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# The Molecular Basis of Antibody Mediated Neutralization of Hepatitis C Virus

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **HCV** | Hepatitis C Virus |
| **E1** | Envelope glycoprotein 1 |
| **E2** | Envelope glycoprotein 2 |
| **Nonstructural** | NS |
| **IRES** | Internal Ribosomal Entry Sequence |
| **NTR** | Non-translated Region |
| **ER** | endoplasmic reticulum |
| **HCVpp** | HCV pseudotyped particles |
| **HCVcc** | cell culture derived HCV |
| **JFH1** | Fulminant cell culture strain of HCV |
| **H77** | Genotype 1 strain of HCV |
| **CLND1** | Claudin-1 |
| **OCLN** | Occludin |
| **SR-B1** | Scavenger Receptor B1 |
| **LDL-R** | Low density lipoprotein receptor |
| **LEL** | Large Extracellular Loop |
| **sE2** | recombinant, soluble E2 |
| **LDL** | low-density lipoprotein |
| **HDL** | high-density lipoprotein |
| **VLDL** | very low-density lipoprotein |
| **HVR1** | Hypervariable Region 1 |
| **HVR2** | Hypervariable Region 2 |
| **EC1** | Extracellular Loop 1 |
| **EC2** | Extracellular Loop 2 |
| **EGFR** | Epidermal Growth Factor Receptor |
| **igVR** | intergenotypic variable region |

| | |
|---|---|
| **DI** | Domain 1 |
| **DII** | Domain 2 |
| **DIII** | Domain 3 |
| **WNV** | West Nile Virus |
| **MAb** | Monoclonal Antibody |
| **HuMAb** | humanized antibody produced in genetically engineered mice |
| **FAb** | Fragment, antigen binding |
| **WT** | wild type |
| **uPA** | urokinase-type plasminogen activator |

ABSTRACT OF THE DISSERTATION

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Hepatitis C virus (HCV) is positive strand, blood-borne, hepatotropic RNA virus that causes chronic infection in ~170 million people worldwide and is the leading cause of liver transplantation in the United States. HCV entry and attachment is mediated by the envelope protein E2 through interaction with several cellular receptors including CD81, scavenger receptor B1 (SRB-1), claudin-1, and occludin, although the exact mechanism by which these receptors facilitate infection remains unclear, largely due to the absence of a structural model of E2. The production of neutralizing antibodies against E2 is thought to be important for controlling HCV infection, likely by blocking virus interaction with these receptors. To better understand the structural and molecular basis of antibody neutralization of HCV, which could be used to inform novel therapeutic or vaccine approaches, we generated a panel of 78 monoclonal antibodies (MAbs) against the E2 protein from HCV genotypes 1 and 2 and assessed their neutralizing activity in vitro. Using this approach and by performing mechanistic studies, we identified three neutralizing MAbs, H77.16, H77.39, and J6.36, that inhibit infection at a post-attachment step. Using a yeast display library of E2 protein variants, we mapped the critical binding residues of these MAbs to distinct regions of the E2 protein: H77.16 binds within the HVR1 and to a conserved CD81 binding region ~125 amino acid residues C-terminal to the HVR1; H77.39 binds to conserved

residues upstream of the hypervariable region (HVR1); and J6.36 binds to amino acid residues

within HVR1 as well a site ~150 amino acids C-terminal to HVR1. Receptor-binding inhibition studies using E2 demonstrated that H77.16 potently inhibits binding to SR-B1, H77.39 potently inhibits binding to SR-B1 and CD81, and J6.36 potently inhibits binding to SR-B1 and modestly inhibits binding to CD81. Further mechanistic studies demonstrated that MAb-mediated neutralization could be enhanced by increases in pre-incubation temperature and time and that these results were likely due to altered epitope exposure on the viral surface. Together, these data provide new insight into the mechanisms by which antibodies neutralize infection of HCV.

Chapter 1

Introduction

**ABSTRACT**

Hepatitis C virus (HCV) is significant cause of morbidity and mortality worldwide. Approximately 80% of exposed individuals develop chronic disease, which is associated with an increased risk of cirrhosis and hepatocellular carcinoma. Despite the financial and emotional burden of this disease, there is no vaccine and treatment options remain limited. The role of the immune response in protection from HCV remains unclear, although vaccination and passive transfer studies suggest that antibodies to the HCV envelope 2 (E2) protein prevent infection. This chapter will provide an introduction to HCV, the mechanisms of monoclonal antibody (MAb) neutralization, and what is known about the antibody response to HCV.

*Epidemiology and importance of human disease*

Hepatitis C virus (HCV) is a hepatotropic RNA virus that infects ~170 million people worldwide (18) and is the leading cause of liver transplantation in the United States (3). HCV is a blood borne pathogen, and in the developed world is primarily transmitted by injection drug use. In developing countries, unsafe therapeutic injections and transfusion with contaminated blood are the main sources of HCV transmission (143). The first six months of HCV infection is considered the period of "acute," often asymptomatic, infection. During this time, ~ 20% of individuals will spontaneously clear infection, whereas the remaining ~ 80% will progress to chronic disease(66), which is associated with an increased risk of cirrhosis and hepatocellular carcinoma (18). Until recently, the only treatment available was a combination of ribavirin and pegylated interferon-$\alpha$, which had a response rate of ~50%, depending on the genotype of infection (45). Recent addition of NS3/4A protease inhibitors (boceprevir and telaprevir) improved response rates in clinical trials, although an increase in side effects also was observed (4, 67, 130, 169). Currently no vaccine is available, the development of which could be invaluable as a prophylactic or therapeutic in high-risk populations or resource-poor countries (146).

HCV is divided into seven genotypes, which share ~30% nucleotide identity. These genotypes are further divided into subtypes, which share ~20% nucleotide identity (7, 53, 145). Furthermore, quasispecies develop in infected hosts as a result of immune pressure, a highly error prone polymerase, and high viral burden (22). Distribution of genotypes is largely geographical, with genotypes 1 and 2 being the most prevalent in the United States(7). Response rates to treatment were previously highly genotype dependent, with genotype 1 being the least responsive to therapy(45), although the new NS3/4A protease inhibitors have dramatically improve response rates in genotype 1 infected patients(4, 67, 130, 169).

*Virus structure, replication and assembly*

HCV is a 9.6kb, positive stranded RNA virus in the family *Flaviviridae*, in the genus *Hepacivirus* (86). HCV is comprised of three structural proteins (core, E1, E2) p7, an ion channel,

and six non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (88). The nonstructural proteins perform many functions, including (i) combining to serve as viral proteases (NS2/NS3, NS3/NS4A), and a helicase (NS3/NS4A); (ii) inhibiting the host immune response (NS3/NS4A, NS5A); (iii) formation of the replication complex in the ER (NS4B); (iv) facilitation of particle replication and assembly (NS5A)(73, 104, 133, 150). The RNA polymerase, NS5B, lacks intrinsic proofreading activity and is thus highly error-prone(104).

HCV virus enters cells via endocytosis through clathrin-coated pits (100) and fuses with the endosomal membrane in a pH dependent manner to release the viral genome into the cytoplasm (13, 17, 25, 64, 79, 80, 153). Several attachment factors have been implicated in HCV entry (see below). After viral fusion, translation of the HCV polyprotein occurs via an internal ribosomal entry sequence (IRES) located in the 5' non-translated region (NTR). The HCV polyprotein is cleaved by both host and viral proteases (104), and the viral non-structural proteins form a replication complex in association with the endoplasmic reticulum (ER) and lipid droplets (108). With the development of an infectious HCV system in tissue culture, the dynamics of viral particle assembly and release are beginning to be studied.

A precise structural understanding of the mature virion remains elusive, largely due to the lack of envelope protein crystal structures as well as the difficulty in producing sufficient quantities of HCV virions for use in cryo-electron microscopy (cryo-EM). One study overcame the latter limitation by using a highly efficient cell-culture adapted strain, which increased titers by ~ 100-fold, combined this with density gradient ultracentrifugation to produce high titer viral stocks. Low-resolution cryo-EM revealed small, spherical particles of about 50 nm in diameter (50). The range of particle sizes, (~31 to >100nm), as well as the average diameter, was consistent with previous analyses of sera from infected patients (70, 85, 131) and cell culture infectious virus (158, 166). Analysis of the cryo-EM data suggests that the range of sizes corresponds to differences in particle populations; smaller particles were non-enveloped and had limited infectivity, particles with the highest infectivity were enveloped and fell into a mid-size range, and rare populations of large particles were multivesicular and less infectious (50).

*Models of HCV Infection*

For many years, the absence of an *in vitro* model for HCV has restricted the study of HCV attachment and entry. Over the past decade, two models for studying HCV infection *in vitro* have been developed: HCV pseudotyped particles (HCVpp) and a cell culture-derived HCV (HCVcc) infectious clone. HCVpp are generated by co-transfection of expression constructs containing the retroviral genes *gag* and *pol,* a marker gene (e.g. Luciferase or GFP), and HCV envelope proteins E1 and E2, into 293T cells. The retroviral genes and marker genes are packaged by the HCV envelope proteins and released into the cell supernatant; HCVpp are capable of sustaining a single round of infection in hepatoma cells (11, 64, 155). The first full-length infectious HCV clone that produced particles capable of full lifecycle infection was derived from a Japanese patient with fulminant hepatitis (JFH-1 strain, genotype 2a)(87, 159). A chimeric J6/JFH1 strain of virus, which replaces JFH-1 core to NS2 with J6 strain (genotype 2a) proteins, produces higher titers of virus and is commonly used in laboratory research (reviewed in (6)). Recently, chimeric viruses expressing structural proteins from genotypes 1, 3, 4, 5, 6 and 7 on a JFH1 backbone with cell culture compensatory mutations have been described (52, 53, 68, 94, 126, 140, 164).  Progress is also being made in the development of a system to study infection in primary hepatocytes, although this system is still limited by a lack of long-term survival and proliferation in culture (129).

To date, a high-throughput, readily tractable small animal model for HCV infection does not exist. In one model, SCID mice are bred to transgenically express urokinase-type plasminogen activator (uPA).  Expression of uPA results in destruction of murine hepatocytes and allows for engraftment of human hepatocytes, which can sustain HCV infection for ~4 weeks (reviewed in (8).  In another model, wild type (WT) mice were lethally irradiated and transplanted with SCID bone marrow and subsequently engrafted with human hepatocytes and infected with virus (65).  Although useful in assessing antivirals, both models require the use of immunodeficient mice, thus precluding studies of the host immune response to HCV.

Advances in the development of an immunologically intact mouse infectious model have come with the identification of claudin-1(CLDN-1) (42) and occludin (OCLN) (128) as critical HCV entry factors; expression of human versions CLDN-1, OCLN, CD81, and scavenger receptor B1 (SR-B1) rendered murine cell lines susceptible to infection(128). Further studies demonstrated that species-specific tropism for human compared to murine cells is determined by expression of human CD81 and human OCLN, but not SR-B1 and CLDN-1 (30, 128). Indeed, adenoviral vectors expressing all four human receptors in immunocompetent mice allowed for entry, but not replication, of cell culture HCV in immunologically intact mice (30). Adaptation of cell culture derived HCV to murine CD81 also has allowed for entry into murine hepatocytes, although only after expression of murine or human versions of SR-B1, CLDN-1 and OCLN, and replication within these cells was not assessed (16).

The only natural animal model for HCV is the chimpanzee. Although chimpanzees are susceptible to HCV, the cost and ethical considerations associated with these animals makes studies costly and challenging. Additionally, unlike humans, only ~40% of chimpanzees progress to chronic HCV, as opposed to ~80% of human subjects, and they do not develop liver disease (54), making vaccination and therapeutic studies difficult to interpret.

**Receptors, Attachment, and Entry**

Despite intense study by multiple groups over the past few years, the precise mechanism of viral attachment and entry remains unclear. Several receptors, including CD81, SR-B1, CLDN-1, OCLN, glycosaminoglycans, and the low-density lipoprotein receptor (LDL-R), have been suggested as important in HCV attachment and entry. The following paragraphs will review these receptors and their function in HCV life cycle.

*CD81*

CD81 is a ubiquitously expressed membrane tetraspanin protein that is thought to interact within a "tetraspanin web" to modulate cell signaling and changes in morphology

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associated with adhesion, synapse formation, fusion, endocytosis and exocytosis. The structure of CD81 has a small extracellular loop between transmembrane domains 1 and 2, and a large extracellular loop (LEL) between transmembrane domains 3 and 4 (reviewed in (82)). Structurally, the LEL is thought to form a "mushroom" shape, with two helices, A and E, forming the "stalk" of the mushroom, and three helices, B, C, and D, forming the head (74). Helices C and D are the most divergent sequences in CD81, distinguishing human CD81 from other tetraspanins and from CD81 found in other species (82).

In 1998, Abrignani's group showed that recombinant, soluble HCV E2 (sE2) bound the (LEL) of human CD81, providing the first description of an HCV receptor (127). Since the initial publication, the ability of sE2 to bind to the LEL of human CD81 has been verified (39, 47, 48, 57, 63, 118, 136). Residue F186 on CD81 is an important residue in the LEL for mediating E2 binding (15, 39, 47, 63), and several other residues (I181, I182, L185, W163, K171, E188, and D196) have all been implicated in E2 binding (15, 39, 47). CD81 is necessary, but not sufficient, for viral entry (13, 26), suggesting that other receptors participate in viral entry. Indeed, it has been proposed that the kinetics of CD81 internalization are too slow to permit viral entry alone (15, 105, 123, 148), further substantiating that HCV infection requires multiple receptors and attachment factors.


*SR-B1*

SR-B1 is a 509 amino acid transmembrane protein that is expressed in a variety of tissues, including the liver, where it is found on nearly all cell types, including hepatocytes, Kupffer cells and sinusoidal epithelium. Structurally, SR-B1 is composed of two transmembrane domains that are bridged by a 407aa extracellular domain of unknown structure. The extracellular domain contains 11 and 9 N-linked glycosylation sites in mice and humans, respectively, which may be important in providing a negatively charged environment to assist with ligand recognition. SR-B1 interacts with a variety of ligands, including low density lipoprotein (LDL), oxidized LDL, acylated LDL, very low density lipoprotein (VLDL), and high density lipoprotein (HDL), and is

thought to be particularly important for the uptake of cholesteryl esters and free cholesterol from HDL (134).

SR-B1 has been shown to bind soluble, recombinant E2 (46, 95, 139), likely via the E2 hypervariable region 1 (HVR1) (46, 139).  SR-B1 also may play a role in the enhancement of HCV infection by HDL, although the mechanism for this phenomenon remains unknown (12, 34, 36, 156, 168).  Recent data has shown that both the C-terminal cytoplasmic tail (35) as well as the extracellular domain of SR-B1 are important in HCV entry (35, 168). Unlike CD81, HCV can utilize both the murine and human SR-B1 receptor for entry (30, 128).

*Claudin-1*

CLDN-1 is a four transmembrane domain tight junction protein that is important in forming a permeability barrier at the apical side of epithelial cells. CLDN-1 contains a large and small extracellular loop (EC1 and EC2, respectively) (42, 141). CLDN-1 co-localizes with CD81, likely via residues I32 and E48 in the EC1 (42, 59, 60), and is believed to act at a post-attachment step (42, 77). Furthermore, inhibition of CD81, SR-B1 and CLDN-1 results in synergistic blockade of infection as compared to inhibition of the individual receptors, suggesting that all three receptors act in a coordinated manner to promote infection (77).  Original reports suggested that CLDN-1 directly bound E1, E2 and E1-E2 (163), but these results have since been challenged (77). Despite a lack of evidence for direct interaction with sE2, blockade of CLDN-1 with anti-CLDN-1 antibodies resulted in decreased binding of E2 and infectious virus to Huh7.5 cells (77), suggesting that CLDN-1 may stabilize HCV interactions with other receptors.

*Occludin*

Like CLDN-1, OCLN is also a four transmembrane tight junction protein with two extracellular loops (141). Occludin was the last receptor identified that appears to be required for HCV entry, as co-transfection of CD81, SR-B1, CLDN-1 and OCLN rendered non-human and non-hepatocyte cell lines permissive to HCV infection. Interestingly, further analysis

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demonstrated that the species specificity of HCV was determined by human OCLN and CD81, but not human SR-B1 and CLDN-1 (30, 103, 128); species specificity of OCLN has been localized to residues A223 and A224 in the second extracellular loop (EC2) (103, 128). OCLN does not appear to directly interact with sE2 (89), but does co-immunoprecipitate structural proteins associated with infectious virus(90).

*Other potential receptors*

In addition to the four well-characterized receptors described above, additional receptors and attachment factors have been suggested to play a role in HCV attachment and entry. These receptors will be reviewed briefly below.

*Heparan Sulfate*. Heparan sulfate is an anionic glycosaminoglycan that is ubiquitously expressed (14). E1 (10), E2 (9) and E1-E2 (154) have been suggested to bind heparin, and E2 binding has been localized to the HVR1, residues within the CD81 binding region (residues 480-487 and 516-530) and residues 412-423 (10). In contrast to SR-B1 and CD81, inhibition studies have shown that heparin blocks infection at a pre-attachment step (56, 93), leading to speculation that heparan sulfate is involved in initial attachment to hepatocytes (21).

*Low Density Lipoprotein Receptor*. Although not one of the four "minimal" receptors required for HCV infection of non-permissive cell lines, low-density lipoprotein receptor (LDL-R) has also been implicated in HCV entry (reviewed in (21)). Recent studies have demonstrated that knockdown of LDL-R reduces HCVcc infection in Huh7.5 cells, whereas over-expression enhances infection (112). Nonetheless, the precise role in attachment and entry remains unknown, although it has been suggested that HCV may utilize LDL-R mediated endocytosis for cell entry (1).

*Epidermal Growth Factor Receptor*. Recently, the Epidermal Growth Factor Receptor (EGFR) was shown to be important HCV for entry. Although unable to bind E2 directly, EGFR was vital for HCVcc infection at a post-binding step, likely by promoting CD81-CLDN-1 interactions, and/or promoting fusion via kinase signaling (93). This receptor, however, does not

limit species-specific infectivity (30, 128), as the human version is not required for infection of murine cells.

*Additional attachment/entry factors*. The C-type lectins L-SIGN and D-SIGN bind E2 protein with high affinity. As such, it has been postulated that these receptors are involved in initial attachment to the sinusoidal endothelial cells prior to transfer to hepatocytes, although direct evidence to support this hypothesis does not exist (reviewed in (167)). Two reports also demonstrate that the tight junction proteins claudin-6 and claudin-9 act as entry co-factors(99, 170), although these results were more significant with HCVpp compared to HCVcc(99). Furthermore, a species-specific requirement for these receptors has not been demonstrated (30, 128)

*The role of apolipoproteins in HCV attachment and entry*

Apolipoproteins are small serum proteins that are associated with lipoproteins, including chylomicrons, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL). Lipoproteins consist of a lipid core surrounded by cholesterol, apolipoproteins, and phospholipids, and are important for transporting triglycerides to host cells and returning excess cholesterol to the liver for excretion (132). Recent studies have suggested that lipoproteins, as well as their associated apolipoproteins, may contribute to HCV virus attachment and entry.

HCV particles generated from serum, when purified on a sucrose density gradient, exhibit heterogeneous density and are most infectious if taken from the lower density fraction (71). As the low-density fractions often contain serum lipoproteins, it was hypothesized that HCV might interact with these components in such a way that augmented infectivity (7, 58). Recent studies have confirmed that infectious HCV interacts directly with apolipoprotein C1 (ApoC1) and apolipoprotein E (ApoE)(102, 112), and that human serum, HDL and ApoC1 enhance HCVpp infection in an SR-B1 dependent fashion (12, 34, 36, 101, 156).

*HCV attachment and entry: a summary*

HCV infection likely requires multiple host attachment factors and receptors acting in concert to allow for internalization of the virus, such as occurs with Coxsackie B virus infection (97). One model of HCV entry involves attachment via heparan sulfate, followed by interactions with CD81, SR-B1, CLDN-1 and OCLN (reviewed in (167)). Indeed, experimental studies have demonstrated that CD81, SR-B1 and CLDN-1 act cooperatively at early post-attachment steps (77). OCLN likely acts at a post-attachment step as well, although its exact function remains poorly understood (reviewed in (167)).  This complex model series of interactions may be necessary to expose the CD81 binding site on E2, which is thought to be obscured by N-linked glycans (62) and the HVR1 (5), in a manner similar to exposure of the CCR5 site of HIV after engagement of the CD4 receptor (78, 135). Which, if any, of these receptors mediates viral internalization remains unknown.  Although further study is needed, recent data suggests that tyrosine kinases, including EGFR, may initiate signaling events required for viral endocytosis (93).

Similar to flaviviruses (86), HCV is thought to undergo clathrin-mediated endocytosis (99) into an endosomal compartment whereby low pH triggers envelope rearrangements required for fusion and release of viral contents into the cytoplasm (11, 17, 25, 64, 79, 80, 153). In contrast to flaviviruses(147), HCV is unable to undergo "fusion from without," indicating both a high degree of virus stability and that steps in the entry pathway are important for priming the endosomal fusion event (100, 153).  Viral fusion is classified structurally into two groups: i) type I fusion requires proteolytic cleavage and rearrangement of a stalk-like protein trimer to expose the fusion peptide, as is seen for the hemagglutanin protein of influenza; ii) type II fusion involves a pH-dependent rearrangement of E protein dimers to form a trimer that exposes the fusion loop, as is seen in flaviviruses and alphaviruses (61).  HCV is predicted to utilize type II fusion based on its genome arrangement and relationship to flaviviruses (40, 76), although experimental support for this hypothesis is lacking.

**E2 glycoprotein**

E2 is a type I membrane glycoprotein (109) that spans amino acids 384-746 of the viral polyprotein(37) and is predicted to be exposed on the surface of the HCV virion (166). Full length E2 protein contains 18 cysteines, which form nine intramolecular disulfide bonds (76), 11 potential N-linked glycosylation sites (51), and three hypervariable regions (HVR1, HVR2, and the intergenotypic variable region (igVR)) (98, 160). E2 also contains both the CD81 binding domain (127), the SR-B1 binding domain (139), and interacts with heparin (9). While prior studies suggested that E1 and E2 formed a non-covalent heterodimer (29, 38, 41, 110, 111, 162), these studies were performed largely with intracellular E1 and E2; a recent study of HCVcc suggests that E1 and E2 are covalently linked on the mature virion (154).

The HVR1 spans the first 27 amino acids (384-411) of the E2 protein (118, 160) and is thought to facilitate viral immune escape from the host humoral response (161), mediate SR-B1-binding (139), and shield the CD81 binding region(5). Antibodies directed to the HVR1 can limit infection (44, 55, 137, 144, 171), however immune pressure generated by anti-HVR1 antibodies also drives mutation in this region, leading to the selection of viral escape mutants (32, 44, 144, 161). Despite the increased propensity for mutation, the conservation of highly basic residues within the HVR1 is critical for viral infectivity, suggesting that HVR1 interacts with anionic molecules during viral attachment and entry(119).

The CD81 binding domain on E2 is comprised of several discontinuous segments between amino acids 396-618, although the exact amino acids contacts remain unclear, with conflicting data in the literature (37, 47, 113, 136, 162). Direct mutational analysis of E1-E2 suggests that the highly conserved residues W420, Y527, W529, G530 and T535 are minimally required for CD81 binding (116), although others may exist. Indeed, some have predicted that the HVR1(113, 136) as well as a short motif at G436 (G436WLAGFLY) (37) participate in CD81-virus binding. The CD81 binding region is well protected on the surface of the virion, as it is shielded by N-linked glycosylations(62).

Despite intense efforts by academic and pharmaceutical laboratories, the crystal structure of E2 remains unsolved. A recent predictive model based on experimental determination of disulfide bonds and the structure of other type II fusion proteins suggests that E2 adopts a three-domain structure similar to flaviviruses.  In this model, the putative domain I (DI) is sandwiched between domain II (DII) and domain III (DIII) and is composed of two overlapping β-sheets with the HVR1 extending from the amino-terminus as an unstructured segment. In this structure, the CD81 binding residues are predicted to localize to the top β-sheet of DI and the amino terminus of DIII.  A group of highly conserved, non-polar residues (502-520) in DII were suggested to form the HCV fusion loop (76), although experimental evidence is required to corroborate this assertion.

Due to the importance of E2 in viral entry and attachment, it is not surprising that antibodies against E2 protein confer protective immunity against HCV *in vivo* (23, 121, 137). Many anti-E2 antibodies have been generated *in vitro*, including those with broad cross-reactivity against multiple genotypes, such as AP33 (114). Table 1 lists the characteristics of MAbs generated by other groups; the MAbs generated from my thesis are discussed in detail in Chapter 2.

**Mechanisms of Antibody Neutralization**

The importance of antibodies in controlling viral infection (reviewed in (117)), and flavivirus infection in particular (reviewed in (124)), are well documented. Antibodies protect against infection by direct binding and inhibition of viral infection (neutralization) as well as by various effector functions, including opsonization, complement-mediated virolysis, and antibody dependent cellular cytotoxicity (reviewed in(20, 117)). The following sections will focus on the direct inhibition of viral infectivity by antibody binding and the stoichiometric requirements needed to achieve this inhibition.

*Pre- and Post-attachment neutralization.* Antibodies can neutralize infection prior to viral attachment to the host target cell (pre-attachment) or after viral attachment (post-attachment)

(reviewed in (75)). Pre-attachment inhibition is posited to be due to coating of the virus such that receptor interactions are inhibited either directly or by steric hindrance (20, 75, 117). Post-attachment inhibition can occur at any number of steps in the viral life cycle after attachment, including: i) blockade of binding to additional receptors after viral attachment, as demonstrated by post-attachment inhibition of HCV infection by anti-CD81 and anti-SR-B1 MAbs (15, 56) as well as MAbs that inhibit E2 binding to those receptors (138), ii) prevention of endocytosis, as has been observed for MAbs V5 and E70 against human papilloma virus (28), iii) inhibition of viral fusion, as has been described for MAb E16 antibody against West Nile Virus (WNV) (107, 151). In some cases, antibodies may block both pre- and post-attachment steps. For example, WNVE16 inhibits fusion at low concentrations yet can block attachment at high concentrations (107, 151).

Stoichiometric requirements for neutralization. The "occupancy" or "coating" model of neutralization states that a critical number of viral epitopes must be bound for neutralization to occur (reviewed in (75, 117)). The ability of an antibody to achieve the minimum occupancy requirement is governed by antibody affinity and the availability of the antibody epitope on the surface of the virion. Antibody affinity is the strength with which an antibody binds its epitope and can be expressed as $K_D=[V][MAb]/[VMAb]$, where $K_D$ is the dissociation constant, [V] is the concentration of virus, [MAb] is the concentration of free MAb, and [VMAb] represents the concentration of MAb bound to virus (75). In physical terms, the affinity determines the number of epitopes bound at a given concentration of antibody (33), with the $K_D$ representing the concentration of MAb needed to bind half of the available epitopes (75). The availability of epitopes is governed by the virus structure; potential epitopes can be obscured beneath the surface of the virion (84), by steric hindrance due to packing of molecular units on the viral surface(125), or by N-linked glycosylation of envelope proteins (62). Additionally, cryptic epitopes can be exposed by conformational changes in the virus, for example, the exposure of the CCR5 binding site of HIV after engagement of the CD4 receptor (78, 135). Thus, some epitopes may not be sufficiently available for antibody to engage the virus above the minimum threshold required

for neutralization (31).  Recent evidence, however, does suggest that hidden epitopes may be transiently exposed due to dynamic changes or "breathing" of the virus particle (31, 83, 84, 92).

**Humoral response to HCV infection**

The role of humoral immunity in HCV infection and the mechanism that is most important for viral neutralization *in vivo* remain largely unknown. During acute infection, the appearance of HCV specific antibodies is markedly delayed as compared to other viruses, with seroconversion occurring roughly 6 to 8 weeks after infection (18, 106), although a recent study of intravenous drug users (IDUs) during acute infection suggests that the early humoral response may not be detected due to strain specificity of the initial antibodies produced (32). Detectable antibodies against E1 and E2 glycoproteins usually appear later in infection, sometimes months after seroconversion, and correspond with an increase in neutralizing antibody titers.  Neutralizing titers increase over time (91, 106), and during chronic infection, some patients produce broadly neutralizing antibodies against heterologous strains of virus(91, 101), although the presence of these antibodies does not directly correlate with viral clearance, possibly due to viral escape from the host response(32, 43, 157).

The importance of anti-E2 antibodies in controlling HCV infection has been suggested by vaccination studies in which chimpanzees immunized with recombinant E1-E2 protein developed protective humoral immunity (23, 137).  Additionally, a study of patients accidentally infected with HCV indicated that the development of an early neutralizing response correlated with an increased probability of clearing infection (121). Anti-HCV antibodies also may function as passive protection, and thus, in theory, could be used as post-exposure prophylaxis. Polyclonal immune sera protects chimpanzees from infection either when pre-incubated with virus, (44, 165), or when administered concomitantly with virus (165).  A greater understanding of the mechanism and function of anti-E2 antibodies in controlling HCV infection may guide the production of future therapies and vaccines against HCV.

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**Conclusions and Rationale**

HCV infects ~170 million people worldwide and is a significant cause of morbidity and mortality (18). Due to a lack of a tractable small animal model and, until recently, a cell culture adapted virus, little remains known about the protective humoral immune response against HCV infection. Neutralizing antibodies against the HCV E2 protein are found in infected patients (91, 101, 106), although the mechanisms by which these antibodies act and the epitopes to which they bind remains poorly understood. The objective of this thesis is to study the molecular mechanism of antibody mediated neutralization of HCV by the generation and characterization of monoclonal antibodies (MAbs) against the E2 protein, with the hope that structural and molecular understanding of neutralization will inform strategies for the development of novel therapeutics and vaccines to treat and prevent HCV infection.

**Figure Legends**

**Table 1.1: Summary of MAbs previously generated against HCV E2**

Summary of neutralizing and non-neutralizing MAbs previously generated against HCV E2. The isotype, species in which the MAb was generated, capacity to inhibit binding to CD81, and ability to neutralize are included, where applicable. N/D: Not done in the studies cited. HuMAb mice: humanized antibody produced in genetically engineered mice.

**Table 1.1: Summary of MAbs previously generated against HCV E2**

| Antibody | Epitope | Isotype | Inhibits CD81 binding? | Species | Neutralization | References |
|---|---|---|---|---|---|---|
| AP33 | L413, I414, N415, T416, G418, W420, H421 | N/D | Yes | mouse | Yes; HCVcc; HCVpp of all genotypes | (49, 113, 114, 118, 149) |
| 11/20c | E2 436-447 | IgG2a | Yes | rat | Yes; HCVpp | (47, 64, 113) |
| 6/16 | HVR1 | IgG1 | No | rat | No | (47, 64) |
| 6/82a | HVR1 | IgG1 | No | rat | No | (47, 64) |
| 7/59 | HVR1 . | IgM | No | rat | No | (47, 64) |
| 9/27 | HVR1 | IgG1 | No | rat | Yes; HCVpp; Neutralization not attenuated by HDL | (12, 47, 64, 156) |
| 7/16b | 436-446 | IgG2a | Yes | rat | No | (47, 64) |
| 9/75 | 524-531 | IgG2a | Yes | rat | No | (47, 64) |
| 3/11 | N415, W420, H421 | IgG2a | Yes | rat | Yes; HCVpp | (47, 64, 149) |
| 1/39 | 432-443 | N/D | Yes | rat | Yes; HCVpp | (47, 64) |
| 2/69a | 432-443 | IgG2b | Yes | rat | Yes; HCVpp | (47, 64) |
| 2/64a | 524-531 | N/D | Yes | rat | No | (64, 113) |
| 6/1a | 464-471 | IgG1 | No | rat | No | (47, 64) |
| 6/41a | 480-493 | IgG2a | Yes | rat | No | (47, 64) |
| 6/53 | 544-551 | IgG2a | Conflicting results | rat | No | (47, 64) |
| 9/86a | Conformational; HVR1 | IgG2b | No | rat | No | (64, 113) |
| H52 | 644-655 | N/D | Yes | rat | N/D | (47) |
| H/53 | Conformational epitope | N/D | No | mouse | N/D | (24, 47) |
| H2 | Conformational epitope | N/D | N/D | mouse | N/D | (29) |
| H60 | Conformational epitope | N/D | No | mouse | N/D | (47, 113, 118) |
| AP320 | 464-471 | N/D | No | mouse | N/D | (113, 118) |
| AP109 | 464-475 | N/D | N/D | mouse | N/D | (152) |
| AP266 | 644-651 | N/D | No | mouse | N/D | (113) |
| AP436 | 464-475 | N/D | No | mouse | N/D | (113) |
| ALP98 | 640-653 | N/D | No | mouse | N/D | (113) |
| ALP1 | 648-651 | N/D | No | mouse | N/D | (113) |
| ALP11 | 644-651 | N/D | | mouse | N/D | (113) |
| HCV #1 | N/D | N/D | Yes | human | Yes; HCVpp | (142) |
| HCV #4 | N/D | N/D | Yes | human | Yes; HCVpp | (142) |
| HCV #7 | N/D | N/D | Yes | human | Yes; HCVpp | (142) |
| HCV #12 | N/D | N/D | No | human | N/D | (142) |
| HCV #13 | N/D | N/D | Yes | human | N/D | (142) |
| 1:7 | W529A, G530A,D535A, G523A | IgG1 | Yes | human | Yes; HCVpp of all genotypes; HCVcc (EC50=60ng/ml) | (2, 69) |
| A8 | W529A, G530A,D535A, G523A | IgG1 | Yes | human | Yes; HCVpp of all genotypes; HCVcc (EC50=560ng/ml) | (2, 69) |
| L1 | N/D | IgG1 | N/D | human | Yes; HCVpp No; HCVcc | (2, 69) |
| L3 | N/D | IgG1 | Yes, | human | N/D | (2) |
| A12 | N/D | IgG1 | Yes, | human | N/D | (2) |
| 1:5 | N/D | FAb | Yes | human | N/D | (2) |
| 1:11 | N/D | FAb | Yes | human | N/D | (2) |
| CBH-2 | Conformational; Binds domain "B" | IgG1 | Yes | human | Yes; HCVpp and HCVcc | (57, 72, 115) |
| CBH-4D | Conformational; Binds domain "A" | IgG1 | No | human | No | (57, 72) |
| CBH-4B | Conformational; Binds domain "A" | IgG1 | No | human | No | (57, 72) |

| CBH-4G | Conformational; Binds domain "A" | IgG1 | No | human | No | (57, 72) |
|--------|-----------------------------------|------|-----|-------|------|----------|
| CBH-17 | N/D; recognizes a linear epitope | IgG1 | No | human | N/D | (57, 72) |
| CBH-5 | G523, P525, G530, D535, N540; conformational | IgG1 | Yes | human | Yes; HCVpp and HCVcc | (57, 72, 115) |
| CBH-7 | N540, W549; conformational | IgG1 | Yes | human | Yes; HCVpp and HCVcc | (57, 72, 115) |
| CBH-8C | Conformational; Binds domain "B" | IgG1 | Yes | human | No | (57, 72) |
| CBH-8E | Conformational; Binds domain "B" | IgG1 | Yes | human | No | (57)[89] |
| CBH-11 | Conformational; Binds domain "B" | IgG1 | Yes | human | No | (57, 72) |
| H | E2 418-431 | IgG1 | N/D | mouse | N/D | (41) |
| All | N/D | IgG1 | N/D | mouse | N/D | (41) |
| I19 | N/D | IgG1 | N/D | mouse | N/D | (41) |
| D32.10 | 297-306 (E1), 480-494 (E2), and 608-622 (E2), | N/D | N/D | mouse | N/D | (122) |
| 4D2 | Conformational | IgG1 | N/D | human | N/D | (27) |
| 7B7 | Conformational | IgG1 | N/D | human | N/D | (27) |
| 1H3 | conformational | IgG1 | N/D | human | N/D | (27) |
| 2H8 | conformational | IgG1 | N/D | human | N/D | (27) |
| 3F12 | conformational | IgG1 | N/D | human | N/D | (27) |
| 2F7 | conformational | IgG1 | N/D | human | N/D | (27) |
| 503 | conformational | IgG1 | Yes | human | N/D | (55) |
| 108 | conformational | IgG1 | No | human | N/D | (55) |
| 291 | N/D | IgG1 | N/D | mouse | N/D | (137) |
| 12AG7 | HVR1 | IgG1 | Yes | mouse | N/D | (137) |
| HCV1 | L413, N415, G418, W420, | IgG3 | N/D | HuMAb mice | Yes; HCVpp from multiple genotypes | (19) |
| 95-2 | L413, N415, W420. | IgG1 | N/D | HuMAb mice | Yes; HCVpp from multiple genotypes | (19) |
| AR2A | N540A | IgG1 | No | Human | Yes; HCVpp and HCVcc | (81) |
| AR3A | S424, G523, P525, G530, D535, N540 | IgG1 | Yes | Human | Yes; HCVpp and HCVcc | (81) |
| AR3B | S424, G530, D535 | IgG1 | Yes | Human | Yes; HCVpp and HCVcc | (81) |
| AR3C | S424, P525, G530, D535, N540 | IgG1 | Yes | Human | Yes; HCVpp and HCVcc | (81) |
| AR3D | S424, G530 | IgG1 | Yes | Human | Yes; HCVpp and HCVcc | (81) |
| AR1A | T416, N417, Y485, V533, N540, W549 | IgG1 | Yes | Human | No | (81) |
| AR1B | N540, P544, P545, G547, W549 | IgG1 | No | Human | No | (81) |
| e20 | W529, G530, D535 | FAb | N/D | Human | Yes; HCVpp and HCVcc | (96) |
| e137 | T416, W420, W529, G530, D535 | FAb | N/D | Human | Yes; HCVpp and HCVcc | (120) |

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Chapter 2


Neutralizing Monoclonal Antibodies against Hepatitis C Virus E2 Protein Bind Discontinuous

Epitopes and Inhibit Infection at a Post-Attachment Step


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**ABSTRACT**

The E2 glycoprotein of hepatitis C virus (HCV) mediates viral attachment and entry into target hepatocytes and elicits neutralizing antibodies in infected patients. To characterize the structural and functional basis of HCV neutralization, we generated a novel panel of 78 monoclonal antibodies (MAbs) against E2 proteins from genotypes 1a and 2a HCV strains. Using high-throughput focus-forming reduction or luciferase-based neutralization assays with chimeric infectious HCV containing structural proteins from both genotypes, we defined eight MAbs that significantly inhibited infection of the homologous HCV strain in cell culture. Two of these bound E2 proteins from strains representative of HCV genotypes 1-6, and one of these MAbs, H77.39, neutralized infection of strains from five of these genotypes. The three most potent neutralizing MAbs in our panel, H77.16, H77.39 and J6.36, inhibited infection at an early post-attachment step. Receptor binding studies demonstrated that H77.39 inhibited binding of soluble E2 protein to both CD81 and SR-B1, J6.36 blocked attachment to SR-B1 and modestly reduced binding to CD81, and H77.16 blocked attachment to SR-B1 only. Using yeast surface display, we localized epitopes for the neutralizing MAbs on the E2 protein. Two of the strongly inhibitory MAbs, H77.16 and J6.36, showed markedly reduced binding when amino acids within hypervariable region 1 (HVR1) and at sites ~100 to 200 residues away were changed, suggesting binding to a discontinuous epitope. Collectively, these studies help to define the structural and functional complexity of antibodies against HCV E2 protein with neutralizing potential.

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**INTRODUCTION**

Hepatitis C virus (HCV) is a blood-borne, hepatotropic virus that infects ~170 million people worldwide. Approximately 70% of infected individuals progress to chronic liver disease, which carries an increased risk of cirrhosis and hepatocellular carcinoma (7). In general, treatment of chronic HCV is complicated by resistance due to extensive genetic diversity. HCV has been classified into seven major genotypes, which differ by ~30% at the nucleotide level (4), and this positive-sense, single-stranded RNA virus has a capacity for rapid evolution of variant viruses during persistent infection. The current treatment, pegylated IFN-a$_{2a}$ and ribavirin, has variable side effects and response rates depending on the virus and host genotype (16). No vaccine is currently available, and pre-clinical development has been hampered by a lack of understanding of which conserved epitopes on the HCV structural proteins should be targeted.

HCV contains a ~9.6kb RNA genome that is translated as a single polyprotein and then cleaved by viral and host proteases into structural proteins (core, E1, E2), p7, and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (39). Viral attachment and entry is mediated by the envelope glycoproteins, E1 and E2. Four attachment or entry receptors that are required for infection of hepatocytes have been identified including CD81 (53), scavenger-receptor B1 (SR-B1) (56), and the tight junction proteins claudin-1 (CLDN1) (14), and occludin (OCLN) (54). The importance of E2 binding to the large extracellular loop of CD81 has been established in vitro (13, 18, 28, 50, 53), and interactions between E2 hypervariable region 1 (HVR1) and SR-B1 have been reported (3, 5, 56). The structural basis of binding of E2 to its cognate cell attachment factors, however, is poorly understood, in part because high-resolution structures of the HCV glycoproteins or intact virion have not been solved.

The role of the humoral immune response in controlling HCV infection in patients remains controversial, as patients with persistent infection develop high-titer antibodies that do not appear to clear infection (reviewed in (7)). Nonetheless, there are emerging data that classes of monoclonal and polyclonal antibodies against HCV have protective activity. Binding to CD81 by soluble forms of E2 (sE2, truncated proximal to the transmembrane domain) is inhibited by

antibodies that also neutralize infection of pseudotyped HCV particles (HCVpp) derived from the structural proteins of multiple genotypes (1, 45). Perhaps more convincing, experiments in chimpanzees and chimeric mice have shown that passive transfer of anti-E2 antibodies protects against infection (15, 37, 64), and immunization with E1-E2 virus-like particles and E2 glycoprotein in chimpanzees induces protective antibodies (10, 29, 37). Moreover, in a comprehensive study of neutralizing MAbs derived from infected patients, MAbs that bound regions comprised of amino acid residues 396–424, 436–447 and 523–540 on E2 neutralized HCVpp derived from multiple genotypes (37). Thus, anti-E2 antibodies apparently can restrict HCV infection, although the exact steps (attachment, entry, or fusion) in the viral entry process that are inhibited and the corresponding E2 binding epitopes have not been elucidated.

To gain more insight into the molecular and structural basis of anti-E2 antibody neutralization of HCV infection, we generated a panel of 78 mouse monoclonal antibodies (MAbs) against soluble, recombinant E2 proteins derived from genotypes 1a (H77 strain) and 2a (J6 strain) HCV strains. These MAbs were analyzed for inhibitory activity against infectious HCV in cell culture and assessed for mechanism of action with respect to inhibition of ligand binding on the cell surface. By combining this functional analysis with a high-throughput yeast surface display mapping strategy, we identified neutralizing MAbs that bound to distinct regions of E2, including MAbs that recognized determinants with discontinuous epitopes with primary sequences greater than 100 amino acids apart. These experiments suggest that neutralizing MAbs blocking distinct stages of the HCV cell entry process recognize discontinuous epitopes on the E2 protein.

**MATERIALS AND METHODS**

*Cells and viruses*. Huh-7.5 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Equitech), non-essential amino acids (Gibco), and antibiotics (penicillin G and streptomycin) at 37°C in a 5% $CO_2$ incubator. SF9 cells were cultured in Grace's Insect cell medium (Gibco) supplemented with 10% FBS at 28°C. HI-5 cells were cultured in Ex-cell media (Gibco) at 27°C. CHO cells were grown in Ham's F12 medium (Gibco) supplemented with 10% FBS (HyClone) at 37°C.

The genotype 2 J6/JFH1/JC1 HCV chimera that expresses luciferase (58) was a generous gift from Apath Inc. The HJ3-5 H77/JFH1 chimera, which expresses the core-NS2 segment of the genotype 1a polyprotein within a genotype 2a background has been described (40, 67). The genotype 1a H77C/JFH1(57), genotype 2a J6/JFH1 (38), genotype 3a S52/JFH1 (23), genotype 4a ED43/JFH1 (57), genotype 5a SA13/JFH1 (32), and genotype 6a HK6a/JFH1 (24) infectious HCV recombinants used in cross-neutralization studies also have been described. Cell culture compensatory and adaptive mutations for the HJ3-5, S52/JFH1, ED43/JFH1, SA13/JFH1 and HK6a/JFH1 chimeras have been described (24, 40, 67).

To generate virus stocks from infectious cDNA clones, plasmids were linearized and RNA transcription was performed using the T7 DNA-dependent RNA polymerase (MEGAscript Kit, Ambion). Infectious HCV RNA (2 mg) was electroporated as described (38), and virus was harvested at 48, 72, and 96 hours, sterile filtered (0.2 mm filter, Corning Inc), and buffered with 10mM HEPES pH 7.2 (Mediatech, Inc.). Virus was stored at 4°C for short-term usage or aliquots were prepared and stored at -80°C. Virus titration on Huh-7.5 cells was performed by $TCID_{50}$ assay as previously described (38).

*Generation of CHO cells stably expressing HCV cell entry factors.* Human SR-BI and CD81 genes were expressed in CHO cells via lentivirus transduction in the context of pTRIP, a self-inactivating lentiviral provirus that expresses no HIV proteins but instead employs an internal cytomegalovirus (CMV) promoter to express cloned genes. An intermediate plasmid, called TRIP-GFP-linker, was generated as a backbone into which SR-BI and CD81 were cloned (all entry

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factor templates were kindly provided by C. Rice, Rockefeller University, NY). TRIP-GFP-linker was generated by amplifying the GFP sequence with the forward oligonucleotide 5'-CGC AAA TGG GCG GTA GGC GTG and reverse oligonucleotide 5'-CTC GAG CTA GTC GAC TTC GAA ACT AGT GCT AGC CCG CGG CTT GTA CAG CTC GTC CAT GCC. This PCR product was digested with restriction enzymes BamHI and XhoI and ligated into the TRIP-GFP plasmid digested with the same enzymes. The human SR-BI sequence was amplified with forward oligonucleotide 5'-CCG CGG ATG GGC TGC TCC GCC AAA GCG and reverse oligonucleotide 5'-GCT AGC CAG TTT TGC TTC CTG CAG CAC from the previously described TRIP-hu-SR-BI plasmid (54) to generate TRIP-GFP-hu-SR-BI-linker. This PCR product was digested with SacII and NheI and ligated into similarly digested TRIP-GFP-linker. The human CD81 sequence was amplified from an expression construct, TRIP-GFP-hu-CD81(56), with forward oligonucleotide 5'-GCT AGC ATG GGA GTG GAG GGC TGC ACC and reverse oligonucleotide 5'-ACT AGT GTA CAC GGA GCT GTT CCG GAT. This PCR product was digested with NheI and SpeI and ligated into similarly digested TRIP-GFP-linker to generate TRIP-GFP-hu-CD81-linker.

Pseudoparticle production was performed as previously described (54) by co-transfection of three plasmids encoding a TRIP provirus containing a transgene, HIV Gag-Pol, and the VSV-G glycoprotein. 293-T cells were seeded at 1.8 x $10^6$ cells/well into a poly-L-lysine (Sigma)-coated six-well plate. Transfection was performed the next day using a total of 1.5 mg of DNA plasmid, with 6 ml of TransIT-LT1 transfection reagent (Mirus). Supernatants were collected 24, 48, and 72 h post-transfection, filtered (0.45-mm pore size), and mixed with 100 ml of 1 M HEPES buffer. All transductions were performed in the presence of 4 mg/ml Polybrene (Sigma). Receptor expression was verified by flow cytometry using the following protocol: cells were lifted using PBS supplemented with 4 mM EDTA and 10% FBS, washed, and pelleted in a V-bottom plate. Cells ($10^5$) were incubated with either 20 mg/ml of mouse anti-hu-CD81 (BD Biosciences) or rabbit anti-hu-SR-B1 (Ab-Cam) for 30 minutes on ice, washed, and then incubated with goat anti-mouse IgG or goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 647 (Molecular Probes). Cells were washed twice and receptor expression was analyzed on a FACSArray flow cytometer

(Becton-Dickinson) using FloJo software (Tree Star).

**Cloning, expression, and purification of recombinant HCV E2.** The E2 protein ectodomain of strains H77 (aa 384-661) (35) or J6 (aa 385-661) was cloned into a baculovirus expression vector (pFastBac derivative) from plasmids containing the structural proteins of H77 (gift of M. Gale, Jr., University of Washington) or the infectious J6/JFH1/JC1 (58) viral genome (gift of Apath, Inc). The baculovirus expression vector adds a honeybee melittin signal peptide at the $NH_2$ terminus and a thrombin-cleavable $His_6$ tag and stop codon at the COOH-terminus. Recombinant baculoviruses expressing HCV E2 ectodomains were generated as described previously (31), amplified in SF9 cells, and used for large scale infection of Hi-5 cells under serum-free conditions. Supernatant was concentrated and buffer exchanged into binding buffer (300 mM sodium citrate, 150 mM sodium chloride, 50 mM sodium phosphate pH 8.0) using a Centramate tangential flow concentrator. E2 was purified by sequential nickel-affinity and size-exclusion chromatography and monodispersed fractions of monomeric protein were collected and used for subsequent studies.

**Generation, purification, and labeling of anti-HCV MAbs**. MAbs were generated by five independent splenocyte-myeloma fusions as described (43). Mice were immunized via an intraperitoneal route with sE2 produced from either genotype 1a (H77) or 2a (J6) HCV strains after complexing with RIBI Adjuvant System (Corixa Corp) or complete Freund's adjuvant (Sigma Chemical). Mice were boosted between two and five times with homologous HCV sE2 protein complexed with either incomplete Freund's adjuvant (Sigma), RIBI Adjuvant System (Corixa), or Sigma Adjuvant system (Sigma), depending on commercial availability, until adequate titers (>1:2500 by ELISA) were achieved. Mice with the highest serum titers were boosted intravenously with purified sE2 (50 mg) three days prior to fusion of splenocytes with P3X63Ag8.53 myeloma cells (12). Hybridomas producing anti-HCV E2 antibodies were identified after binding to *Saccharomyces cerevesiae* yeast expressing sE2 on their surface by flow cytometry, subcloned by limiting dilution, and isotyped by ELISA. For large-scale production, MAbs were generated from ascites or adapted to growth in Hybridoma Serum Free Media (Gibco)

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and purified using protein A or G affinity chromatography (Pierce). In some experiments, MAbs were labeled with Alexa Fluor 647 (Molecular Probes) or NHS-FITC (Pierce) MAb labeling kits according to the manufacturer's instructions.

***Virus neutralization assays***. Neutralization of HCV infection by viruses containing genotype 1a structural proteins (H77/JFH1) was assessed by a focus forming unit (FFU) assay. Serial dilutions of HCV-specific MAb, control MAb (WNV E16 (43)), anti-human CD81 (clone JS81, BD Biosciences), or anti-human SR-B1 (clone 396, Ab-Cam) were pre-incubated with 2.4 x $10^2$ FFU of virus for one hour at 37°C. Virus-MAb mixtures were added to Huh-7.5 cells (1.2 x $10^4$ cells per well) in a 48-well tissue culture plate pre-coated with poly-L lysine (Sigma). After 72 hours, cells were fixed with methanol ($0^o$C), and incubated sequentially with a mouse anti-NS5A (APA-1, 40 ng/ml) (38) (a generous gift of Apath, Inc.) and secondary goat anti-mouse HRP diluted 1:3000 (Sigma). FFU were visualized using the True Blue Peroxidase Reagent (KPL) and quantitated using an S5 Biospot Macroanalyzer (Cellular Technologies Ltd). EC50 values were determined using non-linear regression analysis (Graph Pad Prism 4).

Neutralization of the genotype 2a (J6/JFH1/JC1) HCV was assessed by luciferase assay. Serial dilutions of HCV-specific or control MAbs were pre-incubated with the J6/JFH1/JC1 virus that expresses luciferase ($10^2$ FFU) for one hour at 37°C and then added to Huh-7.5 cells ($10^4$ cells per well) in a 96-well black flat bottom polystyrene-treated microplate (Corning). After 48 hours, cells were lysed and luciferase was detected using the Renilla Luciferase Assay System (Promega) according to the manufacturer's instructions.  EC50 values were determined using non-linear regression analysis (Graph Pad Prism 4).

Neutralization of chimeric viruses with genotype 1a-6a specific core-NS2 sequences was assessed by FFU assay as previously described (55). Briefly, 50 to 400 $TCID_{50}$ of HCV were incubated 1 hour at 37°C with MAb H77.39 or an isotype control and then incubated with cells for 3 hours. After 48 hours, cells were immunostained for NS5A as previously described (23). FFU counting was automated using ImmunoSpot Series 5 UV Analyzer (22). Percent neutralization was calculated by relating FFU counts to mean of six-replicates incubated in the absence of

antibody (virus only). Neutralization data were analyzed as variable slope dose-response curves using GraphPad Prism 4.0 and EC50 values were interpolated by the software.

*Pre-and post virus attachment assays.* To assess the ability of MAbs to inhibit H77/JFH1 virus at pre- and post-attachment steps, FFU assays were modified as follows. For the post-attachment assay, pre-chilled cells were incubated with $4.8 \times 10^2$ FFU of virus for one hour at 4°C. Cells were washed thrice with cold DMEM to remove unbound virus and MAbs (diluted to 50 mg/ml in media and pre-warmed at 37°C) were added and the cells shifted to 37°C. After one hour, a 1:1 MEM-methylcellulose overlay with 4% FBS was added to prevent viral spread. For the pre-attachment assay, $4.8 \times 10^2$ FFU of virus were pre-incubated with 50 mg/ml of media for one hour at 37°C and then added to pre-seeded Huh-7.5 cells.

To assess the ability of MAbs to inhibit J6/JFH1/JC1 at pre- and post-attachment steps, the luciferase assay was modified in the following manner. 48-well tissue culture plates were pre-coated with poly-L lysine (Sigma) and seeded with $1.2 \times 10^4$ cells per well. For the post-attachment assay, $4.8 \times 10^2$ FFU of virus was added to pre-chilled cells and "spinoculated" for 45 minutes at 400 x g at 4°C, followed by a 15 minute incubation at 4°C. Cells were washed and pre-warmed MAbs and methylcellulose were added as described above. For the pre-attachment assay, $4.8 \times 10^2$ FFU of virus were pre-incubated with 50 mg/ml of MAb for one hour at 37°C and then added to pre-seeded Huh-7.5 cells. Cells from both the pre-and post-attachment assay were lysed after 48 hours and transferred to a 96-well black-bottom plate and luciferase was detected using the Renilla Luciferase Assay System (Promega) according to the manufacturer's instructions.

*Cross-reactivity and mapping analysis of MAbs using yeast surface display.* To assess MAb cross-reactivity with other HCV genotypes, the ectodomain of the E2 genes from genotype 1a (H77, amino acids 384 to 660), genotype 2a (J6, amino acids 385 to 664), genotype 3a (UKN 3A13.6, amino acids 385 to 667), genotype 4a (UKN 4.21.16, amino acids 392 to 663), genotype 5a (SA13 NIH, amino acids 384 to 663) and genotype 6a (UKN 6, amino acids 385 to 668) was amplified by PCR with BamH1 and XhoI sites for cloning added at the 5' and 3' ends,

respectively. The PCR products were cloned as downstream fusion proteins to the Aga2 gene in the pYD1 vector (Invitrogen) for expression on the surface of yeast. To determine the relative binding regions on E2 of specific MAbs, COOH-terminal truncation constructs, based on previous studies (41) were generated for genotypes 1a and 2a corresponding to regions I (amino acids 384 to 520 in genotype 1a and 384 to 518 in genotype 2a) or I and II (amino acids 384 to 605 in genotype 1a and 384 to 603 in genotype 2a) and displayed on the surface of yeast.

Expression constructs were transformed into *Saccharomyces cerevisiae* strain EBY100 (17) using the *S.c.* EasyComp transformation Kit (Invitrogen). Individual yeast colonies were grown to logarithmic phase at 30°C in tryptophan-free yeast selection media containing 2% glucose. Protein expression was induced by cultivating yeast for an additional 48 to 72 hrs in tryptophan-free media supplemented with 2% galactose at 20°C. Yeast cells were washed with PBS containing 1 mg/ml BSA (PBS/BSA) and incubated with 40 ml of MAb (neat supernatant or 20 mg/ml purified diluted in PBS) for 30 minutes on ice. Yeast were washed in PBS/BSA, incubated with goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 647 (Molecular Probes) for 30 minutes on ice, washed, and analyzed on a FACSArray flow cytometer (Becton-Dickinson) using FloJo software (Tree Star).

Random mutant libraries of E2 were generated from genotype 1a (H77 strain) and genotype 2a (J6 strain) genes by error-prone PCR using a GeneMorph II random mutagenesis kit (Stratagene). Libraries were ligated into the pYD1 vector and transformed into XL2-Blue ultracompetent cells (Stratagene) with ~5.7 x 10$^5$ and 5.5 x 10$^5$ transformants for genotypes 1a and 2a, respectively. Screening of the libraries for loss of binding variants was performed as described (43, 59). In brief, yeast expressing E2 variants that lost specific binding to individual MAbs were sorted using two-color flow cytometry. To eliminate mutations that abolished surface expression of E2, yeast were stained sequentially with the Alexa Fluor 647-conjugated individual MAb, followed by a FITC-conjugated oligoclonal pool of the cross-reactive MAbs (J6.1, J6.2, J6.16, J6.39, J6.51, and J6.101 for the genotype 1a library and J6.2, J6.14, J6.15, J6.39, J6.51, and J6.99 for the genotype 2a library) on ice for 30 minutes. Yeast that stained positively for the

oligoclonal pool but negatively for the MAb of interest were collected, cultivated, and iteratively sorted. In some cases, sorting was performed using MACS LS magnetic columns (Miltenyi Biotech). In brief, ~$10^7$ yeast cells were pelleted and resuspended in MACS buffer (PBS + 0.5% BSA+ 2mM EDTA) containing a 1:50 dilution of a FITC-labeled MAb of interest for 30 minutes, washed, and then incubated with 10 ml of anti-FITC microbeads (Miltenyi Biotech) on ice for 15 minutes. Yeast were washed and passed over a MACS LS column and the flow-through collected. After four to five rounds, yeast were plated and individual colonies were tested for binding to individual MAbs by flow cytometery. For clones that lost binding to the desired MAb of interest, the plasmid was recovered using a Zymoprep yeast minipreop kit (Zymo Research), transformed into XL1-Blue competent *E. coli*, purified using a QIAprep spin miniprep kit (Qiagen) and sequenced. In cases where more than one mutation was detected, site-specific mutagenesis using the Quick Change II Mutagenesis kit (Stratagene) was used to generate individual mutations within the E2 protein to define the mutant of interest.

**Inhibition of CD81 and SR-B1 binding.** To assess the ability of neutralizing MAbs to inhibit binding of sE2 to CD81 and SR-B1, 50 mg/ml of purified MAb was pre-incubated with 20 mg/ml H77 E2 or J6 sE2 for 30 minutes at 37$^\circ$C. CHO cells expressing HCV receptors were detached with PBS supplemented with 4 mM EDTA and 10% FBS, and washed three times in medium. Cells ($10^5$) were pelleted in a V-bottom plate, resuspended with MAb-protein mixture, and incubated on ice for 30 minutes. Cells were washed and then incubated with a pool of Alexa Flour 647 labeled anti-E2 MAbs (J6.1, J6.2, J6.39, J6.51, H77.30, and H77.34 for the detection of H77 E2; and J6.2, J6.39, J6.51, J6.60, and J6.101 for the detection of J6 E2) for 20 minutes on ice. Cells were washed twice and sE2 binding was analyzed on a FACSArray flow cytometer (Becton-Dickinson) using FloJo software (Tree Star).

**Statistical analysis.** All data was analyzed using Graphpad Prism software (version 4.0). For neutralization assays and receptor-binding assays, an unpaired t-test was used to determine statistical significance.

**RESULTS**

**MAb generation.** Previous studies have demonstrated that HCV-specific monoclonal and polyclonal antibodies, particularly those that recognize the E2 protein, can control HCV infection in vitro and in vivo (10, 15, 33, 37, 47, 62). However, only a few of these antibodies have been characterized for their ability to inhibit at different stages of HCV infection or mapped to epitopes at the amino acid level. To better define the structural basis of antibody neutralization of HCV, we generated a new panel of anti-HCV MAbs by immunizing BALB/c mice with soluble, recombinant E2 protein that was expressed in insect cells and derived from either genotype 1a (H77 strain, amino acids 384-664) or genotype 2a (J6 strain, amino acids 385-664) viruses. After five independent splenocyte-myeloma cell fusions, we subcloned 37 MAbs from genotype 1a-immunized mice and 41 MAbs from genotype 2a-immunized mice, all with reactivity against the E2 structural glycoprotein of HCV **(Table S1).**

**Neutralizing activity of anti-E2 MAbs.** To study the inhibitory capacity of genotype 1a MAbs in cell culture, we utilized an H77-JFH1 chimeric infectious virus that contains genotype 1a core-NS2 sequence in the JFH1 background, with a compensatory Q221L mutation in NS3 (pHJ3-5) (40, 67). For high-throughput screening, we adapted a focus-forming unit (FFU) assay with Huh-7.5 cells such that infectious foci were scored objectively on an ELISPOT reader and the reduction in number of FFU was assessed after pre-incubation of virus with individual MAbs (**Fig 1A**). We performed a single endpoint focus reduction neutralization test (FRNT) using neat antibody supernatant (~10 mg/ml) and identified 9 MAbs that inhibited infection by 40% or greater (**Fig 1B**). Candidate neutralizing MAbs identified above and other selected antibodies were purified by immunoaffinity chromatography and tested for inhibitory activity with a more complete dose-response curve (**Fig 1C**). We demonstrated that five of these MAbs (H77.16, H77.28, H77.31, H77.39 and H77.56) had reproducible neutralizing activity and determined the concentration of MAb at which 50% of foci were inhibited (EC50 value) (**Fig 1C**). Of these MAbs, H77.16 and H77.39 showed the greatest inhibitory activity, with EC50 values of ~3.4 μg/ml and ~1.1 μg/ml, respectively.

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To evaluate the neutralizing activity of MAbs generated against E2 derived from the genotype 2a HCV strain, we utilized a genotype 2a J6/JFH1/JC1 infectious chimera of HCV that contains a Renilla-luciferase reporter gene inserted immediately upstream of NS2A cleavage site (58). All 41 MAbs that bound the genotype 2a E2 protein were purified and assessed for inhibitory activity over a broad range of concentrations to determine the concentration of antibody that reduced luciferase expression by 50% (EC50 value) (data not shown). We identified two antibodies, J6.36 and J6.103 that efficiently neutralized infection, (**Fig 1D**) with J6.36 having an EC50 value below 2 mg/ml. Notably, no significant difference in inhibitory potency of a given neutralizing MAb was observed when the luciferase and FRNT assays were directly compared (data not shown).

*Cross-reactivity of anti-E2 MAbs*. HCV is comprised of six epidemiological important genotypes with ~70% nucleotide identity (4). A better understanding of the specific epitopes that are conserved and recognized by inhibitory antibodies may facilitate the design of future vaccines. To begin to address this, we assessed how genotype variation affected MAb reactivity using recombinant E2 proteins displayed on yeast (1a, H77; 2a, J6; 3a, UKN3a; 4a, UKN4a; 5a, SA13; and 6a, UKN6) and neutralization capacity with chimeric HCV strains (1a, H77; 2a, J6; 3a, S52; 4a, ED43; 5a, SA13; and 6a, HK6a) containing the non-structural proteins (NS3-NS5B) of the genotype 2a JFH1 strain and structural proteins, p7, and NS2 from strains representative of HCV genotypes 1-6.

*(a) Binding to different HCV genotypes*. The ectodomain of E2 from individual strains corresponding to HCV genotypes 1-6 was expressed on the surface of yeast, incubated with MAbs, and analyzed for binding by flow cytometry. Three of the eight neutralizing MAbs were broadly cross-reactive and recognized all five (H77.16 and H77.39) or four of the five (H77.56) heterologous genotypes (**Fig 2 and Table 1**). Three of the neutralizing MAbs (H77.31, J6.36, and J6.103) bound to yeast expressing only the homologous E2.

*(b) Cross-neutralizing potential of MAbs.* As MAb binding capacity to recombinant viral structural proteins does not always directly correlate with neutralizing potential (8), we evaluated

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the inhibitory activity of several of the cross-reactive MAbs against HCV virus of other genotypes. Initially, single endpoint focus reduction assays were performed with high concentrations (50 mg/ml) of purified MAbs generated against genotype 1a or genotype 2a that cross-reacted with genotype 2a or genotype 1a E2, respectively (**Fig 3A and 3B**). Of the cross-reactive MAbs generated against genotype 1a E2, only H77.39 neutralized the genotype 2a virus. Of the cross-reactive MAbs generated against genotype 2a E2, only J6.27 inhibited genotype 1a HCV infection (**Fig 3B and 3C**). This was surprising because J6.27 lacked neutralizing activity against the genotype 2a strain against which it was generated (**Fig 3C**); this pattern of enhanced neutralizing activity of cross-reactive antibodies against the heterologous virus also has been observed with MAbs against distantly related flaviviruses (2, 44). H77.39 inhibited the genotype 2a virus with an EC50 value of ~5 mg/ml (**Fig 3D**), which was comparable to that observed with the genotype 1a virus (see **Fig 1C**). We subsequently tested whether H77.39 neutralized infection of a panel of chimeric viruses that expressed structural proteins from the remaining heterologous HCV genotypes. H77.39 dose-dependently inhibited HCV infection of genotypes 3a, 4a, and 5a but showed reduced activity against a virus containing structural proteins of genotype 6a (**Fig 3E**).

     ***Mechanism of MAb neutralization***. Antibody neutralization may involve different stages of viral infection including attachment, internalization, or fusion (51). To begin to understand how our inhibitory MAbs blocked infection, we performed pre- and post-attachment neutralization assays and binding studies to the CD81 and SR-B1 receptors.

     (a) *Pre- and post-attachment assays.* To identify the stage of infection at which MAbs neutralize infection, we adapted a pre- and post-attachment inhibition assay originally developed for flaviviruses (42, 60, 66). Purified anti-E2 MAb was incubated with virus before or after attachment at 4°C to Huh-7.5 cells, and infection was measured by a single endpoint focus reduction assay. Of the nine neutralizing MAbs tested, three (H77.16, H77.39, and J6.36) significantly reduced infection compared to the negative control MAb (WNV E16) when added after viral absorption to a cell monolayer, suggesting blockade of a post-attachment step (**Fig 4A-D**). Interestingly, both anti-CD81 and anti-SR-B1 MAbs also inhibited infection after viral

adsorption, confirming previous results in Huh-7.5 cells which suggested that HCV binds to CD81 and SR-B1 after initial attachment (6, 25). Inhibition of infection at a post-attachment step by H77.39 was confirmed by performing more complete dose-response curve analysis (**Fig 4E**).

(b) MAb inhibition of sE2 binding to receptors. Given that anti-CD81, anti-SR-B1, and several anti-E2 MAbs all blocked after HCV attached to Huh-7.5 cells, it was difficult to discern whether some antibodies blocked binding to individual HCV receptors. To address this, we developed a binding assay for soluble E2 (sE2) to CHO cells that ectopically expressed human CD81 or SR-B1. CHO cells were transduced with a lentiviral vector encoding CD81 or SR-B1 fused to GFP. Surface staining of intact cells with anti-CD81 and anti-SR-B1 MAbs confirmed high-level receptor expression (**Fig 5A**), as did analysis of cells for GFP fluorescence (data not shown). Binding of genotype 1a (**Fig 5B**) and genotype 2a (**Fig 5C**) sE2 to CD81 and SR-B1 expressing CHO cells (solid histograms), but not control CHO cells (outlined histograms) was confirmed by flow cytometry. To determine whether sE2-CD81/SR-B1 receptor interactions could be disrupted by anti-E2 MAbs, neutralizing or control (anti-WNV E16) MAbs were pre-incubated with sE2, added to wells containing CHO cells expressing CD81 or SR-B1, and loss of binding was assessed by flow cytometry (**Fig 5D**). The neutralizing MAb H77.39 significantly blocked (>70%, P < 0.01) sE2 binding to both CD81 and SR-B1. In comparison, H77.31 also reduced binding of sE2 to both receptors, although inhibition of SR-B1 binding was more modest (~40%, P = 0.04) compared to that seen with CD81 (>80%, P = 0.003). Conversely, J6.36 efficiently inhibited sE2-SR-B1 binding (>80%, P = 0.0002) yet only modestly (~50%, P < 0.05) diminished sE2-CD81 binding. H77.16 and J6.103 blocked sE2 binding to only a single receptor, with both efficiently reducing (>75%, P = 0.0005) binding to SR-B1 (**Fig 5D**). Three neutralizing MAbs, H77.28, H77.56, and J6.27, did not inhibit significantly sE2 attachment to either CD81 or SR-B1, suggesting that these may block an alternate attachment or entry step (**Fig 5E**).

**Epitope localization of MAbs.** To correlate the function of the anti-E2 MAbs with structure of the HCV E2 protein, we localized their epitopes using a previously validated yeast surface display mapping assay (43, 44, 61). Initially, COOH-terminal truncated versions of E2,

based on those described previously (41), were displayed on the surface of yeast and MAbs were tested for immunoreactivity by flow cytometry (**Fig 6 and Table S1**). Neutralizing MAbs showed different requirements for binding. H77.16, H77.39, J6.36, and J6.103 bound to a region bracketed by amino acids 384-520 of genotype 1a and 384-518 of genotype 2a E2 (designated "region I"), whereas H77.28, H77.31, and J6.27 required amino acids 521-605 of genotype 1a or 519-603 of genotype 2a E2 (designated "region II") for binding. In contrast, MAb H77.56 required the full E2 ectodomain (1-664), suggesting that it interacts with amino acids 606-664 alone or requires a conformation of E2 that this region stabilizes. MAbs that neutralized efficiently at a post-attachment step, H77.16, H77.39 and J6.36, all bound to region I of E2.

To localize MAb epitopes more clearly, we used error-prone PCR mutagenesis and yeast surface display to create a library of H77 and J6 E2 variants to define individual amino acid binding residues of neutralizing and non-neutralizing MAbs. Yeast that lost expression of individual MAb epitopes were sorted by flow cytometry and plasmids were recovered, sequenced, and tested for reactivity against a select panel of MAbs (**Fig 7 and Tables 2 and 3**).

H77.39, the most potent and highly cross-neutralizing MAb, showed markedly reduced binding when residues N415 and N417 of E2 were changed (**Fig 7A and Table 2**). Two neutralizing MAbs (J6.36, and J6.103) showed significant loss of binding when a pair of mutations was introduced. J6.36 and J6.103 lost binding with changes in HVR1 and a more distal region of E2; mutation of residues G406, F403, or a combined mutation at residues G397 and R572 abrogated MAb binding. Single mutations of G397 and R572, however, did not affect binding (**Fig 7B and Table 3**). Similarly, H77.16 showed weakly reduced binding when a serine was introduced at residue G406 (**Fig 7A**), but complete loss of binding when residue G530 was altered in combination with G406S. However, complete loss of H77.16 binding also was observed when residue G406 was mutated to an aspartic acid residue.

The neutralizing MAbs that were quantitatively weaker in our neutralization assays, H77.31 and J6.27, showed decreased binding when residues in the putative CD81 binding region (amino acids 523-535 (48)) were changed. H77.31 binding to E2 on yeast was lost when residues

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W529, G530, and D533 were mutated, whereas J6.27 binding was abolished when amino acids A524 and W529 were altered. The remaining two weakly neutralizing MAbs (H77.28 and H77.56) showed reduced binding with changes at residues R543 and C552, respectively (**Fig 7A and Tables 2 and 3**).

Some non-neutralizing MAbs also were mapped. Several non-neutralizing MAbs (H77.27, H77.36, J6.2, J6.6, J6.15, J6.39, and J6.85) shared residues that impacted binding of H77.31 or J6.27 (**Tables 2 and 3**), and a few (J6.2, J6.6, J6.40, and J6.101) had total or partial loss of binding to residue G406, which was identified as an important recognition residue for the neutralizing MAbs H77.16, J6.36 and J6.103. In addition to G406, J6.2, J6.40 and J6.101 recognition was also affected by mutation of residue H621, thus defining another discontinuous epitope, albeit one that is not apparently involved in neutralization (**Fig 7 and Tables 2 and 3**). Additional residues that uniquely affected binding by non-neutralizing MAbs included G470 (H77.14 and H77.23), S440 (J6.60), Y443 (J6.60), and H621 (J6.30).

**DISCUSSION**

In this study, we generated a novel panel of 78 MAbs against the E2 proteins of HCV genotypes 1a and 2a, analyzed them functionally for inhibition of HCV infection, and localized epitopes using yeast surface display of truncated and substituted forms of the E2 protein. We defined MAbs that mapped to distinct regions of E2, neutralized infection at different stages, and differentially affected CD81 and SR-B1 engagement. Our mapping data also suggests a tertiary interaction between the HVR1 and the COOH-terminal membrane proximal regions of E2, which provides new insight into the quaternary structural aspects of neutralization by functionally relevant antibodies.

Prior mapping studies of anti-E2 MAbs have utilized peptide binding (9, 18), phage display (65), alanine scanning mutagenesis of recombinant E1-E2 (33, 37, 49, 62) or E2, (34), or generation of neutralization escape mutants (19) to localize antibody binding sites. In comparison, we used a forward genetic mutational approach coupled with yeast surface display to identify mutants in the context of the entire ectodomain of E2 protein in an unbiased manner. Three of our eight neutralizing MAbs showed loss-of-binding phenotypes with paired amino acid mutations greater than 100 amino acids apart in the linear sequence, suggesting that discontinuous regions of E2 come together to create functionally important antibody epitopes. H77.16 showed a loss-of-binding phenotype when mutations in the HVR1 (e.g., G406S) and the more COOH-terminal residue (G530A) were paired, suggesting that H77.16 binds a conformational epitope. Although complete loss of binding could be achieved with a single less conserved mutation (G406D), the more conserved G406S change required a second mutation at a discontinuous site (G530) for loss-of-binding. This finding, which suggests that the HVR1 interacts with more COOH-terminal residues, is consistent with MAb competition studies with recombinant proteins that suggested that amino acids 396-424, 436-447, and 523-540 comprise an antigenic region (designated "antigenic region-3") within E2 (37), and with sequencing results of MAb AP33 escape variants, which identified non-contiguous amino acid residues (N415 and E655) as factors in the loss of neutralization phenotype (19). Additionally, these data support the recently described model of

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HCV E2 based on the three domain structure of class II E proteins in *Flaviviridae* and *Togaviridae*, which predicts that the HVR1 proximally apposes the proposed HCV Domain I (D1) (36) (**Fig 8B**).

Two other neutralizing MAbs, H77.31 and J6.27, also recognized residues within the third segment of antigenic region-3 (A524, W529, G530 and D533) but did not show a loss-of-binding phenotype when amino acids within segment 1 (396-424) were changed. These two MAbs less potently neutralized infection and were less cross-reactive. In comparison, human anti-HCV MAbs (A8, 1:7, and CBH5) that share epitopes in this region (26, 33) have been characterized as inhibitory and cross-reactive (**Table 4**). Although further analysis is required, the differences in function of the mouse and human MAbs could be related to affinity or possibly, that the human MAbs bind additional sites and do not exclusively recognize the linear epitope centered at residues G523-D535, as was suggested in previous studies (1, 26).

The neutralizing MAbs J6.36 and J6.103 also mapped to a discontinuous epitope, requiring residues within the HVR1 (G397, F403, and G406) and the more COOH-terminal residue R572. Although neutralizing MAbs (9/27 (18, 30) and AP213 (65)) have been mapped to the HVR1, to our knowledge, MAbs that bind residues at or near R572 have not been identified. The MAb 9/27 does not block binding of sE2 to CD81 (18, 30) although it did inhibit HCV VLPs interaction with CD81 (45), suggesting that it also may recognize a conformational or possibly oligomeric epitope.

The MAb in our study with the greatest inhibitory activity, H77.39, localized to two amino acids, N415 and N417, that are highly conserved among all HCV genotypes (48, 65). N415 and N417 were defined previously as possible binding residues for MAbs AP33 and 3/11 (11, 19, 62) (**Table 4**). Residue N417 comprises part of a highly conserved N-linked glycosylation site (20, 21) that is implicated in obscuring antibody-mediated neutralization (27). H77.39, as well as AP33 and 3/11, are thus unique in mapping to an N-linked glycan that is paradoxically hypothesized to impair antibody recognition.

To relate binding epitopes to function, MAbs were tested for their ability to inhibit sE2

engagement with the HCV cognate receptors CD81 and SR-B1. The MAbs J6.36, J6.103, and H77.16, which recognized residues within the HVR1 as well as the more COOH-terminal region, blocked sE2-SR-B1 binding. These results are consistent with data suggesting the HVR1 participates in SR-B1 binding (3, 5, 56), and that the HVR1-specific MAb 9/27 inhibits sE2-SR-B1 interactions (5, 56). Although J6.36 did not map to any of the predicted CD81 binding residues (48), it partially inhibited sE2 binding to CD81. J6.36 could map to additional amino acid residues (within the CD81 binding site) not identified in our study or steric hindrance could mediate this partial inhibition. In the recently modeled E2 structure (36), the J6.36 interaction residues lie in proximity to the HCV D1, which is predicted to contain key CD81 binding residues (36, 48) (**Fig 8B**). Conversely, H77.31, which potently inhibited CD81 binding and maps to residues (W529, G530) involved in CD81 binding (48) partially inhibited SR-B1 engagement despite a lack of contact residues in the HVR1. The inability of J6.103 to inhibit binding to CD81 despite localizing to the same residues as J6.36 could be explained by overlapping but not identical MAb footprints or perhaps differences in affinity of interaction.

Only one MAb, H77.39, potently inhibited sE2 binding to both CD81 and SR-B1. Interestingly, H77.39 did not map to residues within known SR-B1 or CD81 binding regions, suggesting that it may recognize a site that once occupied, can sterically prevent receptor engagement. This concept is supported by studies showing that N415 and N417 can obscure the CD81 and SR-B1 binding sites (11, 27). Finally, the E2 model recently proposed by Krey et al predicts that residues N415-N417 lie at the junction of the HVR1 and D1 (**Fig 8B**), in proximity to both HVR1 and the CD81 binding residues located within *C* and *D* loops of D1 (36, 48).

Pre- and post-attachment neutralization studies provided additional insight into the relative potency of MAbs. Studies with distantly related Flaviviruses have shown that MAbs inhibiting at a post-attachment step tend to have greater inhibitory activity in vitro and in vivo because they require reduced virion occupancy for neutralization (43, 52, 60, 63, 66). Indeed, our three most potent MAbs, H77.16, H77.39 and J6.36, neutralized infection in the post-attachment assay. Nevertheless, J6.103 shared apparent binding epitopes with J6.36, yet did not neutralize

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efficiently when added after attachment. This discrepancy may be explained by J6.36 having additional amino acid contacts not identified in our study.

MAb binding to conserved residues may not directly predict cross-binding or cross-neutralizing capabilities (8 , 59). Despite mapping to highly conserved residues, MAbs H77.31, J.36 and J6.103 failed to cross-react with any other strains tested, and J6.27 was cross-reactive with only two of the strains tested. In comparison, MAb H77.16 was highly cross-reactive, but still did not neutralize heterologous strains. In contrast, H77.39 cross-reacted with genotypes 1-6 and neutralized chimeric virus representative of all strains except genotype 6. The inability of H77.39 to neutralize the genotype 6 chimeric virus may be explained by the presence of a mutation in one of the recognition residues, N417T (24). This mutation is rare in natural HCV isolates (11, 48), but was required for adaptation of the HK6a/JFH1 chimera in vitro (24). Mutations at N415 are rare (11, 48) and attenuating in the context of HCV infection (19).

Generation of an HCV vaccine has been impeded by the lack of a structural understanding of the epitopes on E2 that should be targeted by inhibitory antibodies. Although direct structural confirmation is necessary, our data suggests the existence of discontinuous epitopes that are recognized by antibodies that inhibit CD81 and SR-B1 binding. The yeast surface display antibody mapping data also provides support for a recently proposed structural model of E2 in which the residues comprising the CD81 binding region lie within a single domain of b-pleated sheets that contains the HVR1 as an N-terminal extension (36). The epitopes defined by the MAbs H77.16, J6.36, and J6.103 suggest that the HVR1 might lie in proximity to this domain, creating a conformational epitope (**Fig 8B**), which could be a useful target for vaccines and therapeutic antibodies.

**ACKNOWLEDGEMENTS**

**FIGURE LEGENDS**

**Table 2.1: Binding of MAbs to HCV E2 from different HCV genotypes.** +++, strong binding (40-100%) to yeast expressing E2; +, weak binding (15-40%) to yeast expressing E2; -, no appreciable binding detected. Data is a summary of between 3 and 5 independent experiments.

**Table 2.2: Summary of MAb binding to genotype 1 mutants expressed on the surface of yeast.** Values shown were obtained by dividing the total fluorescence product (percent positive population x mean fluorescence intensity) of a mutant for a given MAb by the total fluorescence product of the wild type E2 for a given MAb. This value was then divided by the total fluorescence product of a mutant for an oligoclonal MAb pool by the total fluorescence product of WT E2 for the same olicoglonal pool (to control for E2 binding) and multiplied by 100. Values in bold indicate complete loss of binding, with reductions in MAb binding greater than or equal to 80% for a given mutation. Underlined values indicate a partial reduction in binding, between 50 and 79%. Values italicized, bolded and underlined show enhancement of binding greater than 500%. The results are the average of three independent experiments for each mutant and each antibody.

**Table 2.3: Summary of MAb binding to genotype 2a mutants expressed on yeast.** Values shown were obtained by dividing the total fluorescence product (percent positive population x mean fluorescence intensity) of a mutant for a given MAb by the total fluorescence product of the wild type E2 for a given MAb. This value was then divided by the total fluorescence product of a mutant for an oligoclonal pool of MAbs by the total fluorescence product of WT E2 for the oligoclonal pool (to control for E2 binding) and multiplied by 100. Values in bold indicate complete loss of binding, with reductions in MAb binding greater than or equal to 80% for a given mutation. Underlined values show partial loss of binding, with a reduction between 50 and 79%. The results are the average of three independent experiments for each mutant and each antibody. Poly-protein amino acid numbering was determined by alignment with the H77 strain using the Sequence Location tool on the Los Alamos HCV database (http://hcv.lanl.gov/cgi-bin/LOCATE/locate.cgi).

**Table 2.4: Previously characterized neutralizing anti-E2 MAbs with available mapping information.** Summary of previously characterized, neutralizing, anti-E2 MAbs.

**Table 2.5**: **Profile of anti-E2 MAbs.** Isotype was determined by ELISA. Cross-reactivity to other genotypes and binding to different regions of E2 was determined by flow cytometric analysis of MAb binding to yeast expressing E2 from different genotypes or truncation mutants of E2, respectively (+++, strong binding (40-100%) to yeast; +, weak binding (15-40%) to yeast; -, no appreciable binding detected). Neutralizing activity at 50 μg/ml was determined by luciferase assay for genotype-2 specific MAbs and by FFU reduction assay for genotype-1 specific MAbs.

**Figure 2.1: Identification of neutralizing anti-E2 antibodies against HCV. A**. Examples of MAb neutralization as judged by a reduction in the number of FFU using the Biospot Macroanalyzer. Spot counts are labeled below each well and well numbers are labeled above. Wells 1 through 8 represent decreasing (3-fold) concentrations of the neutralizing MAb H77.39 (starting concentration of 50 mg/ml). Well 9 shows infection in the absence of MAb, and well 10 is an uninfected well. Data are representative of three independent experiments performed in duplicate. **B.** MAb supernatant was mixed with the H77-JFH1 chimeric HCV for one 1 hour at 37°C and Huh-7.5 cells were infected. Three days later, neutralization was determined by FFU assay. MAb supernatants that decreased the number of FFU to 40% or less (below the solid black line) than the negative control MAb (anti-WNV E122), as well as additional selected MAbs, were purified for testing in full dose-response analysis. Data is pooled from three independent experiments performed in duplicate. **C**. Serial dilutions of genotype 1a specific purified MAbs were mixed with H77-JFH1 chimeric virus and neutralization was assessed. Efficient neutralization was observed for five (H77.16, H77.28, H77.31, H77.39 and H77.56) genotype 1a specific MAbs but not for the negative control MAb (data not shown). EC50 values were calculated after non-linear regression analysis. Data is pooled from of at least three independent experiments performed in duplicate. **D**. Increasing concentrations of purified genotype 2a specific MAbs (J6.36 and J6.103) were mixed with J6-JFH1-JC1-luciferase-expressing virus. At 48 hours, neutralization was assessed in Huh-7.5 cells by monitoring luciferase expression. EC50 values

were calculated after non-linear regression analysis. Data is pooled from at least three independent experiments performed in duplicate. In this Figure, all error bars represent the standard error of the mean.

Figure 2.2: **Identification of MAbs that bind heterologous HCV genotypes using yeast display of E2 protein.** The E2 ectodomain gene from six strains corresponding to HCV genotypes 1-6 was cloned into the PYD1 vector and expressed on the surface of yeast (see Materials and Methods). Yeast expressing HCV E2 were incubated with MAb supernatants and binding was assessed by flow cytometry.  Representative histograms from all neutralizing MAbs (H77.16, H77.28, H77.31, H77.39, H77.56, J6.27, J6.36 and J6.103; solid black histograms) and negative control MAb (WNV E16; unfilled gray histograms) are depicted. Data is representative of three independent experiments.

Figure 2.3: **MAb neutralization of heterologous HCV genotypes.** MAbs that were generated against (**A**) genotype 1a or (**B**) genotype 2a E2 proteins were tested for their ability to neutralize infection of virus from the heterologous genotype. Purified J6 or H77 MAbs (50 mg/ml) were pre-incubated at 37°C with H77-JFH1 (genotype 1a) or J6-JFH1-JC1 (genotype 2a) virus, respectively, and neutralization was assessed as described in Figure 1. **C-E**.  EC50 analysis was performed with (**C**) J6.27 MAb and H77-JFH1 virus (■) or J6-JFH1-JC1 virus (○) or (**D**) H77.39 MAb and J6-JFH1-JC1 virus (○) or (**E**) H77.39 MAb and H77C/JFH1 (■), S52/JFH1 (▼), ED43/JFH1 (♦), SA13/JFH1 (●) and HK6a/JFH1 (□) chimeric viruses. Graphs represent pooled data from at least three independent experiments performed in duplicate (**A-D**) or two independent experiments performed in triplicate (**E**), and error bars represent the standard error of the mean.

Figure 2.4: **Pre- or post-attachment neutralization. A-D**. To determine whether MAbs neutralize HCV infection at a post-attachment step, Huh-7.5 cells were pre-chilled at 4°C and 480 FFU of (**A**) genotype 1a (H77-JFH1) or (**B**) genotype 2a (J6-JFH1-JC1) virus was added to each well for 1 hour at 4°C. After three washes with 4°C DMEM, saturating concentrations of MAbs (50 mg/ml) were added for 1 hour at 37°C and the neutralization assay completed. In comparison, a

standard pre-incubation neutralization test was performed at 37°C, in which (**C**) genotype 1a virus or (**D**) genotype 2a virus and MAb were pre-incubated at 37°C prior to addition to cells. Data shown are the average of three independent experiments, with error bars representing standard error of the mean. Statistically significant difference in neutralization are compared to infection in the presence of a negative control MAb (WNV E16): *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. **E.** To confirm the ability of H77.39 to neutralize infection at both pre-and post-attachment steps, a dose response curve was performed under both pre-and post-attachment conditions as described above using H77/JFH1 virus. Solid squares (■) represent pre-attachment data and clear squares (□) represent post-attachment data. Graphs represent pooled data from at least three independent experiments performed in duplicate, and error bars represent the standard error of the mean.

**Figure 2.5: Inhibition of sE2 binding to CD81 and SR-B1 by neutralizing MAbs. A.** Verification of ectopic CD81 and SR-B1 receptor expression on CHO cells. CHO-CD81 or CHO-SR-B1 cells were incubated with either mouse anti-hCD81 or rabbit-anti-hSR-B1 (black histograms) or an irrelevant MAb (unfilled gray histograms) for 30 minutes on ice. Cells were washed, incubated with the appropriate secondary antibodies, and processed by flow cytometry. **B-C.** Binding of (**B**) genotype 1a (H77) E2 or (**C**) genotype 2a (J6) E2 to CHO-CD81 and CHO-SR-B1 but not WT CHO cells. CHO-CD81 or CHO-SR-B1 (solid black histograms) or WT CHO (unfilled gray histograms) cells were incubated with sE2 and binding was assayed by flow cytometry. Data are representative of at least three independent experiments. **D.** Assessment of inhibition of sE2 binding to CHO-CD81 or CHO-SR-B1 cells by neutralizing MAbs. sE2 was pre-incubated with neutralizing MAbs, added to CHO cells, and binding detected by flow cytometry. Examples of MAbs that inhibit sE2 binding to CD81 preferentially (H77.31), to both CD81 and SR-B1 (H77.39), or only to SR-B1 (J6.103), as well as a negative control MAb (WNV E16) are shown. Histograms are representative of three individual experiments. Solid black histograms represent

sE2 binding in the presence of MAb, red histograms represent sE2 binding in the absence of

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MAb, and shaded gray histograms represent sE2 binding to CHO WT cells. **E**. Graphical representation of sE2 binding to CHO-CD81 and CHO-SR-B1 cells in the presence of neutralizing MAbs. Values were determined by dividing the fluorescence quotient (mean fluorescence intensity x percent positive cells) for E2 binding in the presence of a neutralizing MAb by the fluorescence quotient of sE2 binding to either CHO-CD81 or CHO-SR-B1 cells alone. Asterisks represent statistically significant difference in sE2 binding compared to the negative control MAb, WNV E16: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. Error bars represent the standard error of the mean. Data are pooled from three independent experiments.

Figure 2.6: **Mapping of anti-E2 antibodies using COOH-terminal truncation mutants. A**. Scheme of E2 truncations used for mapping. cDNA containing region I (aa 384-520 and aa 384-518 in E2 of genotypes 1a and 2a, respectively) I and II (aa 384-605 and 384-603 in E2 of genotypes 1a and 2a, respectively), and the full length ectodomain (aa 384-664) were displayed on the surface of yeast. **B**. MAb supernatants were incubated with yeast and assessed for binding by flow cytometry**.** Neutralizing MAbs binding to regions I (H77.16, H77.39, J6.36, and J6.103), II (H77.28, H77.31, and J6.27), and full-length E2 ectodomain (H77.56) are shown. Solid black histograms depict binding of HCV-specific MAbs and gray, unfilled histograms represent binding of a negative control MAb (WNV E16). Histograms are representative of three independent experiments.

Figure 2.7: **Epitope localization of anti-HCV MAbs.** Binding of neutralizing MAbs to yeast expressing E2 protein variants. **A**. Flow cytometry histograms of wild type and loss-of-binding genotype 1a E2 variants (G406D, G406S, N410Y, I411N, N415Y, N417T, W529R, G530A, D533N, R543G, C552S, and G406S + G530A). Representative histograms are shown for the MAbs H77.14, H77.16, H77.28, H77.31, H77.39, H77.56 and WNV E16 (negative control) with WT H77 E2 and each of the variants. Data shown are representative of three independent experiments. Red arrows indicate >80% loss-of-binding of a specific MAb for a given variant.

**B**. Flow cytometry histograms of wild type and loss-of-function genotype 2a E2 variants (G397E, F403L, G406C, A524V, W529C, R572S, H621L and G397E+R572S) with individual neutralizing MAbs. Representative histograms are shown for the MAbs J6.27, J6.36, J6.101, J6.103 and WNV E16 (negative control) with the wild type E2 and each of the variants. Data shown are representative of three independent experiments. Arrows indicate >80% loss-of-binding of a specific MAb for a given variant.

**Figure 2.8: Localization of MAb binding residues on E2**. **A**. Alignment of E2 sequences from HCV genotypes 1-6 with superimposed mapping of MAb binding residues. The sequences of E2 from strains representative of the different genotypes (genotype 1a, H77; genotype 2a, J6; genotype 3a, UKN 3; genotype 4a, UKN4a; genotype 5a, SA513; genotype 6a, UKN 6) used in the yeast mapping studies (**Fig 2)** were aligned. Colored boxes and symbols were used to highlight neutralizing MAb binding residues as follows: red boxes, J6.36 and J6.103; purple boxes, H77.39; blue underscoring, H77.16; green boxes, J6.27; pink circles, H77.31; orange box, H77.28; yellow box, H77.56. **B.** Putative model of structure of the E2 protein with MAb binding regions highlighted. A scheme depicting a possible E2 structure was adapted from Krey et al. (36) to highlight regions involved in MAb recognition. N-linked glycosylation residues are labeled in green and amino acids numbered in black at intervals. b-sheets in D1 are labeled as previously described (36). MAb binding regions are highlighted by colored circles as follows: red circles, J6.36 and J6.103; purple circle, H77.39; light blue circles, H77.16; green circle, J6.27; pink circle, H77.31; orange circle, H77.28; yellow circle, H77.56. **C**. Summary of neutralizing MAbs described in this study. EC50 values (neutralization against homologous virus), cross-reactivity to E2 from different genotypes, inhibition of binding to CD81 and SR-B1, reactivity with different regions of E2, and loss of binding residues are listed. MAb names are color-coded to correspond to panels A and B.

**Table 2.1: Binding of MAbs to HCV E2 from different HCV genotypes**

| MAb | Genotype 1a(H77) | Genotype 2a(J6) | Genotype 3a(UKN 3) | Genotype 4a(UKN4) | Genotype 5a(SA13) | Genotype 6a(UKN6) |
|---|---|---|---|---|---|---|
| J6.1 | +++ | +++ | +++ | +++ | +++ | +++ |
| J6.2 | + | +++ | +++ | +++ | - | - |
| J6.6 | + | +++ | + | - | - | - |
| J6.7 | +++ | +++ | +++ | + | +++ | +++ |
| J6.8 | + | +++ | - | - | - | - |
| J6.9 | +++ | +++ | - | - | +++ | - |
| J6.12 | +++ | +++ | +++ | +++ | - | - |
| J6.13 | - | +++ | - | - | - | - |
| J6.14 | +++ | +++ | +++ | + | + | +++ |
| J6.15 | - | +++ | +++ | +++ | +++ | +++ |
| J6.16 | +++ | +++ | +++ | - | +++ | - |
| J6.21 | +++ | +++ | - | - | - | - |
| J6.23 | + | +++ | - | - | - | - |
| J6.25 | - | +++ | - | - | - | - |
| J6.27 | +++ | +++ | +++ | - | - | - |
| J6.30 | +++ | +++ | +++ | +++ | +++ | +++ |
| J6.33 | +++ | +++ | +++ | +++ | +++ | +++ |
| J6.34 | +++ | +++ | + | + | +++ | + |
| J6.36 | - | +++ | - | - | - | - |
| J6.39 | +++ | +++ | +++ | - | - | - |
| J6.40 | - | +++ | + | - | - | - |
| J6.42 | + | +++ | +++ | +++ | - | - |
| J6.48 | - | +++ | - | - | - | - |
| J6.49 | - | +++ | - | - | - | - |
| J6.51 | + | +++ | + | +++ | - | - |
| J6.56 | +++ | +++ | +++ | + | +++ | +++ |
| J6.58 | +++ | +++ | +++ | +/- | +++ | - |
| J6.60 | - | +++ | - | - | - | +++ |
| J6.62 | +++ | +++ | +++ | + | +++ | + |
| J6.67 | +++ | +++ | + | - | +++ | - |
| J6.68 | + | +++ | - | - | - | - |
| J6.75 | +++ | +++ | +++ | +++ | +++ | +++ |
| J6.76 | - | +++ | - | - | - | - |
| J6.81 | +++ | +++ | +++ | - | +++ | - |
| J6.85 | - | +++ | +++ | +++ | +++ | +++ |
| J6.86 | + | +++ | +++ | +++ | - | - |
| J6.91 | + | +++ | - | - | - | - |
| J6.98 | - | +++ | + | +++ | - | - |
| J6.99 | - | +++ | - | - | - | + |
| J6.101 | +++ | +++ | + | +++ | - | +++ |

| | | | | | | |
|---|---|---|---|---|---|---|
| J6.103 | - | +++ | - | - | - | - |
| H77.1 | +++ | - | - | - | - | - |
| H77.7 | +++ | +++ | +++ | +++ | +++ | +++ |
| H77.8 | +++ | - | - | - | - | - |
| H77.9 | +++ | - | - | - | - | - |
| H77.11 | +++ | - | - | - | - | - |
| H77.12 | +++ | +++ | - | - | +++ | - |
| H77.13 | +++ | - | - | - | - | - |
| H77.14 | +++ | - | - | - | - | - |
| H77.16 | +++ | +++ | +++ | +++ | +++ | +++ |
| H77.17 | +++ | - | - | - | - | - |
| H77.18 | +++ | - | - | - | + | - |
| H77.19 | +++ | - | - | - | - | - |
| H77.22 | +++ | - | - | - | - | - |
| H77.23 | +++ | - | - | - | - | - |
| H77.27 | +++ | +++ | - | - | + | - |
| H77.28 | +++ | +++ | - | - | + | - |
| H77.29 | +++ | +++ | - | - | + | - |
| H77.30 | +++ | - | - | - | - | - |
| H77.31 | +++ | - | - | - | - | - |
| H77.32 | +++ | + | +++ | - | +++ | - |
| H77.33 | +++ | - | - | +++ | - | - |
| H77.34 | +++ | - | - | - | - | - |
| H77.35 | +++ | - | - | - | - | - |
| H77.36 | +++ | +++ | +++ | +++ | +++ | +++ |
| H77.37 | +++ | - | - | - | +++ | - |
| H77.38 | +++ | - | - | - | - | - |
| H77.39 | +++ | +++ | +++ | +++ | +++ | +++ |
| H77.42 | +++ | - | - | - | + | - |
| H77.43 | +++ | - | - | - | - | - |
| H77.44 | +++ | - | - | - | +++ | - |
| H77.45 | +++ | - | - | - | - | - |
| H77.46 | +++ | - | - | - | +++ | - |
| H77.47 | +++ | +++ | - | - | - | - |
| H77.50 | +++ | - | - | - | - | - |
| H77.53 | +++ | - | - | - | +++ | - |
| H77.55 | +++ | - | - | - | +++ | - |
| H77.56 | +++ | +++ | +++ | +++ | - | +++ |

**Table 2.2**. **Summary of MAb binding to genotype 1 mutants expressed on the surface of yeast**

| MAbs | G397E | F403L | G406C | S440P | Y443C | A524V | W529C | E531V | R572S | H617L | G397E+R572S |
|---|---|---|---|---|---|---|---|---|---|---|---|
| J6.1 | 71 | 97 | 100 | 100 | 83 | 83 | 100 | 88 | 100 | 100 | 61 |
| J6.2 | 71 | 100 | **16** | 100 | 93 | 73 | **20** | 68 | *42* | **6** | 56 |
| J6.6 | 64 | 100 | *26* | *41* | 100 | **6** | **4** | 69 | 100 | 100 | 100 |
| J6.15 | 100 | 100 | 100 | 75 | 85 | 66 | **13** | *34* | 100 | 100 | 79 |
| J6.16 | 100 | 100 | 100 | 76 | 100 | 80 | 100 | 86 | 100 | 100 | 100 |
| J6.27 | 74 | 67 | 100 | 90 | 95 | **<1** | **<1** | 77 | 83 | 100 | 80 |
| J6.30 | 85 | 100 | 100 | 100 | 75 | 58 | 88 | 82 | 100 | **3** | 100 |
| J6.36 | 100 | **<1** | **<1** | 60 | 95 | 80 | 100 | 100 | 100 | 100 | **<1** |
| J6.39 | 94 | 91 | 100 | 100 | 83 | 100 | **3** | 63 | 100 | 100 | 81 |
| J6.40 | 98 | 100 | *32* | *32* | 100 | 75 | 63 | 65 | *40* | **15** | 80 |
| J6.60 | 51 | 58 | 100 | **<1** | **<1** | 65 | 90 | 81 | 70 | 73 | 77 |
| J6.75 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| J6.85 | 100 | 100 | 100 | 100 | 91 | 72 | **10** | *27* | 100 | 100 | 80 |
| J6.101 | 83 | 100 | *43* | 100 | 100 | 100 | 57 | 91 | 82 | **3** | 50 |
| J6.103 | 100 | **2** | **<1** | *26* | 100 | 100 | 100 | 100 | 100 | 100 | **<1** |

**Table 2.3: Summary of MAb binding to genotype 2a mutants expressed on the surface of yeast**

| MAbs | G406D | G406S | N410Y | I411N | N415Y | T416I | N417T | G470A | W529R | G530A | G530D | D533N | R543G | C552S | G406S +G530A | G406D +G530D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H77.14 | 100 | 100 | 100 | 100 | 67 | 100 | 58 | <1 | 100 | 91 | 100 | 100 | 66 | 100 | 100 | 100 |
| H77.16 | <1 | 58 | <1 | <1 | 76 | 97 | 100 | 100 | 100 | 97 | 100 | 100 | 100 | 100 | <1 | <1 |
| H77.23 | 100 | 100 | 100 | 100 | 75 | 100 | 61 | <1 | 100 | 100 | 100 | 100 | 89 | 100 | 100 | 100 |
| H77.27 | 77 | 91 | 100 | 97 | 64 | 100 | 80 | 81 | <1 | <1 | 11 | 27 | 84 | 100 | <1 | <1 |
| H77.28 | 96 | 100 | 100 | 74 | 38 | 96 | 57 | 74 | 100 | 61 | 100 | 76 | <1 | >100 | 100 | 100 |
| H77.31 | 77 | 100 | 100 | 100 | 96 | 100 | 65 | 100 | <1 | <1 | 24 | <1 | 100 | 100 | <1 | 100 |
| H77.36 | 97 | 88 | 100 | 92 | 100 | 100 | 98 | 100 | 1.2 | 26 | 100 | 80 | 100 | 100 | 21 | <1 |
| H77.39 | 100 | 100 | 100 | 100 | <1 | 81 | <1 | 61 | 100 | 100 | 100 | 100 | 50 | 100 | 100 | 100 |
| H77.56 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 92 | 100 | 9.5 | 100 | 100 |

**Table 2.4: Previously characterized neutralizing anti-E2 MAbs with available mapping information**

| MAb name | Source of MAb | Binding residues | Neutralization of HCVpp? | Neutralization of HCVcc? | References |
|---|---|---|---|---|---|
| AP33 | Mouse | L413, N415, G418, W420 | Yes; all genotypes | Yes; >80% neutralization with 50ug/ml MAb | (19, 45, 46, 62) |
| 3/11 | Rat | N415, W420, H421 | Yes; genotypes 1, 2, 4, 5, and 6 by >50% | N/D | (18, 30, 45, 62) |
| 9/27 | Rat | 396-407 | Yes; genotype 1 by >99% | N/D | (18, 30, 45) |
| 11/20 | Rat | 436-447 | Yes; genotype 1 by >99% | N/D | (18, 30) |
| 7/16b | Rat | 436-447 | Yes; neutralized HCVpp representative of genotype 1 by >99% | N/D | (18, 30) |
| 2/69a | Rat | 432-443 | Yes; genotype 1 by >99% | N/D | (18, 30) |
| 2/64a | Rat | 524-531 | Yes; genotype 1 by 40% | N/D | (18, 30) |
| 1/39 | Rat | 432-443 | Yes; genotype 1 by 20% | N/D | (18, 30) |
| CBH-5 | Human | G523, P525, G530, D535, N540 | Yes; all genotypes >40% | Yes | (26, 47) |
| CBH-7 | Human | N540, W549 | Yes; genotypes 1, 2 and 4 by >20% | Yes | (26, 47) |
| HCV-1 | HuMAb mice | L413, W420 | Yes; genotypes 1, 2, 3, and 4 | N/D | (9) |
| 95-2 | HuMAb mice | L413, W420 | Yes; genotypes 1, 2, 3, and 4 | N/D | (9) |
| 1:7 | Human | G523, T526, Y527, W529, G530, D535 | Yes; all genotypes | Yes | (33) |
| A8 | Human | G523, T526, Y527, W529, G530, D535 | Yes; all genotypes | Yes | (33) |
| AR2A | Human | N540A | Yes; genotypes 1, 2, 4, and 5 | No | (37) |
| AR3A | Human | S424, G523, P525, G530, D535, N540 | Yes; genotypes 1, 2, 4, and 5 | Yes | (37) |
| AR3B | Human | S424, G530, D535 | Yes; genotypes 1, 2, 4, and 5 | Yes | (37) |
| AR3C | Human | S424, P525, G530, D535, N540 | Yes; genotypes 1, 2, 4, and 5 | Yes | (37) |
| AR3D | Human | S424, G530 | Yes; genotypes 1, 2, 4, and 5 | Yes | (37) |
| e137 | Human | T416, W420, W529, G530, D535 | Yes; genotypes 1, 2, and 4 | Yes | (49) |

**Table 2.5**: **Profile of anti-E2 MAbs**

| MAb | Isotype | Cross-reactivity | Binding to E2 region I | Binding to E2 region II | Binding to Full length E2 | % Neutralization at 50 µg/ml |
|---|---|---|---|---|---|---|
| J6.1 | IgG1 | 1, 3, 4, 5, 6 | - | - | +++ | None |
| J6.2 | IgG1 | 1, 3, 4 | - | - | +++ | None |
| J6.6 | IgG1 | 1, 3 | - | +++ | +++ | None |
| J6.7 | IgG1 | 1, 3, 4, 5, 6 | - | - | +++ | None |
| J6.8 | IgG1 | 1 | - | +++ | +++ | None |
| J6.9 | IgG1 | 1, 5 | - | - | +++ | None |
| J6.12 | IgG1 | 1, 3, 4 | - | - | +++ | None |
| J6.13 | IgG1 | - | - | +++ | +++ | None |
| J6.14 | IgG1 | 1, 3, 4, 5, 6 | +++ | +++ | +++ | None |
| J6.15 | IgG1 | 2, 3, 4, 5, 6 | - | - | +++ | None |
| J6.16 | IgG1 | 1, 3, 5 | - | +++ | +++ | None |
| J6.21 | IgG1 | 1 | - | - | +++ | None |
| J6.23 | IgG1 | 1 | - | +++ | +++ | None |
| J6.25 | IgG1 | - | - | - | +++ | None |
| J6.27 | IgG1 | 1, 3 | - | +++ | +++ | None |
| J6.30 | IgG1 | 1, 3, 4, 5, 6 | - | - | +++ | None |
| J6.33 | IgG1 | 1, 3, 4 ,5, 6 | - | - | +++ | None |
| J6.34 | IgG1 | 1, 3, 4, 5, 6 | - | - | +++ | None |
| J6.36 | IgG2b | - | +++ | +++ | +++ | *95%* |
| J6.39 | IgG1 | 1, 3 | - | +++ | +++ | None |
| J6.40 | IgG1 | 3 | - | - | +++ | None |
| J6.42 | IgG1 | 1, 3, 4 | - | - | +++ | None |
| J6.48 | IgG1 | - | - | - | +++ | None |
| J6.49 | IgG1 | - | - | - | +++ | None |
| J6.51 | IgG1 | 1, 3, 4 | - | - | +++ | None |
| J6.56 | IgG1 | 1, 3, 4, 5, 6 | +++ | +++ | +++ | None |
| J6.58 | IgG1 | 1, 3, 4, 5 | - | - | +++ | None |
| J6.60 | IgG1 | 6 | +++ | +++ | +++ | None |
| J6.62 | IgG1 | 1, 3, 4, 5 | - | - | +++ | None |
| J6.67 | IgG1 | 1, 3, 5 | - | - | +++ | None |
| J6.68 | IgG1 | 1 | - | +++ | +++ | None |
| J6.75 | IgG1 | 1, 3, 4, 5, 6 | - | - | +++ | None |
| J6.76 | IgG1 | - | - | - | +++ | None |
| J6.81 | IgG1 | 1, 3, 5 | - | - | +++ | None |
| J6.85 | IgG1 | 3, 4, 5, 6 | - | +++ | +++ | None |
| J6.86 | IgG1 | 1, 3, 4 | - | - | +++ | None |
| J6.91 | IgG1 | 1 | - | +++ | +++ | None |
| J6.98 | IgG1 | 3, 4 | - | - | +++ | None |
| J6.99 | IgG1 | 6 | +++ | +++ | +++ | None |
| J6.101 | IgG1 | 1, 3, 4, 6 | - | - | +++ | None |

| J6.103 | IgG1 | - | +++ | +++ | +++ | 82% |
|--------|------|---|-----|-----|-----|-----|
| H77.1 | IgG1 | - | - | - | +++ | None |
| H77.7 | IgG1 | 2, 3, 4, 5, 6 | - | +++ | +++ | None |
| H77.8 | IgG1 | - | - | - | +++ | None |
| H77.9 | IgG1 | - | - | - | +++ | None |
| H77.11 | IgG1/2a | - | +++ | +++ | +++ | None |
| H77.12 | IgG1 | 2, 5 | - | +++ | +++ | None |
| H77.13 | IgG2a | - | - | +++ | +++ | None |
| H77.14 | IgG2a | - | +++ | +++ | +++ | None |
| H77.16 | IgG2a | 2, 3, 4, 5, 6 | +++ | +++ | +++ | **_97%_** |
| H77.17 | IgG2a | - | +++ | +++ | +++ | None |
| H77.18 | IgG1 | 5 | - | - | +++ | None |
| H77.19 | IgG2a | - | + | +++ | +++ | None |
| H77.22 | IgG1 | - | - | - | +++ | None |
| H77.23 | IgG2b | - | +++ | +++ | +++ | None |
| H77.27 | IgG1 | 2, 5 | - | +++ | +++ | None |
| H77.28 | IgG1 | 2, 5 | +++ | +++ | +++ | 69% |
| H77.29 | IgG1 | 2, 5 | - | - | +++ | None |
| H77.30 | IgG1 | - | - | - | +++ | None |
| H77.31 | IgG2a | - | - | +++ | +++ | 52% |
| H77.32 | IgG1 | 2, 3, 5 | - | - | +++ | None |
| H77.33 | IgG1 | 4 | +++ | +++ | +++ | None |
| H77.34 | IgG2b | - | - | - | +++ | None |
| H77.35 | IgG1 | - | +++ | +++ | +++ | None |
| H77.36 | IgG1 | 2, 3, 4, 5, 6 | - | +++ | +++ | None |
| H77.37 | IgG1/2b | 5 | - | - | +++ | None |
| H77.38 | IgG2a | - | - | +++ | +++ | None |
| H77.39 | IgG1 | 2, 3, 4, 5, 6 | +++ | +++ | +++ | **_100%_** |
| H77.42 | IgG1 | 5 | - | - | +++ | None |
| H77.43 | IgG2b | - | - | - | +++ | None |
| H77.44 | IgG1 | 5 | - | +++ | +++ | None |
| H77.45 | IgG1 | - | - | - | +++ | None |
| H77.46 | IgG2a | 5 | - | - | +++ | None |
| H77.47 | IgG1 | 2 | - | - | +++ | None |
| H77.50 | IgG1 | - | - | - | +++ | None |
| H77.53 | IgG1 | 5 | - | - | +++ | None |
| H77.55 | IgG2a | 5 | - | - | +++ | None |
| H77.56 | IgG1 | 2, 3, 4, 6 | - | - | +++ | 63% |

**Figure 2.1**

**Figure 2.2**

**Figure 2.3**



**A**

Relative infection — Genotype 2 virus (*J6/JFH1/JC1*)
X-axis labels: WNVE16, CD81, H77.7, H77.12, H77.16, H77.27, H77.28, H77.29, H77.36, H77.39, H77.47, H77.56

**B**

Relative Infection — Genotype 1 virus (*H77/JFH1*)
X-axis labels: WNVE16, CD81, J6.1, J6.2, J6.6, J6.7, J6.8, J6.9, J6.12, J6.14, J6.16, J6.21, J6.23, J6.27, J6.30, J6.33, J6.34, J6.36, J6.39, J6.42, J6.51, J6.56, J6.58, J6.62, J6.67, J6.68, J6.75, J6.81, J6.86, J6.91, J6.101, J6.103

**C**

Relative infection vs log [ug/ml J6.27]
- Genotype 1 (*H77/JFH1*)
- Genotype 2 (*J6/JFH1/JC1*)

**D**

Relative Infection vs log [ug/ml H77.39]
- Genotype 2 (*J6/JFH1/JC1*)

**E**

Relative Infection vs log [ug/ml H77.39]
- Genotype 1 (*H77/JFH1*)
- Genotype 3 (*S52/JFH1*)
- Genotype 4 (*ED43/JFH1*)
- Genotype 5 (*SA13/JFH1*)
- Genotype 6 (*HK6a/JFH1*)

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**Figure 2.4**

**Figure 2.5**

**Figure 2.6**



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**Figure 2.7**

**Figure 2.8**



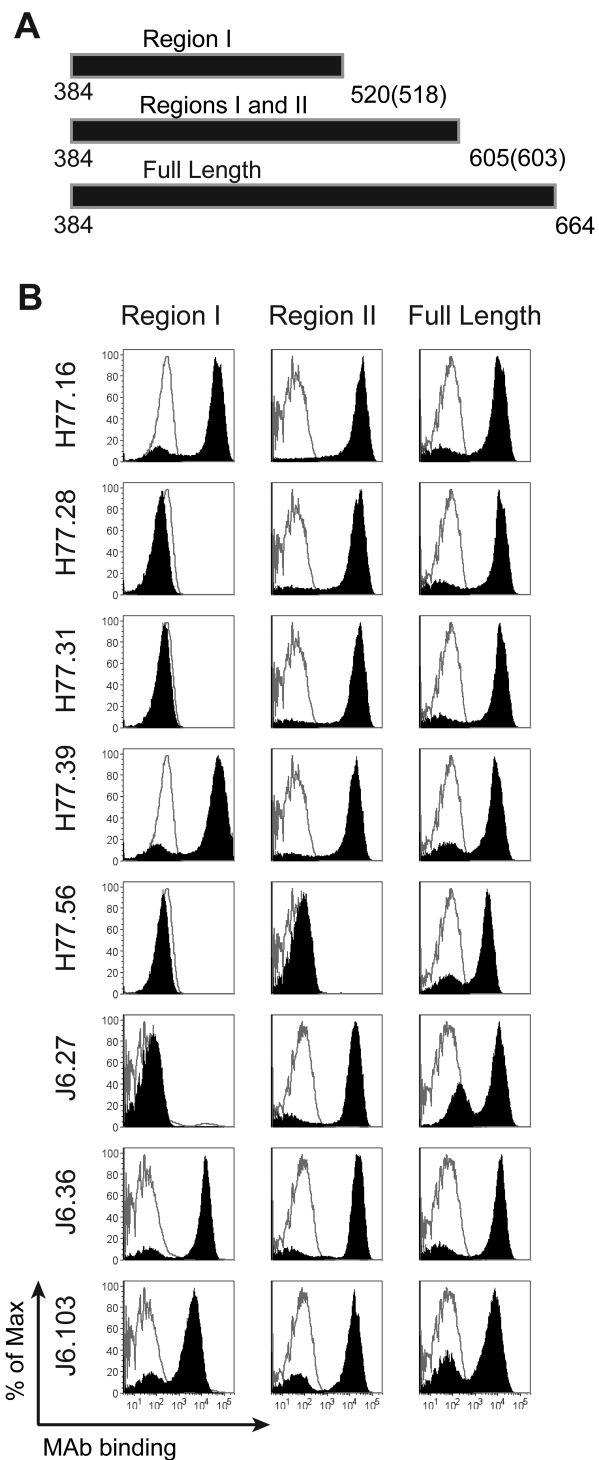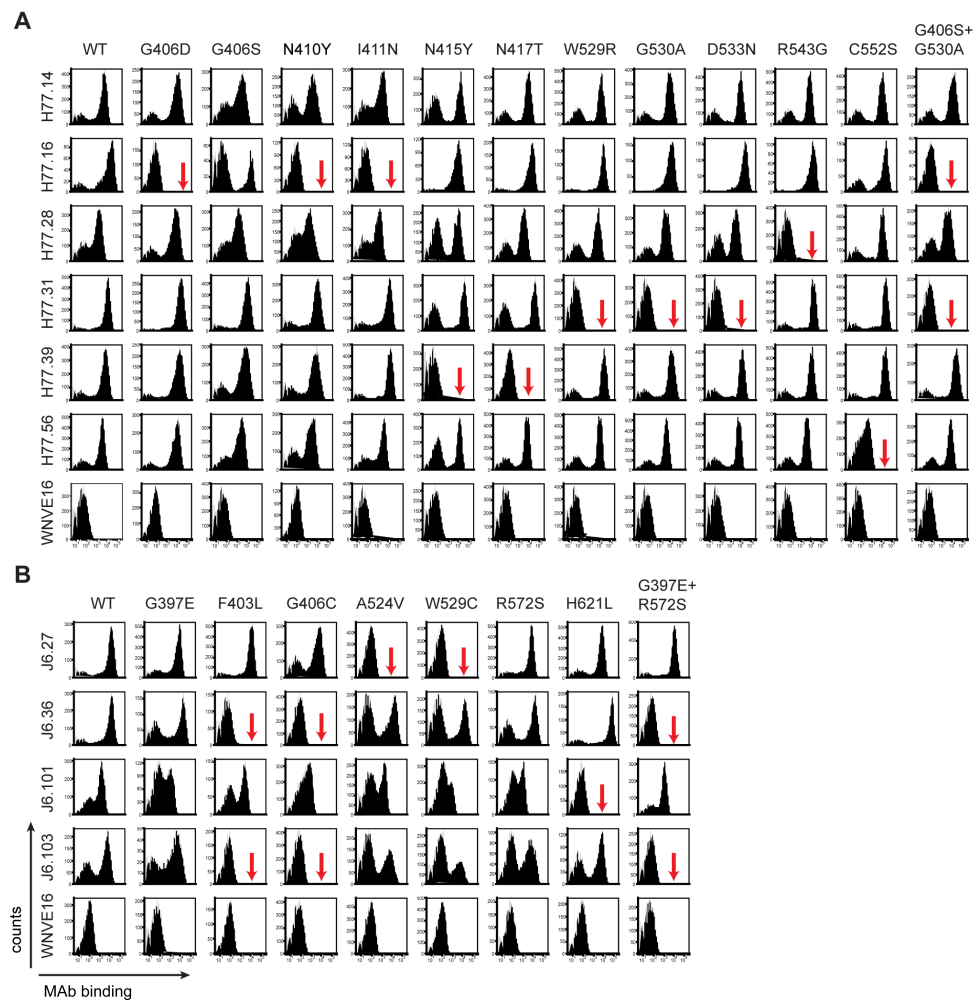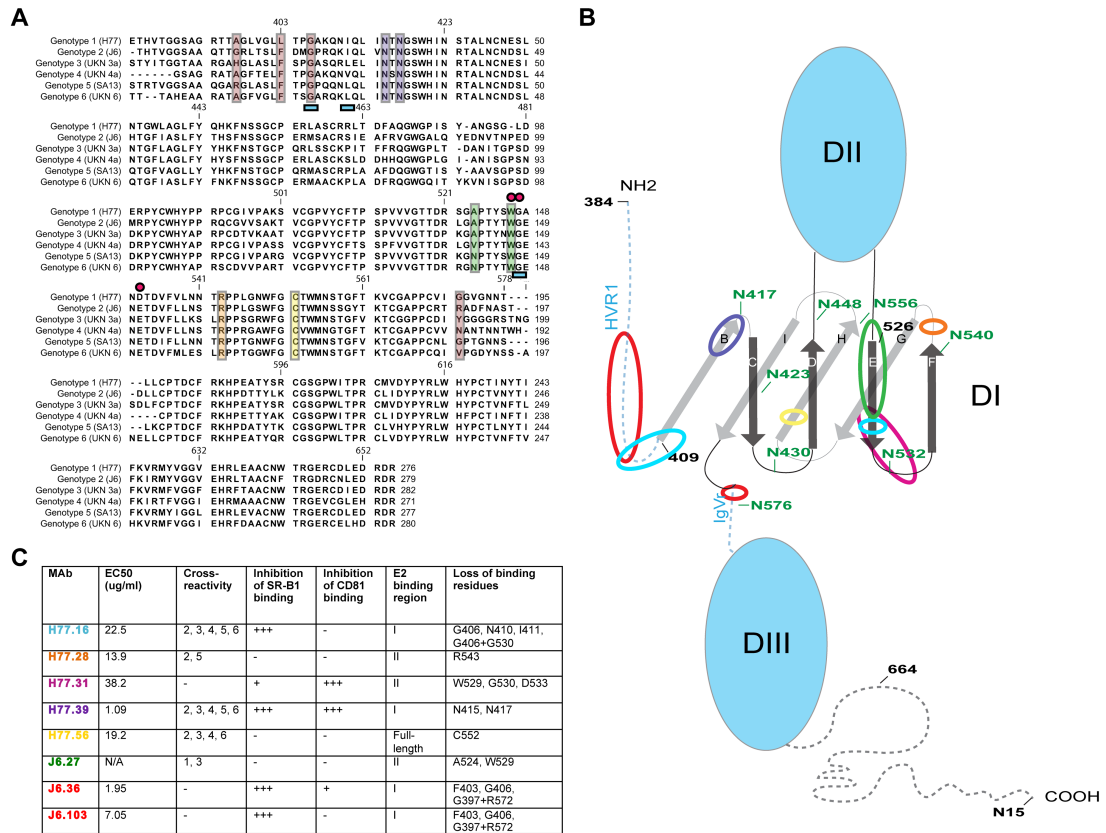| MAb | EC50 (ug/ml) | Cross-reactivity | Inhibition of SR-B1 binding | Inhibition of CD81 binding | E2 binding region | Loss of binding residues |
|---|---|---|---|---|---|---|
| H77.16 | 22.5 | 2, 3, 4, 5, 6 | +++ | - | I | G406, N410, I411, G406+G530 |
| H77.28 | 13.9 | 2, 5 | - | - | II | R543 |
| H77.31 | 38.2 | - | + | +++ | II | W529, G530, D533 |
| H77.39 | 1.09 | 2, 3, 4, 5, 6 | +++ | +++ | I | N415, N417 |
| H77.56 | 19.2 | 2, 3, 4, 6 | - | - | Full-length | C552 |
| J6.27 | N/A | 1, 3 | - | - | II | A524, W529 |
| J6.36 | 1.95 | - | +++ | + | I | F403, G406, G397+R572 |
| J6.103 | 7.05 | - | +++ | - | I | F403, G406, G397+R572 |

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Chapter 3


Hepatitis C virus epitope exposure and neutralization by antibodies is affected by time and

temperature


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**ABSTRACT**

A recent study with flaviviruses suggested that structural dynamics of the virion impact antibody neutralization via exposure of ostensibly cryptic epitopes. To determine whether this holds true for the distantly related hepatitis C virus (HCV), whose neutralizing epitopes may be obscured by a glycan shield, apolipoprotein interactions, and the hypervariable region on the E2 envelope protein, we assessed how time and temperature of pre-incubation altered monoclonal antibody (MAb) neutralization of HCV. Notably, several MAbs showed increased inhibitory activity when pre-binding was performed at $37^{o}C$ or after longer pre-incubation periods, and a corresponding loss-of-neutralization was observed when pre-binding was performed at 4°C. A similar profile of changes was observed with acute and chronic phase sera from HCV-infected patients. Our data suggest that time and temperature of incubation modulate epitope exposure on the conformational ensembles of HCV virions and thus, alter the potency of antibody neutralization.

**INTRODUCTION**

Hepatitis C virus (HCV) is a hepatotropic virus that chronically infects ~170 million people worldwide and results in an increased risk of hepatocellular carcinoma and liver cirrhosis. Until recently, the only available treatment was a combined regimen of ribavirin and pegylated interferon-$\alpha$, which resulted in sustained virologic response in only ~50% of individuals (6). The addition of newly approved NS3 protease inhibitors (boceprevir and telaprevir) to this regimen has improved response rates, although an increase in side effects was noted (1, 24, 50, 66). Given that long-term pharmacological therapy may have limitations in curing HCV-infected individuals, especially in resource-poor parts of the world, there is renewed interest in the development of preventative or even therapeutic vaccines (58). Vaccine development, however, has been hampered by the absence of a tractable small animal model of HCV infection and an incomplete understanding of the correlates of antibody protection in vivo.

HCV is a positive stranded 9.6 Kb RNA virus in the Hepacivirus genus of the *Flaviviridae* family, which also includes globally important pathogens such as Dengue (DENV), West Nile (WNV), yellow fever, and Japanese encephalitis viruses (33). HCV is translated from an internal ribosome entry site (IRES) as a single polyprotein and is cleaved by viral and host proteases into three structural (core, E1, E2) proteins, the ion channel p7, and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (34). Cell culture-produced HCV forms smooth, spherical, enveloped particles that are ~60 nm in diameter (16, 65) with E1 and E2 on the surface. Despite recent predictive models suggesting that HCV E2 protein assumes a three domain structure similar to the E protein of flaviviruses (27), E2 is distinguished from flavivirus E protein by its nine intramolecular disulfide bonds (27), covalent linkage to E1 (62), 11 N-linked glycosylation sites (17, 18), and two hypervariable regions (HVR1 and HVR2) (37, 64). E2 contains binding sites for both the CD81 and SR-B1 receptors (49, 55), and MAbs that block CD81-E2 and SR-B1-E2 interactions prevent infection in cell culture (4, 21, 30, 46, 47, 54, 59).

The role of the humoral response in protection against HCV infection remains controversial, although several studies have suggested that anti-E2 antibodies can limit infection

*in vivo* (14, 23, 30). Antibodies elicited by immunization of chimpanzees with HCV envelope proteins partially protect against viral challenge (15, 38, 51). In the setting of acute infection in humans, antibody responses against the HCV envelope proteins are delayed, with less than 33% of subjects developing neutralizing antibodies at six months (40). Most humans generate a neutralizing antibody response that correlates with viral clearance although chronically infected patients also produce neutralizing antibodies (35). Thus, the presence of neutralizing antibodies in serum does not directly correlate with a viral clearance phenotype. Possible explanations for this phenomenon include: (i) HCV E2 interaction with high-density lipoproteins (HDL) shield virions from recognition by neutralizing antibodies that are present in serum (3, 13, 29), (ii) different functional classes of neutralizing antibodies have distinct inhibitory mechanisms and potencies or (iii) immune pressure drives rapid viral escape from the host humoral response (11, 63)

Antibody-mediated neutralization of *Flaviviridae* family members requires engagement by antibodies with a stoichiometry that exceeds a particular threshold (reviewed by (10)). The number of antibodies bound to the virus particle is governed by the avidity of the antibody for its cognate epitope on the virion, and the number of times that epitope is displayed accessibly on the virion. Antibody avidity determines the fraction of accessible antibody epitopes bound by antibody molecules at a given concentration of antibody (12, 26). For flaviviruses such as WNV, quasi-icosahedral symmetry on the virion surface results in differential display of epitopes, such that the minimum occupancy requirement for a given antibody may never be achieved (reviewed in (9)). Despite this, MAbs that bind to epitopes that are predicted as cryptic can still neutralize infection (36, 43, 57). Recent studies with WNV and DENV have demonstrated that cryptic epitopes can become exposed with increased antibody-virus pre-incubation time or temperature, presumably due to enhanced viral motion (10).

Although HCV E2 is predicted to have a structure similar to flavivirus E proteins (27) and the virion is hypothesized to have an analogous icosahedral organization (65), the association of the virus with apolipoproteins (39, 45), the glycan shield and additional intramolecular and intermolecular disulfide linkages on E2 could impose limits to viral motion and epitope accessibility. To gain more insight into the variation of epitope exposure on the surface of HCV, we studied the effects of temperature and time of antibody-virus incubation on neutralization of HCV using a previously characterized panel of MAbs (54).

**MATERIALS AND METHODS**

**Cells and viruses**. Huh7.5 cells were cultured as previously described (Sabo et al., 2011). Virus stocks of the HJ3-5 H77/JFH1 chimeric virus and the luciferase expressing J6/JFH1/Jc1 virus were generated as described (Sabo et al., 2011) and concentrated using Amicon Ultra tubes (Milipore) with 100 kDa cut-off membranes.

**Quantitative RT-PCR.** RNA was extracted using the RNA Easy Mini kit (Qiagen). qRT-PCR was performed using the Taq-Man one-step RT-PCR master mix reagents (Applied Biosystems) and the following primers and probe located in the 3' untranslated region (3' UTR): Forward primer: 5'-GGC TCC ATC TTA GCC CTA GTC-3'; Reverse primer: 5'-AGT ATC GGC ACT CTC TGC AGT-3'; and probe 6FAM5'-CGG CTC ACG GAC CTT TCA CAG CT3'. Data was analyzed using a 7500 Fast Real-Time PCR system (Applied Biosystems) with 7500 Software (Applied Biosystems, v 2.0.5).

**Antibodies**. The anti-E2 MAbs H77.31, H77.39, H77.46, J6.27, J6.36, and J6.103 were described previously (Sabo et al., 2011). The anti-CD81 antibody (JS-81) was purchased from BD Biosciences. WNV-E16 has been extensively described (Nybakken et al., 2005; Oliphant et al., 2005). HCV immune sera from infected patients (AA, H06, S18, S19, S112, and S154,) have been described previously (Cox et al., 2005; Osburn et al., 2010; Scheel et al., 2008). Negative control serum (SM) was obtained from an HCV-naive donor.

**Neutralization assays**. Neutralization of the chimeric H77/JFH1 virus containing the genotype 1 structural proteins was assessed by focus forming unit (FFU) reduction assay. Serial three-fold dilutions of antibody were pre-incubated with $3.2 \times 10^2$ FFU of HCV for one hour at 37°C and added to a monolayer of Huh7.5 cells in a 96-well plate coated with poly-L lysine (Sigma). Three days later, cells were fixed with ice-cold methanol (0°C) and foci quantified as previously described (Sabo et al., 2011) using a S5 Biospot Macroanalyzer (Cellular Technologies Ltd).

Neutralization of the genotype 2 J6/JFH1/Jc1 virus was determined by luciferase assay as previously described (Sabo et al., 2011). Serial three-fold dilutions of antibody were pre-incubated with $5 \times 10^2$ FFU of virus for one hour at 37°C and added to monolayers of Huh7.5 cells in a 96-well black bottom plate (Corning). Luciferase expression was detected after 48 hours as previously described (Sabo et al., 2011), according to the manufacturer's instructions.

To assess the role of temperature on MAb activity, neutralization assays were modified as follows. Serial dilutions of antibody were pre-incubated with HCV (multiplicity of infection (MOI) of 0.05) for one hour at 4°C, 37°C, or 40°C. In most experiments, antibody-virus mixtures were added to monolayers of Huh7.5 cells and infection assessed as described above. In some experiments with H77.39 and J6.36, antibody-virus mixtures were chilled, added to pre-chilled monolayers of Huh7.5 cells, and "spinoculated" at 1500 rpm for 45 minutes at 4°C. After three washes with ice cold DMEM containing 10% FBS, the temperature was raised to 37°C, and infectivity assessed by luciferase assay or FFU assay 48 hours or 72 hours later, respectively. Relative infectivity was determined after comparison to infectivity of HCV incubated at the same temperature in parallel in the absence of antibody.

To assess the role of incubation time on antibody potency, neutralization assays were modified as follows. Serial dilutions of antibody were pre-incubated with HCV at an MOI of 0.05 for one hour at 37°C to achieve baseline neutralization. Antibody-virus mixtures were then either directly added to monolayers of Huh7.5 cell or incubated for an additional 2, 4 or 8 hours prior to infection of cells. Infectivity was assessed by luciferase assay or FFU assay 48 hrs or 72 hrs later, respectively. Relative infectivity was determined after comparison to infectivity of HCV incubated under the same conditions in parallel in the absence of antibody.

**Immunoprecipitation assays.** To assess for changes in MAb-virus binding at different temperatures, 50 µg/ml of J6.36, negative control MAb (WNV-E16) or medium alone were pre-incubated with 500 FFU of HCV (J6/JFH1/Jc1) for one hour at 4°C, 37°C or 40°C. Subsequently, 50 µl of protein G Sepharose (Pierce) was added, and the slurry mixed overnight at 4°C. Sepharose beads were pelleted at 1,000 x g for 2 minutes and washed six times each with 1 ml

of PBS. Pellets were lysed and RNA was extracted using the Qiamp viral RNA mini kit (Qiagen) and quantitated by qRT-PCR.

To assess for changes in MAb-virus binding over longer pre-incubation periods the immunopreciptation assay was modified as follows. MAbs J6.36, WNV-E16 or medium alone were pre-incubated with 500 FFU of virus for one hour at 37°C and protein G Sepharose was then added immediately and MAb-virus slurries mixed at 4°C, or MAb-virus mixtures were incubated for an additional 2, 4, or 8 hours prior to the addition of protein G Sepharose and transfer to 4°C for mixing. Protein G Sepharose beads were pelleted and washed, and HCV RNA was extracted and quantified by RT-PCR.

**Surface plasmon resonance.** The dissociation constants of MAbs H77.39 and J6.36 for sE2 from genotypes 1a (H77) and 2a (J6) were determined by surface plasmon resonance on a Biacore T100 instrument. Approximately 500 response units (RU) of H77.39, J6.36 and negative control murine MAb (anti-H2-K$^b$) were covalently coupled to a CM5 sensor chip using amine chemistry. Increasing concentrations of monomeric, soluble E2 (4 to 1024 nM) were flowed over the chip in 10mM HEPES, 150 mM sodium chloride, 3 mM EDTA, and 0.005% polysorbate 20 (HBS-EP) at 4°C and 37°C. Binding and dissociation phases were each carried out at 40µl/min for 180 seconds and the chip was regenerated with 60-second pulses of 0.1 M acetate pH 4.2 and 1 M sodium chloride. All curves were reference subtracted from a flow cell containing the negative control MAb. Maximum response units were plotted versus concentration and this curve was fitted to determine $K_D$ at each temperature. Antibody J6.36 is not cross-reactive so its affinity for the genotype 1a sE2 was not determined.

**Statistical Analysis.** Data was analyzed using GraphPad Prism software, version 4.0. Comparison of dose response curves was assessed by an F-test. Viral co-immunoprecipitation assays and EC50 values were analyzed using an unpaired t-test.

**RESULTS**

**Temperature alters the neutralization potency of anti-E2 MAbs.** Studies with distantly related flaviviruses have suggested that virus "breathing" occurs with increased temperature allowing differential exposure of epitopes and altered antibody binding and neutralization (10, 36). As HCV envelope proteins have a greater number (9 versus 6) of intramolecular disulfide bonds (19, 27), which could rigidify the structure, we initially assessed whether temperature changes altered infectivity of an H77/JFH1 chimeric HCV. A one hour incubation at 4$^o$C, 37°C, 40°C and 43$^o$C had relatively small (~2-fold) effects on HCV infectivity (**Fig 1A).** To assess how temperature altered MAb-mediated neutralization, serial dilutions of two previously described neutralizing anti-E2 MAbs, H77.39 and J6.36 (54) were incubated with a genotype 2a J6/JFH1/Jc1 luciferase reporter virus (54) for one hour at 4°C, 37°C, or 40°C. To ensure that only the pre-incubation temperature was being evaluated, virus-MAb mixtures subsequently were bound to cells at 4°C, and unbound virus and MAb was washed away prior to raising the temperature to 37$^o$C for infection. Neutralization by H77.39 and J6.36 was abolished at 4°C and improved at 40°C (**Fig 1B and C**). Comparison of the EC50 values at 37°C and 40°C also was statistically different (**Fig 1B and C**, right panels, $P$ < 0.03). As expected, neutralization of HCV infection was not observed at 4°C, 37°C, or 40°C with the negative control MAb (WNV-E16), which binds to WNV E protein (data not shown). The effects of temperature on MAb neutralization could not be attributed to virus aggregation, as enhanced neutralization at higher temperatures also was observed with Fab fragments (**Fig 1D**), and the neutralization curves did not show a characteristic triphasic curve that is reported in studies reporting antibody-virus aggregation (60). Of note, and in contrast to experiments with intact IgG, we omitted the wash step in Fab fragment neutralization assays prior to HCV infection, as washing resulted in a complete loss of inhibitory activity, likely secondary to the loss of avidity and resulting detachment of Fab fragments from the virion (data not shown). Thus, excess Fab fragments were present beyond the initial incubation (at 4$^o$C or 40$^o$C) period and throughout the experiment (at 37$^o$C), which explains why the 4$^o$C condition inhibited infection as opposed to that observed with intact H77.39 IgG (**Fig 1B**).

**MAb-virus but not E2 binding is reduced at lower temperatures.** *To assess whether the temperature-dependent difference in MAb neutralization of HCV reflected a change in affinity, the $K_D$ of antibody interaction with soluble E2 ectodomain (sE2) was measured at 4°C and 37°C by surface plasmon resonance (SPR). Monovalent affinities were calculated using steady-state analysis between genotype 1a (H77) and genotype 2a (J6) sE2 and H77.39, and genotype 2a (J6) sE2 and J6.36. Notably, the $K_D$ values were not substantially different at 4°C and 37°C (Table 1). As no appreciable dissociation was observed between sE2 and immobilized H77.39 and J6.36 MAb, kinetic parameters were not defined although a qualitative assessment indicated extremely long half-lives at both temperatures (Fig 2A and B). These data suggest that the altered MAb neutralization profiles at different temperatures were not due to large-scale effects on E2 binding.* Consistent with this, prolonged pre-incubation periods at 4°C up to 24 hours only partially affected neutralization by H77.39 and J6.36 (**Fig 2C**), and did not alter infectivity of the J6/JFH1/Jc1 luciferase reporter virus in the absence of MAb (**Fig 2D**). In comparison, pre-incubation of cells at 4°C for one hour with anti-CD81 MAb efficiently neutralized HCV infection (**Fig 2C**).

We hypothesized that the greater MAb neutralization potency at higher temperatures was due to enhanced epitope exposure and capture. To test whether temperature affected the physical interaction of HCV virions with MAbs, immunoprecipitation studies were performed. J6/JFH1/Jc1 virus was pre-incubated with J6.36 MAb at 4°C, 37°C, or 40°C for one hour, virus-MAb complexes were immunoprecipitated with protein G Sepharose, and levels of viral RNA were analyzed by qRT-PCR. As expected, in all cases, greater amounts of HCV were immunoprecipitated by J6.36 MAb than the negative control WNV-E16 MAb (**Fig 3A**), establishing the specificity of the assay. Significantly more virus was precipitated when the pre-binding step was performed at 37°C or 40°C compared to 4°C **(Fig 3A,** $P < 0.01$). Despite the enhanced MAb neutralization at 40°C relative to 37°C (see **Fig 1**), we did not observe a statistical difference ($P > 0.2$) in virus precipitated by J6.36 MAb after pre-binding at 37°C compared to 40°C; this apparent discrepancy could reflect the slight decrease in stability of virus at 40°C,

which is not accounted for in this experiment.

**Increased incubation time at 37°C enhances MAb-virus binding and neutralization.** Increasing the pre-incubation period also can enhance MAb potency, possibly due to changes in epitope accessibility as the virus samples alternate ensembles of conformations over time (10). To assess whether longer incubation times augmented MAb neutralization of HCV, serial dilutions of H77.39 and J6.36 MAbs were incubated for one hour at 37°C to achieve baseline equilibrium binding, and MAb-virus mixtures were either added immediately to Huh7.5 cells (time 0) or incubated for an additional 2, 4 or 8 hours prior to infection. Increasing the pre-incubation period consistently improved neutralization potency (**Fig 4A and 4B**), with a significant reduction in EC50 values occurring after 8 hours of pre-incubation (**Fig 4C and 4D,** $P < 0.05$). To determine whether this effect was due to enhanced MAb-virus binding, complexes were formed for up to 8 hours prior to immunoprecipitation, and viral RNA was quantified by qRT-PCR. Notably, the amount of virus precipitated was increased after 2, 4 and 8 hours of additional pre-incubation at 37°C (**Fig 4E,** $P < 0.05$).

**Increased incubation time and temperature broadly improves antibody function against HCV.** MAbs J6.36 and H77.39 were mapped previously to residues within and adjacent to the hypervariable region 1 (HVR1) on the E2 protein (54). To determine whether temperature and time-dependent effects also altered neutralization of MAbs mapping to different sites with distinct potencies, we tested a panel of neutralizing and non-neutralizing MAbs for functional changes with increased pre-incubation temperature (40°C) or time (12 hours) (**Fig 5**). The most potent of the additional MAbs tested, H77.16, localizes to residues in the HVR1 (G406, N410, I411) and the CD81 binding region (G530) (54), and showed enhanced neutralization with changes in temperature and time during pre-incubation. This improved activity was reflected by a shift in neutralization curves (F test, $P < 0.0001$) and EC50 values (unpaired t-test, $P < 0.01$) (**Fig 5A**). In comparison, the more weakly neutralizing MAbs J6.27 and H77.31, which map to residues in the CD81 binding region, or J6.103, which maps to the HVR1 and the intergenotypic variable

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region (27, 37, 54), showed little enhancement of neutralization under the conditions tested (**Fig 5B,** *P* > 0.2). The only non-neutralizing MAb tested showing improved neutralization with increased temperature or time was H77.46 (**Fig 5C,** *P* < 0.001).

**Neutralization potency of immune sera from HCV-infected patients is improved by incubation at higher temperature and prolonged time.** To further assess the relevance of increased temperature or incubation time on antibody neutralization, we analyzed its impact on polyclonal antibody from sera from acute or chronically HCV-infected patients. Enhanced neutralization after pre-incubation at 40°C for one hour or 37°C for 12 hours was observed with sera from acutely infected patients with genotype 1 (S154) or chronically infected individuals with genotype 1 (S112, S18, S19, H06) and genotype 4 (AA) (**Fig 6A and B,** P < 0.0001, F test). As expected, no specific increase in neutralization with time or temperature change was observed with sera from an uninfected individual (SM). Thus, polyclonal anti-HCV antibody present in immune sera behaved in a manner similar to MAbs of defined specificity with respect to the effects on neutralization of prolonged time or elevated temperature exposure.

**DISCUSSION**

Prior studies with distantly related flaviviruses have established that antibody-mediated neutralization of infection is modulated by several factors including avidity of binding and the availability of the epitope on the virion surface (reviewed in (9, 48)). Nonetheless, some antibodies neutralize infection despite binding epitopes that ostensibly are obscured on the virus, at least based on contemporary high-resolution structural models (10, 31, 32, 36). This has led to the concept of virus "breathing" in which structural perturbations on the virion surface allow antibody to bind cryptic epitopes, a phenomenon that can be promoted by changes in temperature (10, 32, 36) and time of interaction (10, 53). Although it is closely related to flaviviruses, it was unclear whether similar principles apply, as HCV contains additional intramolecular and intermolecular cysteine bonds on its envelope proteins that could rigidify structure and restrict movement. Indeed, unlike flaviviruses, HCV is resistant to inactivation at low pH in solution, and does not undergo plasma membrane fusion from without (61). These observations may be explained by recent evidence suggesting covalent linkage between the glycoproteins on the virion surface, a feature that would further rigidify the viral structure (62). Nonetheless, in our study, we showed that increasing the time and temperature of pre-incubation enhanced neutralization of HCV by monoclonal and polyclonal antibodies, suggesting that epitope accessibility can change in a manner analogous to that observed with flaviviruses.

Cryo-electron microscopy studies with the related DENV demonstrated that incubation of virions at physiological temperature ($37^{o}$C) induced alterations in the virion icosahedral structure (36) that expose cryptic epitopes allowing for antibody binding and neutralization (10). In the absence of high-resolution structural information on the HCV envelope proteins or virions, we assessed functionally whether changes in temperature impacted HCV stability or antibody neutralization, presumably by altering epitope accessibility. As prolonged incubation in solution over a range of temperatures ($4^{o}$ to $43^{o}$C) only minimally impacted infectivity (see also (7, 25)), HCV appears relatively stable, suggesting it does not readily undergo irreversible structural changes. Consistent with this, and in direct contrast to the related flaviviruses, HCV can be stored

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at 4$^o$C for several weeks without appreciable loss of infectivity (7) and exposure to a pH 5.0 acidic solution does not expose domains on the envelope proteins that result in adventitious fusion and virus inactivation (61). Nonetheless, prolonged time and elevated temperature likely promoted changes in the ensemble of virion conformations, as significantly different neutralization profiles were observed with some but not all anti-HCV antibodies. The changes in neutralization at different temperatures reflect altered epitope exposure and not modified binding kinetics or virion aggregation, as SPR experiments showed minimal change in affinity between 4$^o$C and 37$^o$C and neutralization by monovalent Fab fragments of HCV antibodies also was enhanced at higher temperatures. Together, with our immunoprecipitation experiments showing that increased amounts of virus are precipitated at 37°C compared to 4°C, these data suggest that HCV undergoes some structural perturbations over time, and this results in altered epitope capture and antibody neutralization.

The CD81-binding site on HCV E2 may be shielded by HVR1 and a subset of N-linked glycans (N417, N423, N448, and N532), as deletion of HVR1 or site-specific substitutions that abolish N-liked glycosylation sites augments the inhibitory capacity of antibodies disrupting E2-CD81 interactions (2, 22). Because of this, we anticipated that increasing the time and temperature of interaction would facilitate exposure of antibody epitopes proximal to the CD81 binding site region, resulting in improved neutralizing activity. While we observed enhanced potency of neutralization by MAbs (H77.16, H77.39 and J6.36) mapping to HVR1 or contiguous regions, we did not observe this effect for weakly neutralizing MAbs (H77.31 and J6.27) that localize to the CD81 binding region. The failure to observe enhanced inhibitory activity by E2-specific MAbs that interfere with CD81 binding may reflect a requirement for more significant structural shifts for complete epitope exposure, analogous to the those required to reveal the CCR5 binding epitope on HIV gp120 (28, 52). Indeed, previous studies have demonstrated that anti-CD81 antibodies inhibit infection at a post-attachment step, indicating that CD81 is not completely engaged by the virus directly after attachment (5, 20, 54). Interestingly, the MAb J6.103 also did not demonstrate enhanced potency, despite mapping to the same residues as a

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MAb that did, J6.36 (54), possibly due to variations in the MAb footprint that are not apparent from our epitope mapping by yeast surface display; indeed, J6.36 and J6.103 have different receptor-blocking capabilities (54), suggesting these MAbs are not functionally equivalent.

While many humans develop a neutralizing antibody response that correlates with viral clearance, chronically infected patients also produce neutralizing antibodies (35). Thus, the presence of neutralizing antibodies in serum does not directly correlate with a viral clearance phenotype. Our studies show that elevated temperature modulates the potency of neutralizing anti-HCV antibodies, a finding that may have implications for an improved understanding of the correlates of antibody protection for HCV: (i) the inherent neutralizing capacity of antibody in serum could change substantially depending on assay conditions; and (ii) fever occurs as a collateral effect of therapy with pro-inflammatory pegylated IFN-$\alpha$ (67). It is tempting to speculate that the fever response associated with therapy could improve the efficiency of antibody neutralization.

Generation of a vaccine for HCV in part, has been hampered by the lack of a structural understanding of the virus. Although direct structural studies of the HCV virion are required to corroborate our findings, our studies suggest that HCV has a dynamic component analogous to flaviviruses (10, 36). Nonetheless, portions of CD81 binding region on E2 likely remain shielded, making this region a challenge for molecular and immunological targeting. Further study and identification of the host factors that modulate antibody neutralization against HCV will likely inform strategies to contain and control infection through induction of more potently inhibitory humoral responses.

**FIGURE LEGENDS**

**Table 3.1: Kinetic parameters of MAb binding to sE2 at different temperatures.**
Estimates of the $K_D$ were determined by equilibrium binding fits of E2 to H77.39 and J6.36 at 4°C
and 37°C degrees. Fold difference was calculated by dividing the $K_D$ determined for each MAb-E2
pair at 4°C by the $K_D$ determined at 37°C.

**Figure 3.1: Temperature alters the neutralizing capabilities of anti-E2 MAbs. A.**
Chimeric H77/JFH1 HCV was incubated for one hour at the specified temperatures, added to
Huh7.5 cells, the temperature was raised to 37°C, and infectivity assessed 72 hours later by FFU
assay. Results of FFU assays are pooled from three independent experiments performed in
triplicate. Asterisks represent statistically significant differences in infectivity: *, $P < 0.05$; **, $P <$
0.01; and ***, $P < 0.001$. **B-D.** Serial dilutions of (**B**) H77.39 MAb, (**C**) J6.36 MAb, or (**D**) H77.39
Fab fragments were pre-incubated with J6/JFH1/Jc1 luciferase reporter virus for one hour at
either 4°C (♦), 37°C (●) or 40°C (■). For **B** and **C**, MAb-virus mixtures were incubated at the
indicated temperature, chilled, and then added to pre-chilled Huh7.5 cells to allow for attachment.
Cells were then washed thrice (removing excess antibody) and the temperature raised to 37°C for
the duration of the assay. For **D**, Fab-virus mixtures were incubated at the indicated temperature
and then added to pre-chilled Huh7.5 cells to allow for attachment. Infectivity was assessed by
luciferase assay 48 hours later. Dose response curves (*left panels*) are displayed relative to the
infectivity of HCV pre-incubated at the indicated temperature in the absence of antibody.
Comparison by F-test demonstrated a statistically significant difference between the curves in all
cases ($P < 0.001$). The EC50 values at 37°C and 40°C were determined by nonlinear regression
analysis and asterisks represent significant differences: *, $P < 0.05$; **, $P < 0.01$; and ***, $P <$
0.001. Graphs are pooled from at least three independent experiments performed in duplicate.
Error bars represent the standard error of the mean.

**Figure 3.2: MAb-E2 binding kinetics are not altered by changes in temperature. A-
B.** SPR analysis of MAb binding to soluble E2 at different temperatures. Examples of
sensograms (*inset*) and graphs of the steady-state fit of E2 binding to H77.39 at 4°C (**A**) and

37°C (**B**) are shown. Points represent maximum resonance units (RU) at various concentrations of E2 and the solid line represents the steady-state fit of the maximim RU values. **C.** Effect of time of incubation at 4°C on MAb neutralization. MAbs (50 μg/ml of H77.39, J6.36, anti-CD81 (positive control) and WNV-E16 (negative control) were incubated with J6/JFH1/Jc1 luciferase reporter virus at 4°C for one hour and added directly to Huh7.5 cells (T=0, white bars), or incubated for an additional 12 (T=12, black bars) or 24 hours (T=24, grey bars) at 4°C prior to infection. Cells were infected for one hour at 4°C, washed at 4°C to remove unbound virus, the temperature was raised to 37°C, and infectivity detected by luciferase assay 48 hours later. Infectivity is displayed relative to conditions in the absence of MAb. Graphs are pooled from three independent experiments performed in duplicate. Asterisks represent statistically significant differences in infectivity compared to cells infected in the absence of antibody at the specified time point: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.  **D**. Effect of time of incubation at 4°C on infectivity in the absence of MAb. J6/JFH1/Jc1 luciferase reporter virus was incubated at 4°C for one hour (T=0) or an additional 12 (T=12) or 24 hours (T=24). Infectivity is expressed as relative light units (RLU). Data are pooled from three independent experiments performed in triplicate. Infectivity is not significantly different at T=12 and T=24 compared to T=0. Error bars represent the standard error of the mean.

**Figure 3.3: Changes in temperature alter MAb-virus binding.**  J6/JFH1/Jc1 luciferase reporter virus was pre-incubated for one hour at 4°C (white bars), 37°C (grey bars), or 40°C (black bars) with J6.36 MAb, a negative control MAb (WNV-E16) or medium alone, precipitated with protein G Sepharose beads, and the quantity of virus was assessed by qRT-PCR.  Data are pooled from at least three independent experiments performed in duplicate. Fold change is calculated compared to viral RNA detected in the absence of antibody. Asterisks represent statistically significant differences: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Error bars represent the standard error of the mean.

**Figure 3.4: Incubation time alters MAb neutralization and binding to HCV. A-D.** Neutralization analysis. Serial dilutions of (**A**) H77.39 or (**B**) J6.36 were incubated with

J6/JFH1/Jc1 luciferase reporter virus for one hour at 37°C to achieve a baseline equilibrium. Samples were added either immediately to Huh7.5 cells (time 0,■) or incubated for an additional 2 (T=2,□), 4 (T=4,Δ), or 8 (T=8, ●) hours at 37°C prior to infection of cells. Infectivity was determined by luciferase expression 48 hours later. Dose response curves are statistically different (F-test, $P < 0.0001$) and are displayed relative to the infectivity of HCV incubated for the indicated time interval in the absence of MAb. EC50 values of (**C**) H77.39 and (**D**) J6.36 at each time point were determined by non-linear regression analysis. Asterisks represent significant differences in EC50 values compared to T=0: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. **E.** Immunoprecipitation assays. J6.36 and J6/JFH1/Jc1 virus were pre-incubated for one hour at 37°C to achieve baseline equilibrium binding. Protein G Sepharose beads were added immediately (T=0) or at 2, 4, and 8 hours (T=2, 4 and 8, respectively) and bound virus was quantitated by qRT-PCR. Fold change was determined compared to background binding of virus to beads alone. Asterisks represent significant differences in fold change in virus precipitated at T=0 compared to other times: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Graphs are pooled from at least three independent experiments performed in duplicate and error bars represent the standard error of the mean.

**Figure 3.5: The effects of increased incubation time and temperature on additional neutralizing and non-neutralizing MAbs.** Serial dilutions of additional purified (**A**) strongly, (**B**) weakly, or (**C**) non-neutralizing MAbs were incubated with either J6/JFH1/Jc1 reporter virus (J6.103) or H77/JFH1 virus (H77.16, H77.31, H77.46, J6.27) for one hour at 37°C or 40°C. Virus-MAb mixtures were then immediately added to cells (T=0) or incubated for an additional 12 hours (T=12) at 37°C prior to infection of cells. Dose response curves are displayed relative to the infectivity of HCV in the absence of antibody for each of the conditions tested and represented by the following symbols: ■, T=0 h, 37°C; ▼, T=0 h, 40°C; ▲, T=12 h, 37°C. Graphs are pooled from at least three independent experiments performed in duplicate. Error bars represent the standard error of the mean. Asterisks represent significant differences in the EC50 value compared to that at time 0, 37°C: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Figure 3.6: Kinetic and temperature-dependent enhancement of neutralization by sera from HCV infected patients. A.** Chronic phase sera from HCV infected patients (H06, AA) or control sera (SM) was serially diluted and incubated with genotype 2a J6/JFH1/Jc1 luciferase reporter virus for one hour at 37°C or 40°C. Sera-virus complexes were then added directly to Huh7.5 cell monolayers (T=0) or incubated for an additional 12 hours at 37°C prior to infection of cells (T=12). Infectivity was assessed by luciferase expression 48 hours post-infection. Comparison of dose response curves by F test demonstrated a significant difference ($P < 0.0001$) in neutralization under different conditions by H06 and AA, but not by negative control sera (SM). **B.** Acute or chronic phase sera from HCV infected patients (S154, S112, S18 and S19) or control sera (SM) was serially diluted and incubated with H77/JFH1 chimeric virus for one hour at 37°C or 40°C. Sera-virus complexes were added to pre-seeded Huh7.5 cells (T=0) or incubated for an additional 12 hours at 37°C prior to infection of cells (T=12). Infectivity was assessed by FFU assay 72 hours post-infection. Comparison of dose response curves by F test demonstrated a significant difference ($P < 0.0001$) in neutralization under different conditions with S154, S112, S18 and S19 sera, but not with negative control serum (SM). Dose response curves are displayed relative to the infectivity of HCV in the absence of antibody for each of the conditions tested and represented by the following symbols: ■, T=0 h, 37°C; ▼, T=0 h, 40°C; ▲, T=12 h, 37°C. Graphs are pooled from at least three independent experiments performed in duplicate and error bars represent the standard error of the mean.

**Table 3.1. Kinetic parameters of MAb binding to sE2 at different temperatures**

| | $K_D$ (4°C) | $K_D$ (37°C) | Fold difference |
|---|---|---|---|
| H77.39-H77 E2 (1a) | $1.66 \times 10^{-7}$ | $4.69 \times 10^{-8}$ | 3.54 |
| H77.39-J6 E2 (2a) | $2.50 \times 10^{-7}$ | $4.84 \times 10^{-8}$ | 5.17 |
| J36-J6 E2 (2a) | $4.25 \times 10^{-8}$ | $8.91 \times 10^{-9}$ | 4.77 |

# Figure 3.1

# Figure 3.2

**A**



**B**



**C**



**D**



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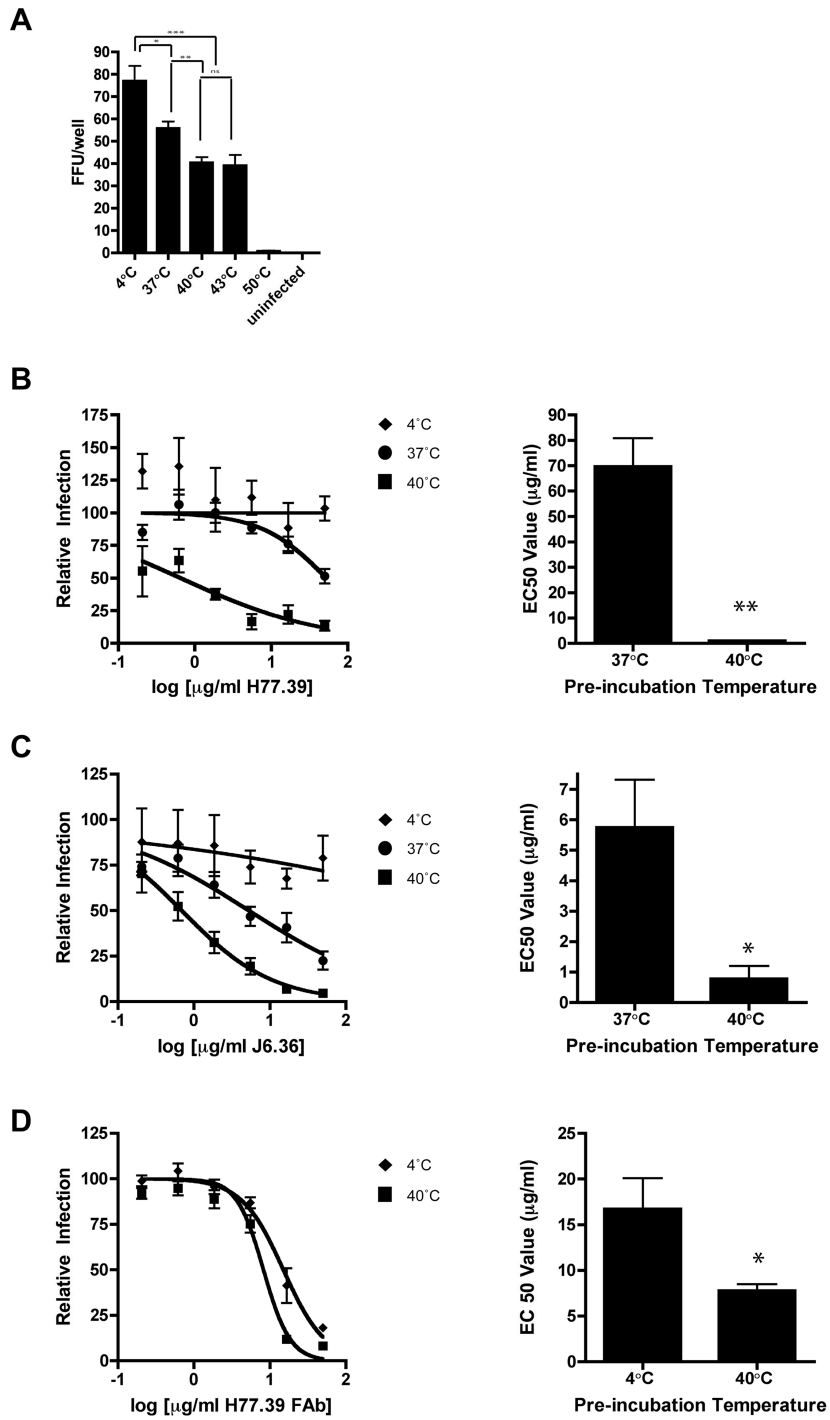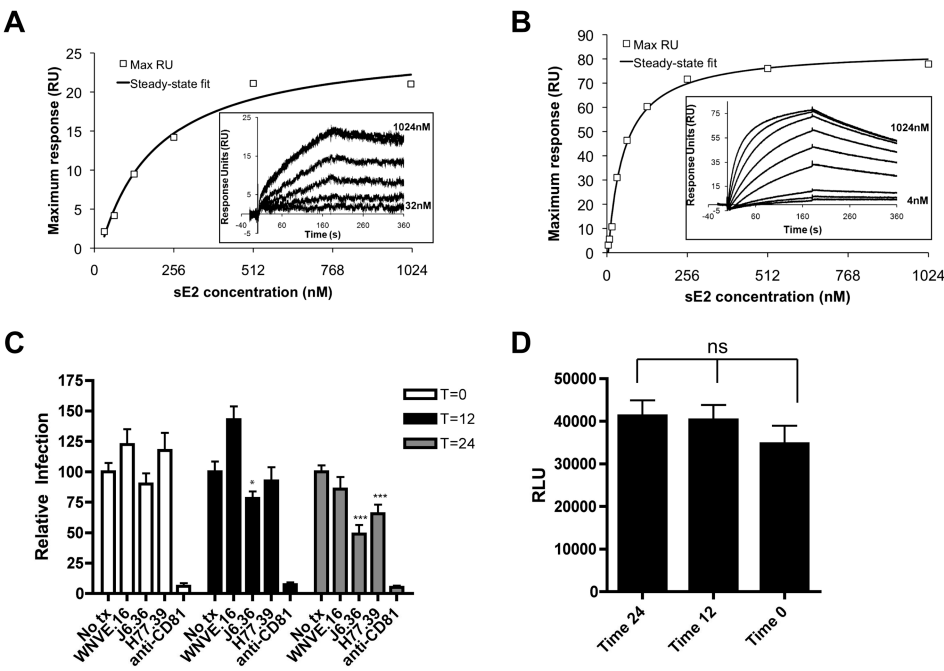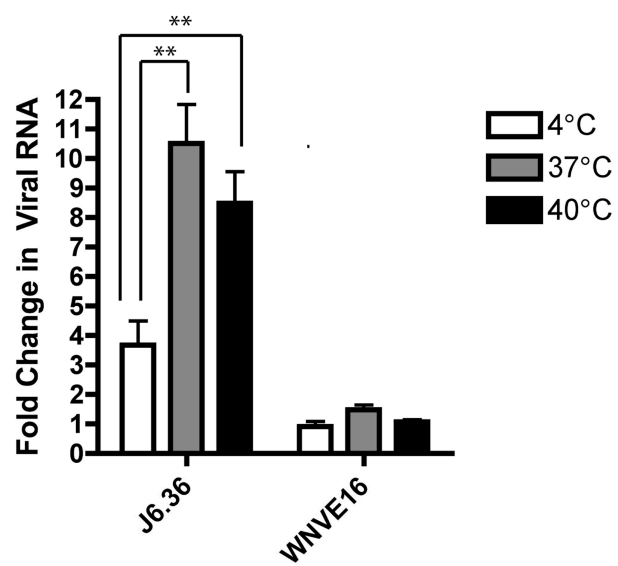
**Figure 3.3**

**Figure 3.4**

**Figure 3.5**

**Figure 3.6**

**A**    Genotype 1



**B**    Genotype 2



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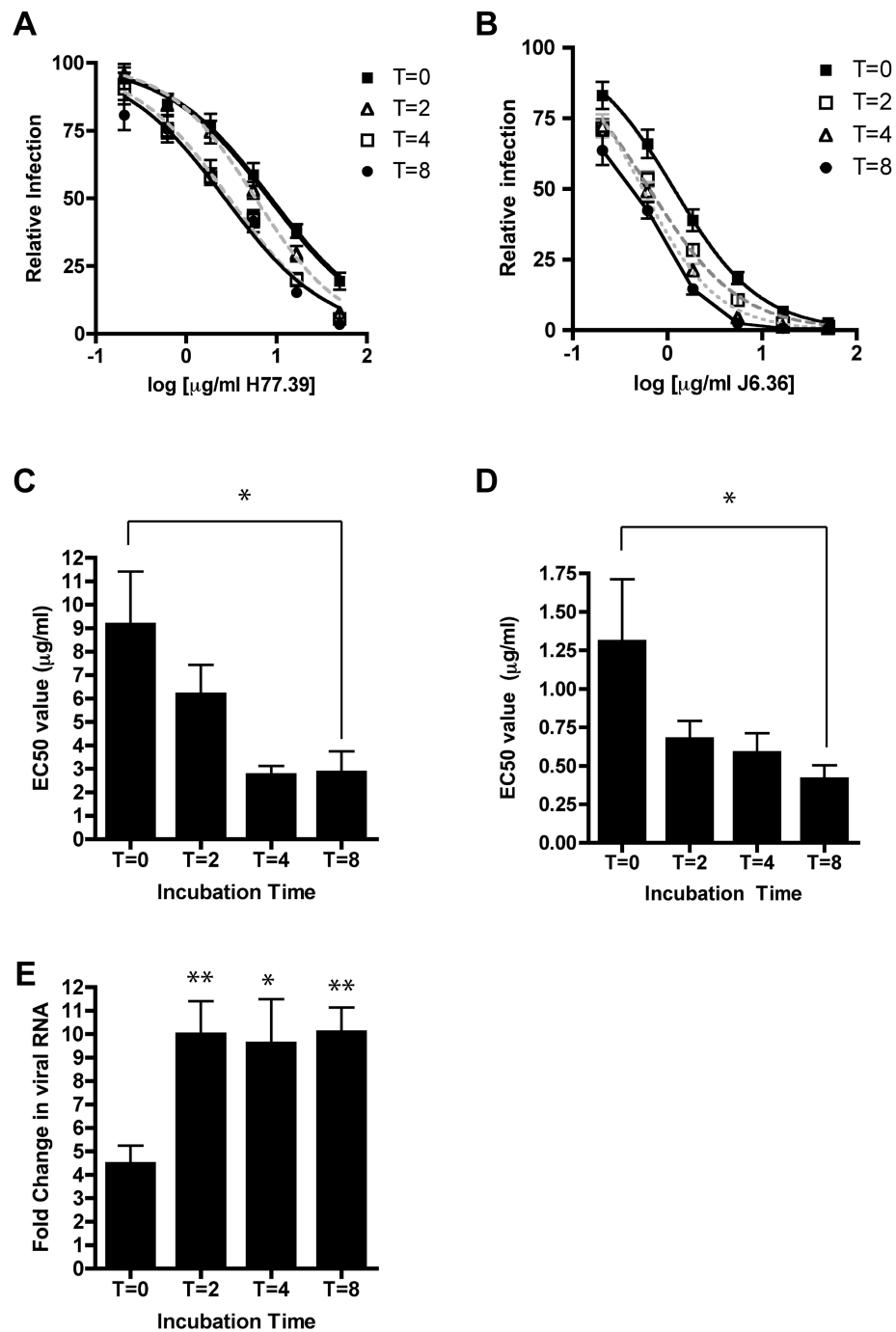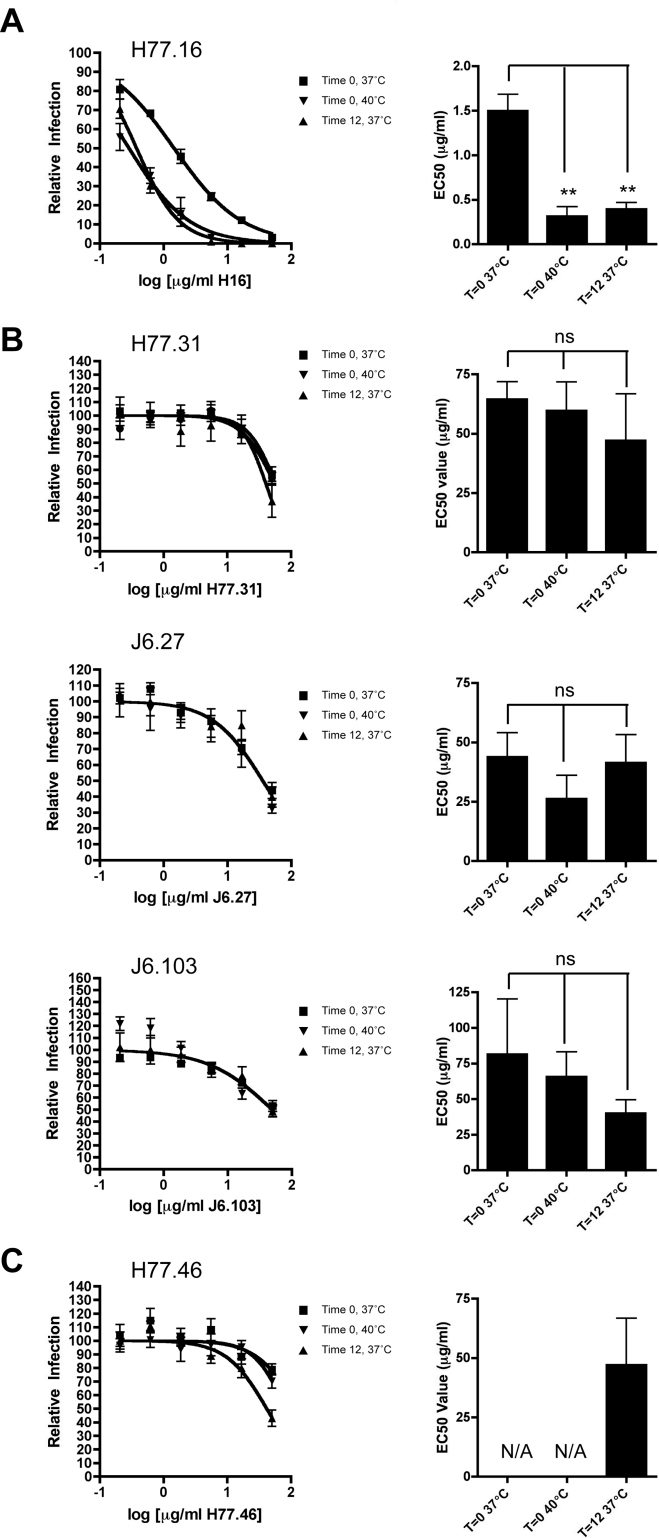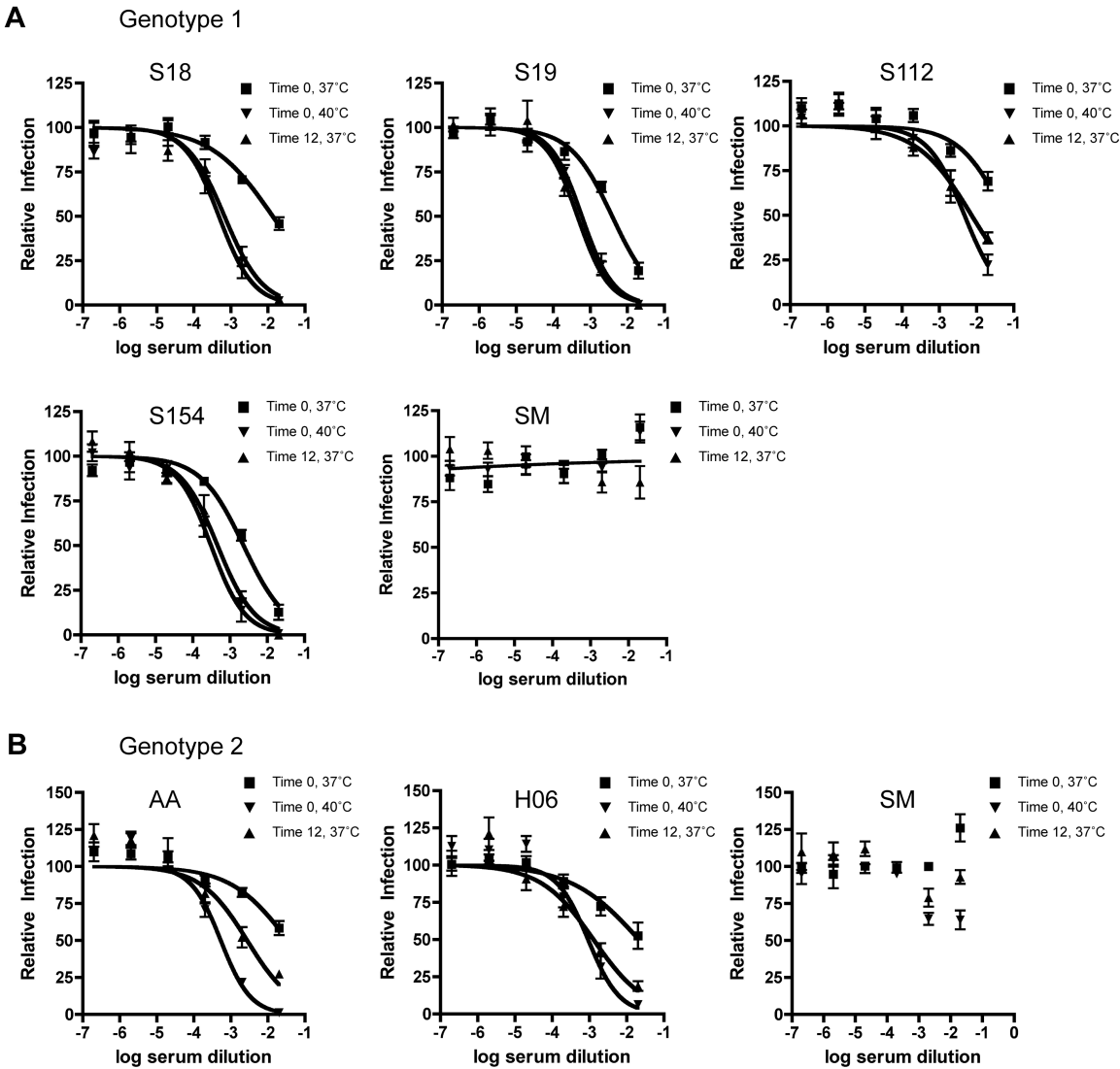## REFERENCES

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Chapter 4

Discussion

**ABSTRACT**

Although MAbs directed against HCV-E2 have been described previously (see Table 1, Chapter 1), analysis has been limited by a restricted culture system, the lack of an E2 crystal structure, and the absence of a tractable small animal model. In particular, the study of antibody neutralization largely have been limited to a single replicating strain or HCVpp, and extensive studies of pre- and post-attachment inhibition have not been performed. Furthermore, mapping of critical binding residues has been performed largely by site-directed alanine mutagenesis, which precludes the discovery of novel or conformational epitopes. In this thesis, we have generated a large panel of mouse anti-E2 MAbs, characterized their neutralizing capabilities, and mapped their critical binding residues using a novel library of E2 variants displayed on the surface of yeast. These studies have led to an enhanced understanding of the mechanics of antibody-mediated neutralization of HCV, as well as the structure of E2. An in depth analysis of these advances, as well as potential future studies, are discussed in the following sections.

**Anti-E2 MAbs neutralize at both pre- and post-attachment steps**

HCV entry is a complex process, requiring multiple attachment factors and receptors (11). In theory, neutralizing MAbs may block at one of several steps during and after viral attachment, yet few studies of post-attachment neutralization have been performed. Studies with distantly related flaviviruses suggest that MAbs inhibiting at a post-attachment step are highly potent, possibly due to the lower occupancy requirement required by these to MAbs block key entry steps (44). We hypothesized that identification of HCV-specific MAbs that neutralized at a post-attachment step, and mapping of their critical binding residues, would provide new insight that could inform future vaccine or therapeutic antibody development. Here, I identified three MAbs against HCV E2 (H77.16, H77.39 and J6.36), which neutralize at a post-attachment step. All three MAbs mapped to regions near or within the HVR1, suggesting that the HVR1 remains accessible after viral attachment and that MAbs that bind to this region can disrupt later steps in the entry pathway.

Post-attachment neutralization of HCV may prevent fusion or other requisite receptor interactions after attachment. Indeed, anti-SR-B1 and anti-CD81 antibodies inhibit infection after viral attachment to hepatocytes (3, 6, 18, 25, 56), thus suggesting two possible post-attachment steps at which HCV entry can be blocked. Coupled with antibody mapping data, determination of the mechanism of neutralization would aid in dissecting the precise steps of HCV entry and the critical E2 epitopes required for infection. To distinguish MAbs that prevent viral fusion from those that block earlier steps in viral entry, an attachment and entry assay with labeled MAbs could be performed as described in Thompson et al (50). In brief, virus is pre-bound to cells at 4°C, fluorescently labeled MAbs are subsequently added, the temperature is raised to 37°C to allow entry, and cells are assessed by fluorescence confocal microscopy. MAbs that inhibit viral fusion will be visualized intracellularly, whereas those that block infection at a post-attachment step but prior to entry will remain bound to virus on the cell surface. Alternatively, purified virus can be labeled directly with fluorescent dye, as described for other flaviviruses(36), which would allow for direct tracking of the virus in the presence and absence of MAb.

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If no MAbs that prevent viral fusion directly were identified in our panel, it may be necessary to generate additional MAbs for further study. One limit to our panel is that MAbs were generated and screened against sE2 and not infectious virus. Although successful, such an approach might limit the detection of conformational epitopes expressed only on whole virus; indeed, some MAbs that neutralize potently at a post-attachment step and recognize conformational epitopes exclusively on the virion and not on soluble West Nile Virus E protein have been identified (55). Thus, identification of novel epitopes utilized in viral entry and fusion might require further generation of MAbs against conformational forms of HCV. One such approach, described below, would involve generating B cell hybridomas from the lymphocytes of HCV infected patients and screening for MAbs by either viral trap ELISA or by neutralization assay.

**Neutralizing MAbs block binding to CD81 and SR-B1**

Although SR-B1 is well recognized as an HCV co-receptor (47), prior to our studies only one MAb, 9/27, had been shown to inhibit sE2-SR-B1 interactions (2, 47). Here, we identify five MAbs (H77.16, H77.31, H77.39, J6.36 and J6.103) that inhibit sE2 binding to SR-B1 by varying degrees; furthermore, three of these (H77.31, H77.39 and J6.36) also inhibited E2 binding to CD81, suggesting the CD81 binding region and HVR1 are in close proximity. Interestingly, the most potent inhibitor of sE2 binding to SR-B1 and CD81, H77.39, mapped to residues outside both the HVR1 and the CD81 binding motif (e.g., residues N415 and N417) (42). The ability of H77.39 to inhibit post-attachment binding to two HCV co-receptors, suggests the discovery of an "Achilles heel" for HCV. Although the CD81 binding domain is well protected (1, 20), H77.39 subverts these protective mechanisms by localizing to less shielded residues that are not directly involved in CD81 binding, yet still allow for antibody-mediated inhibition of CD81 binding. It is tempting to speculate that viral escape from H77.39 would be difficult due to i) the level of conservation of residues N415 and N417 between genotypes(19, 42); ii) poor viral fitness of cell culture viruses harboring mutations at N415(14); iii) my data demonstrating that H77.39 blocks

two HCV co-receptors, possibly limiting the ability of the virus to escape by altering receptor usage(21). Generation and assessment of variants resistant to H77.39 neutralization, however, will be necessary to begin to address these hypotheses.

One caveat to our receptor binding studies is they assess receptor interactions with sE2, rather than whole virus. E2 likely exists in close association with E1 on the viral surface (53), and receptor binding sites may be exposed differently on the virion compared to sE2. Indeed, we did not observe sE2 binding to CLDN-1 and OCLN in our unpublished studies, suggesting that both receptors may bind either conformational epitopes or alternate, surface exposed viral proteins. Indeed, a recent study demonstrating that OCLN could immunoprecipitate components of whole virus suggests that OCLN interactions require whole virus(32). To overcome these limitations, a receptor-binding assay using infectious virus or HCVpp will need to be developed. Attempts in our lab to study HCV-receptor binding utilized CHO cells expressing CD81, SR-B1, CLDN-1 or OCLN. Unfortunately, high levels of background binding of HCV to WT CHO cells made interpretation of the results of these assays challenging. Although sE2 displays no background binding to WT CHO (27, 46), HCV virus is known to associate with apolipoproteins and other serum components (34, 40) that may result in non-specific adsorption to CHO cells. Reduction of background may require identification of a cell line that shows less non-specific interaction with virus.


**Neutralization of infectious HCV representative of multiple genotypes**

HCV is highly divergent between genotypes, particularly in the HVR1 (48), as a result of it's error prone polymerase, chronic infection, geographic distribution, and high rate of replication (9, 35). Identification of target residues conserved between genotypes will be important in informing the design of novel therapeutics that could be directed at multiple HCV genotypes. To screen for MAbs with cross-binding capacity, we expressed E2 representative of all six HCV genotypes on the surface of yeast and screened for MAb binding by flow cytometry. Only 13 MAbs bound E2 derived from all six HCV genotypes, of which only 2 neutralized infectious virus,

suggesting that the conserved epitopes recognized by the other 11 MAbs are either not involved in viral entry or poorly accessible on the surface of the virion.

Of the two neutralizing MAbs with broad cross-binding potential, only H77.39 neutralized HCV strains representative of multiple genotypes. Until recently, only one strain of HCV (JFH1, genotype 2a) (31) had been successfully propagated in cell culture, and analysis of MAb neutralization of different genotypes was limited to HCVpp (4, 22, 28, 41, 43, 49), which are not believed to recapitulate the structural characteristics of infectious HCV(38, 53). To our knowledge, H77.39 is the first described MAb that neutralizes replicating virus representative of five out of six HCV genotypes. MAb H77.39 neutralized infection of chimeric viruses expressing structural proteins of genotypes 1, 2, 3, 4 and 5 in a dose-dependent manner, suggesting that the residues to which H77.39 maps (N415 and N417) are conserved and accessible on all genotypes. H77.39 did not neutralize chimeric virus representative of genotype 6, however this may be explained by the presence of a mutation at residue N417, which was necessary for cell culture adaptation(17), but is rarely observed in clinical isolates(19, 42).

As described above, H77.39 potently neutralizes at a post-attachment step and inhibits binding to SR-B1 and CD81. Furthermore mutation of its binding residue (N417) impairs viral fitness(14). Based on these findings, the H77.39 epitope would seem an excellent target for the host immune response. Interestingly, all previously characterized MAbs that localize to residue N417 were generated in mice (H77.39, AP33) or rats (3/11), raising the possibility that this epitope is not targeted or at best, sub-dominant in the human immune response. Indeed, previous studies with WNV demonstrate that the murine humoral response generates highly neutralizing antibodies targeted to the DIII lateral ridge epitope, whereas the human response produces less inhibitory antibodies that recognize the DII fusion loop(37); whether there are differences in epitope targeting of HCV between mouse and human remains to be explored. Interestingly, none of the published neutralizing MAbs generated from human lymphocytes map to residues N417 (see Table 4 in chapter 2 or in (46)), although to date too few human MAbs have been studied to draw general conclusions about the human humoral response to E2, and further studies are warranted.

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One difficulty in studying the human antibody response in general has been the inability to generate stable human lymphocyte hybridomas.  Instead, two alternative approaches have been used with other pathogens(5, 51): phage display of Fab fragments or EBV transformation of memory B cells and/or plasmablasts. Recently, however, an efficient novel fusion partner for human lymphocytes derived from either peripheral blood or lymph nodes has been described (23).  In collaboration with the Trakht laboratory at Columbia University, we have identified several strongly neutralizing human MAbs against HCV (data not shown). In future studies, the Trakht and Diamond laboratories plans to characterize anti-E2 human MAbs generated from the lymphocytes of intravenous drug users (IDUs) who rapidly clear infection and have strong and broadly cross-neutralizing serum antibody responses (7, 39).

**Mapping of neutralizing MAbs provides novel insights into the structure of E2**

A major limitation in the study of neutralizing MAb-HCV interactions is the lack of an atomic resolution structure of E1, E2, or E1-E2.  Previous mapping studies of anti-E2 MAbs utilized an ELISA-based peptide mapping approach (4, 12) or alanine scanning of recombinant E1E2 by ELISA (22, 28, 43, 49).  Although useful for some MAbs, this method is most effective for a subset of antibodies that bind linear determinants. Furthermore, there is also a concern that the structure that short peptides adopt in solution or the solid phase may not reflect the structure of a more complex domain or intact protein. Finally, these studies rely on alanine scanning and thus, are biased in the selection of potential mutants. In the studies described in my thesis, I utilized a novel, unbiased mapping strategy in which random mutations were introduced into recombinant E2 displayed via a linker on the surface of yeast and loss of binding mutants were identified by flow cytometry. Using this strategy we identified two neutralizing MAbs, H77.16 and J6.36, which map to residues over 150 amino acid residues apart; H77.16 maps to residues in the HVR1 (G406, N410, I411) and the putative CD81 binding region (G530) and J6.36 maps to the HVR1 (G397, F403, G406) as well as residue R572. In the context of a recently proposed model of E2 (26), this mapping data suggests that the HVR1, which is predicted to be an

unstructured extension of DI, actually lies between the putative D1 and DIII in the conformational virion. Localization of the HVR1 between DI and DIII, both of which contain portions of the CD81 binding motif, also supports previous studies suggesting that the CD81 binding region is obscured by the HVR1 (1).

A major limitation to our mapping strategy is that E2 is expressed on the yeast in the absence of E1. E1 and E2 are predicted to form a heterodimer and be tightly linked on the viral surface (53), although the conformation of these proteins as a heterodimer is unknown. Discovery of MAbs that map to residues within both proteins would provide insight into the structure of E1-E2 as well as allow for the identification of novel epitopes. Mapping could be performed by generation of viral escape mutants, which would also allow for the identification of conformational epitopes.

Generation of viral escape mutants also could provide important information regarding the mechanism of viral escape from immune pressure. Recent studies with influenza have demonstrated that viral escape can be mediated by changes in receptor affinity resulting from mutations distant to the MAb binding site, such that MAb binding is preserved but neutralization is lost (21). Given its requirement for multiple receptors, it is possible that HCV can escape immune pressure by mutating amino acid residues that alter receptor usage. Indeed, although HCV is initially attenuated in HVR1 deletion mutants, titers eventually rise suggesting the incorporation of adaptive mutations that compensate for the inability to interact with SR-B1 (13, 45). To directly address whether HCV can evade antibody neutralization by altering receptor usage, escape mutants could be made with different MAbs. Mutations that result in loss of neutralization could then be cloned into E2 and MAb binding to the E2 variants assessed. Mutations that result in a loss of MAb neutralization yet retain binding to the complementing mutant E2 protein would suggest that viral escape was due to mutations that did not directly alter MAb binding. Testing for changes in receptor binding and affinity could then be used to assess the mechanism of escape by variants of interest.

**Exposure of E2 epitopes and neutralization is altered by changes in temperature**

Antibody neutralization of flaviviruses is governed by both MAb affinity and epitope availability (10, 24). Although it would be predicted that epitopes hidden in the conformational structure of the virus would not be sufficiently available for neutralization, multiple studies with both enveloped and non-enveloped viruses have suggested that cryptic epitopes can become transiently exposed, suggesting that viral particles are in constant, dynamic motion (8, 29, 30, 33). Recent studies with flaviviruses demonstrate that increasing pre-incubation time and temperature enhances exposure of obscured epitopes, resulting in improved MAb function (8, 33). Although a member of the *Flaviviridae*, HCV has unique properties that could limit viral movement, including 15 predicted N-linked glycosylation sites on E1 and E2 (15, 16), 9 intramolecular disulfide bonds (26), and covalent interactions between E1 and E2 (53). In these studies, we demonstrate that, despite these constraints, antibody neutralization of HCV is enhanced when pre-incubation time and temperature are increased, suggesting that HCV does undergo some degree of dynamic motion or changes in conformation akin to those described for flaviviruses (8, 33). Interestingly, two anti-HCV E2 MAbs, H77.31 and J6.27, which map to a linear epitope in the CD81 binding region, did not demonstrate enhanced neutralizing capability under any conditions tested, suggesting that a conformational change, rather than small transient movements, are involved in exposure the CD81 binding site. Indeed, Tscherne et al have demonstrated that HCV can only undergo "fusion from without" after prior binding to the target cell, suggesting that HCV undergoes significant conformational changes during attachment and entry (52). Cryo-EM studies and resolution of the envelope glycoprotein crystal structures will be critical in proving these hypotheses.

We confirmed that increased pre-incubation time and temperature enhanced the neutralizing capabilities of acute and chronic phase sera from HCV infected patients, suggesting that the time spent circulating in the bloodstream prior to productive infection, as well as the presence or absence of fever, may modulate the potency of the humoral response *in vivo*. Whether this can be extrapolated to other viruses or to *in vivo* models remains to be tested,

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although it is tempting to speculate that one function of fever (which also can be induced by interferon-α treatment) (57) is to enhance the effectiveness of the MAb response. Furthermore, these results suggest that *in vitro* neutralization studies may not be entirely predictive of antibody efficacy *in vivo*. Indeed, previous studies in our laboratory have demonstrated that effector functions significantly modulate *in vivo* protection of antibodies (54).

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