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

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A B S T R A C T
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, 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 °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.

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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, which resulted in sustained virologic response in only ~50% of individuals (Bowen and Walker, 2005). 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 (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011; Zeuzem et al., 2011). Given that long-term pharmacological therapy may have limitations in curing HCV-infected individuals, especially in resource-poor settings, there is renewed interest in the development of preventative or even therapeutic vaccines (Strickland et al., 2008). 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 (Lindebach et al., 2007). 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) (Lindenbach and Rice, 2005). Cell culture-produced HCV forms smooth, spherical, enveloped particles that are ~60 nm in diameter (Gastaminza et al., 2010; Yu et al., 2007) 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 (Krey et al., 2010), E2 is distinguished from flavivirus E protein by its nine intramolecular disulfide bonds (Krey et al., 2010), covalent linkage to E1 (Vieyres et al., 2010), 11 N-linked glycosylation sites (Goffard et al., 2005; Goffard and Dubuisson, 2003), and two hypervariable regions (HVR1 and HVR2) (McCaffrey et al., 2007; Weiner et al., 1991). E2 contains binding sites for both the CD81 and SR-B1 receptors (Pileri et al., 1998; Scarselli et al., 2002), and MAbs that block CD81-E2 and SR-B1-E2 interactions prevent infection in cell culture (Bartosch et al., 2003; Hadlock et al., 2000; Law et al., 2008; Owsianka et al., 2001, 2008; Sabo et al., 2011; Tarr et al., 2006).
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 (Farci et al., 1996; Houghton and Abrignani, 2005; Law et al., 2008). Antibodies elicited by immunization of chimpanzees with HCV envelope proteins partially protect against viral challenge (Forns et al., 2000; Meunier et al., 2011; Puig et al., 2004). 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 (Netski et al., 2005). Many humans generate a neutralizing antibody response that correlates with viral clearance although chronically infected patients also produce neutralizing antibodies (Logovnioff et al., 2004). 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) shields virions from recognition by neutralizing antibodies that are present in serum (Bartsch et al., 2005; Drexux et al., 2006; Lavillette et al., 2005), (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 (Dowd et al., 2009; von Hahn et al., 2007).

Antibody-mediated neutralization of Flaviviridae family members requires engagement by antibodies with a stoichiometry that exceeds a particular threshold (reviewed by (Dowd et al., 2011)). 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 epitopes bound by antibody molecules at a given concentration of antibody (Dowd and Pierson, 2011; Klasse and Sattentau, 2002). For flaviviruses (e.g., WNV), the envelope proteins are arranged with T=3 icosahedral symmetry on the virion surface and displayed in three distinct environments defined by their proximity to the 2-, 3-, or 5-fold axis of symmetry. Because of this, the minimum occupancy requirement for neutralization by a given antibody may never be achieved (reviewed in (Diamond et al., 2008)). Despite this, MAbs that bind to epitopes that are predicted to be cryptic can still neutralize infection (Lok et al., 2008; Oliphant et al., 2006; Stasny et al., 2006). 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 (Dowd et al., 2011).

Although HCV E2 is predicted to have a structure similar to flavivirus E proteins (Krey et al., 2010), the organization of the HCV virion and exposure of specific epitopes under conditions of dynamic motion remains unclear. Furthermore, the association of the HCV with apolipoproteins (Meunier et al., 2008; Owen et al., 2009), the glycans shield, and additional intramolecular and intermolecular disulfide linkages on E2 also could impose limits on 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 (Sabo et al., 2011).

**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 (Dowd et al., 2011; Lok et al., 2008). As HCV envelope proteins have a greater number (9 versus 6) of intramolecular disulfide bonds (Gubler et al., 2007; Krey et al., 2010), 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 °C, 37 °C, or 40 °C and 43 °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 (Sabo et al., 2011) were incubated with a genotype 2a J6/JFH1/Jc1 luciferase reporter virus (Sabo et al., 2011) 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 °C for infection. Neutralization by H77.39 and J6.36 was abolished at 4 °C and improved at 40 °C (Figs. 1B and C). Comparison of the EC50 values at 37 °C and 40 °C also was statistically different (Figs. 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 describing antibody-virus aggregation (Thomas et al., 1986). 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 °C or 40 °C) period and throughout the experiment (at 37 °C), which explains why the 4 °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 MAbs, kinetic parameters were not defined although a qualitative assessment indicated extremely long half-lives at both temperatures (Figs. 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 h only partially restored 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. 3), 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. 3, *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
Fig. 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 raised to 37 °C, and infectivity assessed 72 h 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 virus and 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 h 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 (right panels) 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.
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 (Dowd et al., 2011). 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 h prior to infection. Increasing the pre-incubation period consistently improved neutralization potency (Figs. 4A and B), with a significant reduction in EC50 values occurring after 8 h of pre-incubation (Figs. 4C and D; *P < 0.05). To determine whether this effect was due to enhanced MAb-virus binding, complexes were

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Table 1

<table>
<thead>
<tr>
<th>Kinetic parameters of MAb binding to sE2 at different temperatures.</th>
<th>$K_D$ (4 °C)</th>
<th>$K_D$ (37 °C)</th>
<th>Fold difference</th>
</tr>
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<tr>
<td>H77.39-H77 E2 (1a)</td>
<td>1.66 x 10^{-7}</td>
<td>4.69 x 10^{-8}</td>
<td>2.82</td>
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<tr>
<td>H77.39-J6 E2 (2a)</td>
<td>5.28 x 10^{-7}</td>
<td>1.28 x 10^{-8}</td>
<td>3.74</td>
</tr>
<tr>
<td>J36-J6 E2 (2a)</td>
<td>4.25 x 10^{-9}</td>
<td>8.91 x 10^{-10}</td>
<td>1.00</td>
</tr>
</tbody>
</table>

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.

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Fig. 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 maximum 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 or 24 h (T=12, gray bars) or for an additional 12 h (T=24, black bars) at 4 °C prior to infection. Cells then were infected with MAb-virus complexes for one hour at 4 °C, washed at 4 °C to remove unbound virus, and infectivity detected by luciferase assay 48 h later. 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.

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Fig. 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 (gray 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.
formed for up to 8 h prior to immunoprecipitation, and viral RNA was quantified by qRT-PCR. Notably, the amount of virus precipitated was increased after 2, 4 and 8 h 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 (Sabo et al., 2011). 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 h) (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) (Sabo et al., 2011), 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

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**Fig. 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 h 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 h (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.
Fig. 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 h (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.
H77.31, which map to residues in the CD81 binding region, or J6.103, which maps to the HVR1 and the intergenotypic variable region (Krey et al., 2010; McCaffrey et al., 2007; Sabo et al., 2011), 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 of HCV was observed after pre-incubation at 40 °C for one hour or 37 °C for 12 h with sera from patients acutely infected with genotype 1 (S154) or chronically infected with genotype 1 (S112, S18, S19, H06) and genotype 4 (AA) (Figs. 6A and B, \( P < 0.0001 \), F test). However, sera from several of the patients (S154, S112, S18 and S19) infected with genotype 1 failed to cross-neutralize the genotype 2 HCV strain, indicating the inhibitory effect was antigen-specific (data not shown). Also, 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 (Diamond et al., 2008; Pierson et al., 2007)). Nonetheless, some antibodies neutralize infection despite binding epitopes that ostensibly are obscured on the virion, at least based on contemporary high-resolution structural models (Dowd et al., 2011; Lewis et al., 1998; Li et al., 1994; Lok et al., 2008). 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 (Dowd et al., 2011; Li et al., 1994; Lok et al., 2008) and time of interaction (Dowd et al., 2011; Ruprecht et al., 2011). 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 restrict motion and exposure of hidden epitopes. Indeed, unlike flaviviruses, HCV is resistant to inactivation at low pH in solution and does not undergo plasma membrane fusion from without (Tscherne et al., 2006). These observations may be explained by recent evidence suggesting covalent linkage between the glycoproteins on the virion surface (Vieyres et al., 2010), a feature that would affect structural protein packing, limit viral breathing, and impact transient exposure of some epitopes. Nonetheless, in our study, we showed that increasing the time and temperature of pre-incubation enhanced neutralization (Dowd et al., 2011). 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°C to 43°C) only minimally impacted infectivity (see also (Ciesek et al., 2010; Kim et al., 2011)), 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 at 4°C for several weeks without appreciable loss of infectivity (Ciesek et al., 2010) and exposure to a pH 5.0 acidic solution does not expose domes on the envelope proteins that result in adventitious fusion and virus inactivation (Tscherne et al., 2006). 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°C and 37°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, which 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-linked glycosylation sites augments the inhibitory capacity of antibodies disrupting E2-CD81 interactions (Bankwitz et al., 2010; Helle et al., 2010). 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 (Kwong et al., 1998; Rizzuto et al., 1998). 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 (Bertaux and Dragic, 2006; Haberstroh et al., 2008; Sabo et al., 2011). Interestingly, the MAb J6.103 also did not demonstrate enhanced potency, despite mapping to the same residues as a MAb that did, J6.36 (Sabo et al., 2011), 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 (Sabo et al., 2011), 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 (Logvinoff et al., 2004). 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-α (Zeuzem et al., 2000). 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 (Dowd et al., 2011; Lok et al., 2008). Nonetheless, portions of the 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.

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 (Millipore) 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 TTC ATC TTA GCA CTA GTC-3′; Reverse primer: 5′-AGT ATC GGC ACT CTC TGC AGT-3′; and probe 6FAM-5′-CGC CCG GAC CCT 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 (J5-81) 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 × 10^5 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 fixed 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 × 10^5 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 h 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 min 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 h or 72 h 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 h prior to infection of cells. Infectivity was assessed by luciferase assay or FFU assay 48 h or 72 h 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 MAbs-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 1000 × g for 2 min 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 MAbs-virus binding over longer pre-incubation periods the immunoprecipitation 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 h 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 quantitated 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-Kβ) were coupled covalently to a CM5 sensor chip using amine chemistry. Increasing concentrations of monomeric soluble E2 (4 to 1024 nM) were flowed over the chip in 10 mM 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 40 μl/min for 180 s 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 KD 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.

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References


