\text{off}}$ the rate constant of dissociation, $K_D$ the equilibrium dissociation constant, and $K_a$ the equilibrium association constant.

![Graph showing binding kinetics and affinities of IgG](image)

**TABLE 1.** $K_D$ measurements for E2 binding by HC-1 and AM mutants

|          | $K_D$(nM) | $K_D^{\text{wt}}$ | $K_D^{\text{Matured}}$ | $K_{\text{on}}$(M$^{-1}$s$^{-1}$) | $K_{\text{off}}$(s$^{-1}$) |
|----------|-----------|-------------------|-------------------------|-------------------------------|--------------------------|
| wt       | 21.1      | 1                 | 1.781e5                 | 3.755e-3                      |
| VH1.48   | 0.45      | 47                | 8.763e4                 | 3.941e-5                      |
| Vk1.9    | 0.22      | 96                | 3.837e4                 | 8.389e-6                      |
| VH1.48-Vk1.9 | 0.45 | 47                | 4.928e4                 | 2.233e-5                      |
| VHVK3.42 | 0.06      | 351               | 1.840e5                 | 1.103e-5                      |
| VHVK3.64 | 0.41      | 52                | 8.619e4                 | 3.522e-5                      |
| VHVK3.67 | 0.86      | 25                | 1.382e5                 | 1.183e-4                      |

When 293T cells were transfected with env-deficient proviral genome only (supplemental Fig. S1D, left panels) compared with specific binding when 293T cells were transfected with an expression plasmid encoding HCV 1a H77C E1E2 (supplemental Fig. S1D, right panels), nuclear staining was observed in both sets of 293T cells when the cells were counterstained with Hoechst nuclear stain H33342 (supplemental Fig. S1D, lower panels, blue).
Affinity Maturation of HCV-neutralizing Antibody

than by SPR or by flow cytometry on the yeast surface. Affinity data from KinExA validated the improvement of affinity-matured clones over WT (Fig. 4C). Again, the most dramatic change was the slowing of the $K_D$. The best clone was HC-1.VHVk.42, which gave a $K_D$ of 59 nM, corresponding to a 351-fold improvement over WT HC-1 (Fig. 4D). An experiment was also performed with an E2 concentration over 200-fold above the measured $K_D$, to produce a receptor-controlled data curve and accurately measure the actively binding E2 concentration. The 1b34 E2 input concentration was 50 nM; HC-1.Vk1.9 was employed for this analysis. The active receptor concentration was found to be 1.63 nM with a 95% confidence interval between 1.19 and 2.07 nM (supplemental Fig. S1D). This represented ~3% of the previously assumed value. This finding places the $K_D$ values obtained by SPR as less accurate because they were measured against a mixture of native (estimated at 3% by KinExA), misfolded, and aggregated E2. Nonetheless, a relative increase in affinity was observed by both methods. Measurements by KinExA are more in agreement with ELISA. For example, the $K_D$ for 1b34 is 24 nM by ELISA (Fig. 1B) and 21 nM by KinExA. This is probably because the ELISA measurements are determined against more native E2 as expressed in cell lysate.

Characterization and Neutralization of Affinity-matured HC-1 Mutants—The neutralization potency of the affinity-matured HC-1 mutants relative to the wild type was investigated against a panel of 1a and 1b HCVpp isolates. Each antibody was employed with titrated concentrations ranging from 0.01 to 100 µg/ml. Consistent with the affinity improvement of the 1b34 E2 antigen, the HC-1 affinity-matured mutants also expressed improved neutralization activity against HCVpp1b34. Compared with WT HC-1, which had an IC$_{50}$ value of 47.78 µg/ml against HCVpp1b34, the affinity-matured IgG exhibited an IC$_{50}$ in a much lower range of 1.61 to 3.86 µg/ml. The HC-1 mutant Vk1.9 showed the best neutralization potency, with an IC$_{50}$ of 1.61 µg/ml. This is a reduction of 30-fold as compared with WT HC-1 (Fig. 5C). The improvement of VH1.48 and Vk1.9 indicate that both the VH and Vκ chains of HC-1 contribute to neutralization as well as to binding E2. We also noticed that although the $K_D$ of VH1.48 differed considerably from that of other mutants, the neutralization activity did not show much difference. This suggests that beyond a certain range there is no apparent correlation between the actual $K_D$ and the IC$_{50}$. Similar experiments were carried out with other 1a and 1b HCVpp isolates. Although we only performed the affinity maturation on subtype 1b34, we also observed a marked improvement of neutralization by affinity-matured HC-1 IgG against all of the tested isolates. Interestingly, HCVpp 1A20.8, previously not neutralized by WT HC-1, was neutralized and bound more effectively by all of the affinity-matured IgG except Vk1.9 (Fig. 5, A and B). A summary of the results in the form of a comparison of neutralization IC$_{50}$ of the affinity-matured antibody to the parental HC-1 is presented in Fig. 5C. Overall, VHVk3.64 was the best clone with respect to neutralization potency and breadth. We also assessed the neutralizing activity of the affinity-matured HC-1 IgG on the genotype 2a HCVcc (45, 46). The neutralizing capability was determined by the inhibition of JFH1 E2 expression in infected Huh7.5 cells detected by a GNA capture ELISA of the cell lysate. The neutralization IC$_{50}$ value for WT HC-1 against 2a HCVcc was 0.05 µg/ml. In comparison with the WT, all of the affinity-matured HC-1 IgG demonstrated improved neutralization, as shown in Fig. 5D and summarized in Fig. 5E. The most potent antibody for 2a HCVcc neutralization was VHVk3.64, which showed a greater than 5-fold reduction in the IC$_{50}$ and a 9-fold reduction in the IC$_{90}$ value.

Of note is the maximum neutralization observed against 1a20.8 HCVpp with HC-1 affinity-matured mutants at 100 µg/ml is at 80% (Fig. 5B), although the IC$_{90}$ values against 2a HCVcc is <0.1 µg/ml (Fig. 5E), with maximum neutralization at 100% (Fig. 5D). A significant contribution to the different neutralization activities is that they are against two different isolates. However, we have observed similar difference IC$_{50}$ values between HCVpp and HCVcc of the same isolate, in which greater sensitivity and maximum neutralization are observed with HCVcc (19). The basis for the global differences between HCVcc and HCVpp is not known but could reflect differences in how these particles are assembled and released, leading to differences in the relative copy number of the incorporated viral glycoproteins, in amino acid alignment, and in altered accessibility of these epitopes. This could include differences in E2 glycosylation, with HCVpp and HCVcc affecting the surface exposure of their epitopes.

Breadth of Neutralization against Different HCV Genotypes—To better evaluate the breadth of reactivity of the HC-1 affinity-matured mutants, we investigated HCVpp bearing E1E2 glycoprotein from different HCV genotypes. An HCVpp panel of genotypes 2a, 2b, 3a, 4, and 5 was studied for binding and neutralization. The three most potent mutants, VH1.48-Vk1.9, VHVk3.42, and VHVk3.64, along with the WT HC-1, were chosen for this investigation. The ELISA data on binding activity showed that all of the affinity-matured IgG possessed significantly improved binding to the UKN panel compared with the parental HC-1 (Fig. 6A). Similarly, for neutralization, the affinity-matured IgG showed markedly improved potency relative to the WT HC-1 on all of the genotypes, as exemplified by reactivity against UKN4.11 HCVpp (Fig. 6B). The best clone of improvement was VHVk3.42. It decreased the IC$_{50}$ for HCVpp UKN3a by more than 15-fold and close to 10-fold for UKN4.11. It is worth noting that, although the VH1.48-Vk1.9 clones showed obvious improvement in the binding of UKN3a and UKN2a, the neutralization potency did not improve correspondingly. The binding of these affinity-matured IgG was also tested by GNA capture ELISA of the lysate, with E1E2 representing these genotypes. A summary of the results in the form of a comparison of neutralization activity and binding of the affinity-matured HC-1 IgG to the WT is presented in Fig. 6C.

HCVcc Escape from HC-1 and Related Affinity-matured Clones—Because of the rapid evolution of HCV quasispecies, a significant challenge for antibody-mediated neutralization is the emergence of resistant HCV escape mutants. We implemented an approach utilizing an in vitro escape selection designed to maximize the likelihood of escape variants by subjecting HCVcc to increasing concentrations of the selection antibody from 0.05 to 100 µg/ml (25). R04, an hmAb to human
cytomegalovirus, was used as a control in this study. Triple color fluorescence staining, which included HC-1, anti-NS3, and Hoechst 33342, was employed to observe virus infection and escape (Fig. 7A and B). Cells infected by virus were revealed by fluorescence-labeled anti-NS3 staining, and the escape mutants would lose HC-1 staining. When WT HC-1 reached 10^8 cells/ml, 2–3% of the cells were infected by virus without evidence of escape after three rounds of selection, i.e., cells stained with anti-NS3 and with HC-1 (Fig. 7A). After the fourth round of selection at 10 µg/ml, no infected cells were observed, indicating complete virus elimination (Fig. 7D). We subjected the virus that survived at 10 µg/ml HC-1 to higher concentrations ranging from 10 to 100 µg/ml. The results revealed that, in the presence of ≥40 µg/ml WT HC-1, complete virus elimination occurred (Fig. 7C). We also observed that at 10 µg/ml HC-1 to repetitive rounds of antibody-neutralization followed by amplification of the surviving virus by passage of infected cells in the absence of HC-1. At the third round, cell infectivity still remained at 2–3%. The cells treated with R04 (10 µg/ml) showed >80% infectivity (Fig. 7D). No HC-1 escape mutants were observed during the whole course of this selection, as all the NS3-positive cells could be detected by HC-1. For comparison, two affinity-matured HC-1 IgG, VH1.48-Vk1.9 and VHVk3.64, were chosen and subjected to the same study. No virus survival could be detected in cells at a concentration of 1 µg/ml for either IgG (Fig. 7C). Lowering the HC-1 variant IgG concentration to 0.1 µg/ml resulted in virus infection of 4–5% of the cells. But after several passages, complete virus elimination was achieved. A comparison of the WT and affinity-matured mutants with respect to HCVcc escape is presented in Fig. 7D.
DISCUSSION

Broadly neutralizing antibodies to HCV are usually directed against conformational epitopes on the E2 glycoprotein that contain contact residues within the CD81 binding regions (20, 22–24, 47, 48). For immunotherapeutic applications, a critical question is the likelihood that immune pressure from the selected antibody could lead to viral escape. For example, CBH-2 is another hmAb recognizing a broadly conserved epitope in domain B that contains residues Gly-530 and Asp-535 (47). However, a single amino acid substitution at residue 431 in a naturally occurring variant located at a considerable distance from other CBH-2 contact residues, located at positions 530 and 535, results in complete escape from CBH-2-mediated neutralization in a genotype 1aH77c virus (47). In addition, these domain B antibodies exhibited different neutralization patterns against a panel of sequential HCV variants isolated from a patient over several decades of chronic hepatitis (36, 47). Some hmAbs consistently neutralized the sequential isolates, whereas others, including CBH-2, neutralized with waxing and waning patterns suggestive of virus escape from neutralization. In this study, HC-1 consistently neutralized the sequential HCV variants (36, 47). (Note that HC-2, as identified in our earlier studies (36, 47) is the same antibody as HC-1 by Ig sequence analysis (data not shown).) Furthermore, HC-1 is not associated with escape viral variants (25). We therefore chose HC-1 for affinity maturation by random mutagenesis via yeast surface display. Several clones with improved binding affinity relative to WT were isolated. The higher affinity resulted in the improvement of neutralization potency and breadth against both HCVpp and HCVcc. While investigating HC-1 neutralization against different genotype 1a and 1b isolates, we observed significant variation in the neutralization potency and binding affinity. Notably, HC-1 failed to neutralize HCV 1A20.8 and poorly neutralized HCVpp 1b34. Interestingly, the neutralization efficiency was correlated with the binding affinity.

We employed a strategy of mutating in parallel the VH and Vk chains of the HC-1 IgG separately and subsequently combining the optimized mutants. For this purpose, three different yeast mutagenic libraries were generated and screened for improved affinity using HCV 1b34 E2 antigen in a range of dilutions. Several clones with improved affinity were isolated from all three libraries. Sequence analysis showed that mutations in both the VH and Vk regions similarly contributed to affinity improvement. For the VH clone, G100R in the VH CDR3 and T56I in the VH CDR2 were the sites of consensus change (supplemental Table S1); for Vk, the relevant residue was S31R at Vk-CDR1 (supplemental Table S2). The selected HC-1 mutants, VH.1.48 and Vk1.9, also contained these mutations. Although combining the affinity-improved VH and Vk mutants might produce an augmented binding effect, it could also lower the affinity, as observed in a study by other researchers (49). This decrease in affinity is possible because the beneficial mutations in VH or Vk may not match each other, and combining them thus ultimately compromises the affinity. This was not the case when VH.1.48 and Vk.1.9 were combined into a single clone, HC-1 VH.1.48-Vk.1.9. The affinity, as measured by flow cytometry, displayed a 58-fold improvement in comparison with the wild type. This corresponded to a 16-fold improvement relative to VH.1.48 alone and a 22-fold improvement over Vk.1.9 alone, indicating the additive contributions of the mutations. Moreover, our strategy of constructing the combination VHVk-Mut library (Figs. 2C and 3A) followed by selecting for

![Figure 6](image_url)

**FIGURE 6. Reactivity of HC-1 affinity-matured mutants against different HCV genotypes.** A, dose-dependent binding by WT HC-1 and affinity-matured IgG to HCV UKN4.11.1 E1E2 lysate by ELISA. B, dose-dependent neutralization of HCVpp UKN4.11.1 by WT HC-1 and its affinity-matured mutants. For experiments A and B, error bars indicate the range in replicate studies. C, neutralization of different genotypes HCVpp by WT HC-1 and its affinity-matured mutants as determined by IC_{50} values. Binding affinity (K_D) was measured by ELISA against a panel of genotype 2a, 2b, 3a, 4, and 5 HCV E1E2 lysates.
Affinity-improved clones also avoided the pitfall of a possible lowering of affinity when VH and Vk mutants are combined (49). From the VHVk-Mut library, we isolated three clones containing mutations in both VH and Vk, which exhibited further affinity improvement over that achieved by the VH or Vk mutants alone. All of these clones contained the consensus mutations found in the VH- or Vk-only mutants, which also verified the additive effect of the consensus mutations.

After conversion of the affinity-matured scFv to IgG, SPR analysis was employed to study the on- and off-rate between these IgG and E2 antigen. Compared with WT, all of the affinity-matured IgG showed some increase in the on-rate, $K_{on}$ (in the best case, a 3.7-fold increase), but the most dramatic change was the slowing of the off-rate, $K_{off}$, which ranged from 4.2- to 12.7-fold slower than with WT. To confirm the affinity measurements, another solution affinity method, KinExA, was used. This method enabled us to calculate the functional E2 percentage in the total E2, and the result showed that only 3% was active in the purified E2. This brings into question the validity of the affinity measurements by flow cytometry and SPR, as they were performed against a combination of native and misfolded E2 in the purified E2 preparation. Nonetheless, the results from KinExA confirmed the substantial improvement in binding affinity of the affinity-matured clones. Again, the most dramatic difference was the slowing of the $K_{off}$. HC-1.VHVk.42 showed a 351-fold improvement over WT HC-1. Because HC-1 was found in a patient with a comparably long and well controlled HCV infection, it might have undergone long-term somatic hypermutation of the natural in vivo maturation (20, 22). Here, using the in vitro affinity maturation method, we further improved its affinity for E2.

In a study of affinity-matured Fab clones of a neutralizing Fab to HIV by targeted diversification of the CDR-H2 loop, it was reported that for affinity-matured mutants, not all mutants with improved affinity showed improved neutralization activities (50). For our study, all matured HC-1 IgG clones with affinity improvements also exhibited improved neutralization potencies against HCVpp 1b34. The best clone improved about 30-fold as evaluated by IC50. In determining the neutralization profile of the HCV 1a and 1b subtypes on the panel of HCVpp,
we observed that the affinity-matured IgG exhibited an improvement in broad neutralization against other subtypes. Even compared with the successful neutralization of HCV 1a and 1b isolates by WT HC-1, the affinity-matured clone had about 3–5-fold lower IC₅₀ values. More impressively, HCV 1A20.8, which was not neutralized by WT HC-1, was neutralized by the affinity-matured IgG clones. It is interesting to note that Vk1.9 barely showed improvement in neutralization against HCV 1A20.8, although for the majority of isolates the Vk1.9 clone showed improved neutralization. The results agreed well with the data on biochemical binding to HCV 1A20.8 E1E2 cell lysate in which Vk1.9, the same as WT, did not show binding. When we examined neutralization across all of the HCV genotypes, almost all of the affinity-matured antibodies exhibited improvement over the WT. This is consistent with the improvement in the binding. In addition, we investigated the effectiveness of the affinity-matured HC-1 IgG in neutralizing the HCVcc system. Again, the HC-1 mutants showed improvement in neutralization relative to WT. In summary, the broad reactivity of human HC-1 affinity-matured antibodies was observed in binding and neutralization assays against different isolates of HCVpp and HCVcc.

Because of the rapid evolution of HCV quasispecies, another challenge for antibody-based therapy is the emergence of resistant HCV escape mutants. Mutation arising in the neutralization epitope may lead the virus to escape control by neutralizing antibodies. To obtain new insights on the mechanisms of escape, a method was implemented that allowed escape variants to be amplified for detection by propagating infectious 2a HCVcc under increasing concentrations of a neutralizing antibody. We believe that this approach appears to recapitulate the evolution of viral antigenic determinants under immune pressure in humans. In a separate study that included CBH-2, one of the escape mutants from CBH-2 contained a mutation at D431G (25). The induction of the escape mutant at D431G observed naturally occurring variant at this site, D431E, that was not neutralized by this antibody (47). Propagating 2a HCVcc under the selective pressure of WT HC-1 or affinity-matured HC-1 antibodies yielded no viral escape mutants. More importantly, both WT HC-1 and affinity-matured HC-1 at critical concentrations eliminated the virus. Furthermore, the affinity-matured IgG HC-1 needed 100-fold less antibody concentration to achieve complete virus elimination. Finally, these findings indicate that the HC-1 epitope is highly conserved across different HCV genotypes and subtypes. These characteristics make the HC-1 epitope of interest for vaccine design. One issue, which will be addressed in future studies, is whether all of the affinity-matured clones kept the same contact points as the WT or whether there was some epitope shifting during the affinity-maturation process. In summary, the HC-1 affinity-matured antibodies characterized in this article are potential candidates, singly or in combination with other human anti-HCV antibodies, for passive immunization to complement other pharmaceutical measures against HCV infection.

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