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Selection Pressure from Neutralizing Antibodies Drives Sequence Evolution during Acute Infection with Hepatitis C Virus

Kimberly A. Dowd¹, Dale M. Netski¹, Xiao-Hong Wang³, Andrea L. Cox^{1,2}, and Stuart C. Ray¹

¹Department of Medicine, Johns Hopkins Medical Institutions, Baltimore, MD, 21205, USA

²Department of Oncology, Johns Hopkins Medical Institutions, Baltimore, MD, 21205, USA

³Southwest Hospital, Third Military Medical University, Chongqing, Peoples Republic of China, 400038

Abstract

Background & Aims—Despite recent characterization of hepatitis C virus-specific neutralizing antibodies, it is not clear to what extent immune pressure from neutralizing antibodies drives viral sequence evolution in vivo. This lack of understanding is particularly evident in acute infection, the phase when elimination or persistence of viral replication is determined and during which the importance of the humoral immune response has been largely discounted.

Methods—We analyzed envelope glycoprotein sequence evolution, and neutralization of sequential autologous hepatitis C virus pseudoparticles in eight individuals throughout acute infection.

Results—Amino acid substitutions occurred throughout the envelope genes, primarily within the hypervariable region 1 of E2. When individualized pseudoparticles expressing sequential envelope sequences were used to measure neutralization by autologous sera, antibodies neutralizing earlier sequence variants were detected at earlier time points than antibodies neutralizing later variants, indicating clearance and evolution of viral variants in response to pressure from neutralizing antibodies. To demonstrate the effects of amino acid substitution on neutralization, site-directed mutagenesis of a pseudoparticle envelope sequence revealed amino acid substitutions in hypervariable region 1 that were responsible for a dramatic decrease in neutralization sensitivity over time. In addition, high-titer neutralizing antibodies peaked at the time of viral clearance in all spontaneous resolvers, while chronically evolving subjects displayed low-titer or absent neutralizing antibodies throughout early acute infection.

Conclusions—These findings indicate that during acute hepatitis C virus infection in vivo, virus-specific neutralizing antibodies drive sequence evolution and, in some individuals, play a role in determining the outcome of infection.

Introduction

The World Health Organization estimates that 170 million persons are infected with the hepatitis C virus (HCV) worldwide, of which four million are in the United States.^{1,2} While ~30% of acute HCV infections are spontaneously resolved, the majority progress to chronic infection. Persistent viremia can lead to complications such as cirrhosis and hepatocellular carcinoma, making HCV a major cause of liver disease worldwide.^{3,4}

Correspondence: Stuart C. Ray, M.D., Center for Viral Hepatitis Research, Johns Hopkins University School of Medicine, 855 North Wolfe St, Suite 530, Baltimore, MD 21205, phone: (410) 614-2891, fax: (410) 614-7564, sray@jhmi.edu.

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The HCV genome encodes a mutation prone polymerase, resulting in the existence of the virus as a quasispecies, defined as a collection of genetically related but distinct viral variants.⁵ The capacity of the virus to mutate continually most likely contributes to the establishment of chronicity, as variants that escape immune responses have a survival advantage. Little is known regarding the role of HCV-specific neutralizing antibodies (nAb) in modulating HCV pathogenesis or driving viral sequence evolution. Establishment of HCV glycoprotein-bearing retroviral pseudoparticles (pp) has only recently allowed for detailed studies of the nAb response,^{6,7} the majority of which used HCVpp expressing heterologous envelope sequences, usually the reference strain H77.^{8–13} The considerable heterogeneity of HCV, particularly within the envelope genes, may distort results from heterologous assays, resulting in an under-representation of nAb responses. To date, only a small number of neutralization studies have used HCVpp expressing autologous, or person-specific, envelope sequences: two in the setting of single-source HCV outbreaks,^{14,15} and four studies of the nAb response in patient H, a well-studied individual from whom the H77 reference strain originated.^{11,13,16,17}

During the acute phase, antibodies to heterologous HCV envelope proteins have been shown to appear later and at lower titers compared to antibodies directed against non-structural proteins, suggesting that nAbs may play only a minor role in spontaneous resolution.^{10,13} However, the rapid evolution and greater variability of the envelope genes compared to the rest of the HCV genome suggests that the circulating viral quasispecies is modulated by ongoing humoral immune pressure. It is possible that the use of autologous HCVpp is necessary to detect strain-specific antibodies appearing during acute infection. In support of this hypothesis, autologous HCVpp studies reported correlations between nAb responses in acute HCV with both control of viremia¹⁴ and spontaneous resolution,¹⁵ associations not reported with heterologous antigen-based assays.

To assess the impact of immune pressure exerted by HCV nAb responses on viral sequence evolution, we measured neutralization of subject-specific HCVpp in an autologous setting. Our results provide strong evidence that HCV nAb responses in acute infection have a direct impact on viral sequence evolution and that spontaneous resolution of the virus may be associated with the magnitude of the nAb response.

Materials and Methods

Participants

Blood samples were obtained from consenting HCV-infected adults participating in a prospective study of young intravenous drug users as previously described.¹⁸ At each visit, participants were provided counseling to reduce the risks of drug use. Blood was drawn for isolation of serum, plasma, and PBMC in a protocol designed for monthly follow-up. Serum and plasma were stored at -80°C . The study protocol was approved by the institutional review board of the Johns Hopkins School of Medicine

HCV envelope sequences and nAb responses were studied in eight subjects (Table 1). All subjects were initially infected with a genotype 1a virus, except s11, who was infected with genotype 1b. Subjects s11 and s26 did not demonstrate clear outcomes of their primary infections (described in results), however their secondary infections resulted in persistence and clearance, respectively.

HCV testing protocol

Details of the HCV testing protocol have been described elsewhere.¹⁸ Briefly, serum or plasma samples were tested for the presence of HCV-specific antibodies using the commercially available Ortho version 3.0 ELISA (Ortho Clinical Diagnostics) to identify seroconverters.

Then, HCV RNA testing was performed on samples collected prior to seroconversion to determine the onset of viremia, and after seroconversion to evaluate the outcome of infection. Quantitative RNA detection was performed with the COBAS AMPLICOR HCV Monitor version 2.0 and the COBAS TaqMan 48 assays (both from Roche Molecular Systems) which have limits of detection of $2.8 \log_{10}$ IU/mL and $1.2 \log_{10}$ IU/mL, respectively. Qualitative RNA detection was performed with the COBAS AMPLICOR Hepatitis C Virus Test version 2.0 (Roche Molecular Systems), which has a limit of detection of $1.7 \log_{10}$ IU/mL. Negative samples were confirmed using either the Roche qualitative test or the Taqman assay. Strain-specific HCV clearance was defined as the presence of anti-HCV antibody with HCV RNA undetectable in serum or plasma specimens from at least two consecutive visits that were obtained at least 300 days after the initial detection of viremia. Persistence was defined as the persistent presence of anti-HCV with HCV RNA detectable in serum or plasma specimens that were obtained at least 300 days after initial viremia, and additionally confirmed by phylogenetic analysis of viral sequences throughout follow-up.¹⁸ The date of initial viremia was estimated as the midpoint between the last HCV RNA negative specimen and the first HCV RNA positive specimen. For subjects who entered the study as HCV RNA positive, HCV antibody negative, that date was considered the initial infecting date. When applicable, the date of clearance was estimated as the midpoint between the last HCV RNA positive specimen and the first HCV RNA negative specimen. Genotype was determined by performing phylogenetic analysis on Core-E1 region sequences as previously described.^{18,19}

Cloning of HCV sequences

Hemigenomic HCV clones—The 5.2-kb region from the 5' UTR to the NS3/NS4a junction was amplified from serum or plasma, and archived as ~40 individual clones, as described previously.²⁰ For each test-visit, 20–34 clones were sequenced through a 450 nt region spanning the E1E2 junction for hypervariable region 1 (HVR1) consensus determination and clonal sequence analysis.

E1E2 clones—A region including the last 27 aa of Core through the end of E2 was PCR amplified from hemigenomic clones with HCV-specific primers (Supplementary Table 1). PCR products were cloned into the expression vector pcDNA3.2/V5/Dest (Invitrogen) using Gateway technology. Sequences were assembled into contigs using Aligner (CodonCode Corporation). All sequences have been deposited into Genbank/EMBL/DDBJ with accession numbers _pending_.

E1E2 Site directed mutagenesis clones—Mutagenesis clones were created with the QuikChange® II XL site-directed mutagenesis kit (Stratagene). Primers were designed using the Stratagene webtool [<http://www.stratagene.com/qcprimerdesign>]. PCR reactions were performed according to the manufacturer's instructions.

Pseudoparticle production and neutralization assays

Pseudoparticles containing the luciferase reporter gene were generated as described elsewhere. 7×10^{13} Pooled virus collected at 48 and/or 72 hours was either used immediately for transduction of target cells or stored at -80°C . Neutralization assays were performed as previously described.²¹ Briefly, two-fold dilutions of heat-inactivated serum/plasma samples were incubated with pp for 1 hour at 37°C , added to Hep3B hepatoma cells in quadruplicate wells of a 96-well plate for 5 hours, followed by measurement of luciferase activity 72 hours post-infection. Pseudoparticle infection resulting in luciferase activity was measured in terms of relative light units (RLUs) in the presence of test-plasma (RLU_{test}) versus average infection in the presence of two or three separate HCV-negative specimens ($\text{RLU}_{\text{control}}$). Percent neutralization was calculated as $100 \times [1 - (\text{RLU}_{\text{test}}/\text{RLU}_{\text{control}})]$. Results are reported as % neutralization, or as 50% inhibitory dilution (ID_{50}) values, the dilution of test-plasma that

resulted in $\geq 50\%$ decrease in pp infectivity. As a control, all test-plasma neutralized murine leukemia virus (MLV) pp $\leq 30\%$.

Statistical Analysis

P values and R^2 values were determined with SigmaStat (Systat Software, Inc.), using either linear regression analysis or a Student *t* test. A *P* value of $<.05$ was considered significant.

Results

Study Subjects

We obtained E1E2 envelope sequence data and assessed autologous nAb titers in eight subjects during acute HCV infection (Table I). For each subject, sequence information was obtained for at least two test-visits separated by 2.1–25.2 months, with the first visit representing initial or early viremia, between 0.2–2.9 months after the estimated date of infection (Figure 1). Envelope sequences were used to create HCVpp, and the ability of autologous plasma samples to neutralize the resulting HCVpp was assessed (Figures 2 and 3).

To verify if sequential sequences amplified from each subject represented ongoing evolution from an initial infecting virus, genotyping and clonal sequencing were performed on Core-E1 and E1–E2 amplicons, respectively, from test-visits and additional visits, one of which represented first detectable viremia. Sequence analysis confirmed that for six of eight subjects, the viral quasispecies at initial HCV RNA detection was phylogenetically related to virus detected throughout follow-up, confirming a single, distinct infection.

Subjects s11 and s26 demonstrated “unclear” clinical courses in which phylogenetically distinct viral strains were detected at subsequent visits. Genotype 1b virus was detected in s11 from initial viremia until 14.9 months, however at the following visit (16.0 months), genotype 1a virus was detected. Similarly, genotype 1a virus was detected in s26 from initial viremia until 2.6 months, followed by detection of genotype 1b at the subsequent visit (3.4 months). For s11, both test-visits occurred during the primary infection (0.2 and 9.3 months), therefore E1E2 sequences represent genotype 1b variants. However, s26 test-visits correspond to time points during both the primary and secondary infections (0.5 and 3.4 months), and therefore represent genotypes 1a and 1b, respectively. Because of the lack of an HCV RNA negative visit separating the primary and secondary infections, both superinfection and clearance followed by reinfection remain possibilities. We therefore refrain from referring to s11 and s26 as spontaneous resolvers and omit them from any associated analyses.

Sequence changes accumulate in envelope genes during acute infection

Amino acid substitutions that occurred between test-visits are shown in Figure 1. For each subject, E1E2 sequences are depicted by horizontal lines, with substitutions represented by connecting vertical lines. With the exception of s13 (1.3 month) test-visit, for which we were unable to produce infectious HCVpp, each sequence represents the envelope clone used for assessing autologous neutralization. Sequences from s26, which represent genotype 1a and 1b variants, are shown to illustrate the degree of heterogeneity between subtypes, but were omitted from analyses of sequence evolution. Substitutions were scattered throughout E1E2, however most were located within HVR1. The number of mutations that occurred between sequenced visits ranged from 2 (s11) to 16 (s28), however infrequent sampling and different sampling intervals among the subjects precludes any detailed analyses of mutation rates.

Since the envelope clones used to create HCVpp are single viral isolates from each visit, we assessed how well the sequences represented the corresponding quasispecies. Since the majority of substitutions occurred within HVR1, E1E2 amplicons from ≥ 20 hemigenomic

clones were used to generate an HVR1 consensus for each test-visit. Sequence comparisons demonstrated that 12 of 16 HCVpp clones exactly matched their corresponding consensus and that the remaining four only differed by ≤ 2 amino acid residues (Supplementary Figure 1). In summary, multiple amino acid substitutions occurred throughout the envelope genes, primarily within HVR1, during acute infection with HCV. Sequence analysis confirmed that our HCVpp envelope clones represented the corresponding viral quasispecies.

Neutralization of autologous HCVpp shows ongoing escape from the humoral immune response

HCVpp infectivity levels in Hep3B target cells are shown in Figure 2, compared to mock (empty vector) and MLV envelope controls. Because titrations of selected HCVpp, as well as comparisons of freshly harvested versus freeze-thawed virus (resulting in ~50% decrease in infectivity) gave equivalent results these levels of infectivity were sufficiently high to ensure that the antibody concentration rather than the pp concentration in the assay was limiting.

Neutralization by acute-phase plasma specimens was measured using autologous HCVpp (Figure 3). For subjects with sequential HCVpp, neutralization was detected against the earlier HCVpp variant prior to, or at a greater magnitude than, the later HCVpp variant. In multiple subjects (s18, s28, s29, and s110), neutralization of the later HCVpp variant corresponded with the emergence of that sequence in the quasispecies. Clonal sequencing available for s29 (13.7 month) revealed that the HVR1 consensus at this additional visit was identical to the 1.9 month consensus, demonstrating that the HVR1 remained unchanged for the first ~14 months of infection and implying that the initial changes must have occurred between 13.7 and 24.7 months. These sequencing results help to explain the lack of detectable nAbs for the first 24 months, and demonstrate an association in s29 between HVR1 evolution and the appearance of nAbs. Overall, these results provide evidence of viral sequence evolution occurring as a direct response to immune pressure from nAbs.

Measurement of autologous neutralization is more sensitive than heterologous neutralization

We next assessed whether measuring neutralization of autologous antigens would result in greater sensitivity compared to heterologous antigens. Neutralization by sequential plasma samples from all eight subjects was measured against HCVpp expressing an H77 envelope sequence. Figure 4 compares ID₅₀ neutralization titers against heterologous H77-HCVpp and autologous HCVpp (expressing the earliest sequence variant). With few exceptions, neutralization of autologous HCVpp resulted in higher titers when compared to heterologous H77-HCVpp. To quantitate the difference in neutralization sensitivity for each subject, we calculated geometric mean titers (GMT) for heterologous (H77) and autologous (early variant) HCVpp neutralization results. Subtracting $\log_{10}(\text{GMT}_{\text{heterologous}})$ from $\log_{10}(\text{GMT}_{\text{autologous}})$ provided a measure of the difference in neutralization sensitivity between HCVpp variants. A trend was detected between the difference in GMT values and the similarity of the autologous and heterologous HCVpp sequences, such that heterologous neutralization sensitivity decreased as subject-specific E1E2 sequences diverged from the H77 sequence ($R^2 = 0.33$, $P = .14$) (Figure 5). When the analysis was limited to subjects infected with a genotype 1a variant (eliminating s11, for whom the H77 strain was a subtype mismatch), the correlation became significant ($R^2 = 0.78$, $P = 0.02$). Subjects who displayed only modest differences between heterologous and autologous neutralization results (s28, s29, s110), represented the autologous E1E2 sequences most closely related to H77 (see boxed points in Figure 5).

The association between neutralization sensitivity and sequence similarity, however, was only consistent when comparing heterologous neutralization to neutralization of the earlier autologous HCVpp variant. Figure 6 shows all neutralization results for s110, demonstrating

that neutralization of the later autologous HCVpp variant (9.2 month) is actually less sensitive than neutralization of heterologous H77-HCVpp. This phenomenon was also observed for s18, s28 and s29. These results suggest a hierarchy such that nAb responses against autologous (early) > heterologous-subtype-matched > autologous(late) HCVpp. Specifically, measuring neutralization of the initial infecting virus, before immune pressure has had a chance to drive sequence evolution, provides maximum detection of the nAb response in a given subject. In comparison, heterologous neutralization provides an “average” representation, while autologous (late) variants represent viruses that have specifically evaded the initial nAb response.

Mapping amino acid substitutions involved in nAb escape

For s110, we attempted to identify the amino acid substitutions responsible for the difference in neutralization of early and late HCVpp variants. Neutralization by 9.2 month plasma was significantly higher against the earlier 2.9 month HCVpp variant (ID₅₀ 1:800) than the contemporaneous 9.2 month HCVpp variant (ID₅₀ undetectable at 1:50, reported as 1:25) (Figure 7A). Our results suggest that this decrease in neutralization sensitivity is due to amino acid substitutions resulting in escape from the nAb response. Site directed mutagenesis was used to create a panel of clones in which amino acids present in the earlier, 2.9 month envelope sequence were introduced into the later, 9.2 month envelope sequence in an effort to restore neutralization sensitivity (Figure 7B). Mutant and wild-type HCVpp were then assessed for neutralization by 9.2 month serum. Mutants B and E separately showed significant increases in neutralization sensitivity, and when these mutations were combined to create mutant F, neutralization reached 73%, almost restoring the level seen against the 2.9 month pp clone (89%). In terms of ID₅₀ values, mutant F restored neutralization from undetectable (as seen against the 9.2 month pp) to a titer of 1:125. Although considerably less than the titer of 1:800 seen against the 2.9 month variant, a significant amount of the change in neutralization sensitivity over time could be attributed to the three HVR1 amino acid substitutions in mutant F (K384T, K408R, and S405P). In conclusion, we used sequential autologous HCVpp to successfully map in vivo neutralization determinants.

Spontaneous resolving subjects display higher neutralization titers compared to chronic progressors

Autologous neutralization profiles of the three subjects who spontaneously resolved their viremia (s18, s110, s117) were strikingly different from the three subjects who progressed to chronic infection (s13, s28, s29) (Figure 3). The resolvers all possessed high-titer nAbs (>1:1000) which peaked at the time of viral clearance. In contrast, all chronically evolving subjects possessed low or absent nAb titers until well into acute infection, with a maximum titer of 1:800 detected for s28 and s29 after more than 24 months into infection, well beyond the acute phase. Taken together, these results suggest a role for nAbs in driving sequence evolution and in a proportion of subjects, spontaneous resolution of viremia.

Discussion

In this investigation, we measured neutralization against autologous HCVpp expressing envelope variants cloned during acute infection. Producing HCVpp from sequential time points for multiple subjects allowed us to assess the impact of nAbs on envelope sequence evolution, thereby demonstrating neutralization escape occurring in vivo during early infection. Several aspects of the study are noteworthy: 1) earlier sequence variants were neutralized by autologous plasma prior to neutralization of later variants showing that nAbs are responsible for envelope sequence changes over time, 2) neutralization measured against heterologous H77-HCVpp was less sensitive than neutralization measured against autologous HCVpp, and 3) for the six subjects in this study with clearly defined outcomes, spontaneous resolution was associated

with high-titer nAbs, while persistent viremia was associated with low-titer or absent nAbs during the acute phase.

The role of the humoral response in controlling HCV infection remains controversial, with the preponderance of evidence being negative based on delayed humoral responses, incomplete protection in animal studies,^{22,23} and evidence of cell-to-cell spread of HCV in tissue culture.²⁴ However, the extreme genetic diversity of HCV envelope genes²⁵ coupled with the widespread use of heterologous HCV antigens may disproportionately affect studies of anti-HCV antibody responses. This may explain why detectable humoral responses to HCV have been detected later than expected when compared to other infections,²⁶ and why they appear later than HCV-specific cellular immune responses in some studies.²⁷ In a previous study, the use of sequential autologous envelope antigens from a single individual showed continual neutralization escape during chronic infection, illustrating an effect of nAbs on viral sequence evolution in vivo and demonstrating the advantages of using autologous antigens to study the humoral response.¹⁶

Collectively, a sampled quasispecies will demonstrate preferred, or consensus, amino acid residues at each position, while random sampling will produce viral variants with point mutations differing from the consensus.²⁸ Sequence evolution in response to nAb pressure is evident in substitutions that occur at the population sequence level, as opposed to substitutions only ever present in a minority of variants. A key aspect of this investigation was the ability to control for this variable through sequence analysis. By extensive sequencing of the HVR1 region at each test-visit, we were able to confirm that our HCVpp envelope sequences closely or exactly represented the majority circulating variant at the sampled time point.

The use of autologous HCVpp to assess nAb titers is another important feature of this investigation, since as previously mentioned, a majority of prior studies measured neutralization in a heterologous setting. This report represents the largest collection of autologous HCVpp clones analyzed to date. Importantly, for seven of the eight subjects studied, infectious HCVpp were generated for at least two sequential time points in acute infection. This provided a unique opportunity to assess escape from the nAb response over time, which has previously only been investigated in a single subject during chronic infection, and never during acute infection.¹⁶ Neutralization of HCVpp expressing earlier envelope variants was detected prior to, or at a greater magnitude than, neutralization of HCVpp expressing envelope variants from later in infection. The results imply that pressure from nAbs drives sequence evolution and effectively clears circulating viral variants, followed by replacement with escape variants when viremia persists. This is not entirely surprising, since it is well known that HVR1 mutates at a faster rate than the rest of the HCV genome and represents the major target of nAbs.²² However, as previously discussed, a significant role for nAbs in acute HCV infection has been questioned. Our study also demonstrated that the use of autologous HCVpp provided a more sensitive measure of neutralization than heterologous HCVpp. Taken together, these findings suggest that continual neutralization of HCV occurs routinely during acute infection, and that this neutralization is not accurately measured with the use of heterologous antigens.

Since escape from nAbs can be detected with autologous HCVpp, we further attempted to identify precise amino acid residues that were responsible. In one subject, neutralization of mutants with autologous plasma identified three residues in HVR1 that were responsible for a significant loss in neutralization sensitivity. It is unlikely that all substitutions in the envelope genes are directly related to nAb escape; for example, T-cell epitopes identified in both E1 and E2^{16,29–31} have been observed to mutate in a manner consistent with both immune escape³² and reversion to restore replicative fitness.^{33,34} Additionally, the structures of the HCV envelope proteins are not known, making it difficult to determine if and how distal amino acids may interact upon protein folding. These considerations imply that mapping neutralization

determinants is a somewhat daunting task, however our results provide a proof-of-concept for the use of autologous HCVpp. Current investigations are ongoing to identify invariant neutralizing epitopes for use in vaccine design. In particular, a specific domain of E2 has been identified as containing potentially useful neutralization epitopes.³⁵

Our study subjects included three spontaneous resolvers and three chronic progressors. All resolvers possessed high-titer nAb responses that peaked at the time of viral clearance, while all chronic progressors possessed low-titer or absent nAb responses throughout the acute phase. It was surprising that 100% of the resolvers had such robust responses. Interestingly, the three clearance subjects maintained viremia for lengthy periods before clearance (19, 11.9 and 15.3 months for s18, s110, and s117, respectively), whereas spontaneous resolution is typically reported to occur in the first few months. It is possible that a subset of resolvers with “late clearance” profiles are associated with high nAb titers. As previously mentioned, some former studies of more “classic” resolvers did not detect nAbs during the acute phase, albeit using heterologous HCVpp. Also differing from previous studies is the detection of nAbs at very early visits (s11, s26, and s28), which then decline to undetectable levels before rising again later in infection. We postulate that this may represent a brief response dominated by IgM isotype antibodies, and is supported by the finding that in s26, the initial visit at which nAbs are detected (0.5 months) was prior to HCV seroconversion, as assessed by a commercial ELISA that is not specific for IgM.

In summary, our findings demonstrate a role for nAb responses in driving envelope sequence evolution in acute HCV infection, and identified spontaneous resolving subjects characterized by high-magnitude nAb responses. We show that autologous HCVpp provide a more sensitive measure of nAb responses as compared to heterologous HCVpp, and represent a unique tool for mapping neutralization determinants in vivo. Studies with autologous HCVpp will further elucidate the role of nAbs in HCV infection and have the ability to enhance the ongoing search for neutralization epitopes suitable for vaccine development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GMT	geometric mean titer
HVR1	hypervariable region 1
MLV	murine leukemia virus
nAb	neutralizing antibody
pp	pseudoparticle
RLU	relative light units

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Figure 1.

E1E2 amino acid substitutions occur during acute HCV infection. E1E2 envelope sequences, depicted as horizontal lines, are shown aligned to the block diagram representing the corresponding region of the HCV polyprotein. Each horizontal line represents a sequence used to generate HCVpp for autologous neutralization studies*, with connecting vertical lines representing amino acid substitutions occurring between test-visits of a given subject. Substitutions were determined using the initial test-visit from each subject as the reference. For clarity, the amino acid sequences of the HVR1 region of E2 are shown on the far right. Outcome refers to the primary infection (s11 and s26 did not have clear outcomes as described in the text). Sequences from s18 consistently maintained a 2 aa insertion at the beginning of HVR1. Numbers refer to the HCV polyprotein location according to the reference strain H77.

*For s13 (1.3 months), an infectious HCVpp clone was not isolated and the depicted sequence represents a consensus of six envelope clones from that visit.

Figure 2.

HCV pseudoparticle infectivity levels. HCVpp were produced in HEK-293T cells, harvested, and immediately used to infect target Hep3B cells. Infectivity levels, as determined by measuring luciferase activity, are reported in relative light units (RLU). Results of two separate HEK-293T cell transfections are reported as the mean + SD. For positive and negative controls, pp expressing the murine leukemia virus (MLV) envelope protein or no envelope protein (Mock) were used, respectively.

Figure 3.

Autologous neutralization during acute HCV infection. HCVpp expressing envelope sequences from sequential timepoints* in acute infection were incubated with two-fold dilutions of autologous plasma, and results reported as ID₅₀ titers. If 50% neutralization was not detected at the starting plasma dilution of 1:50, the result was recorded as one half this value, a titer of 1:25. Results for each subject represent a single experiment or median values from two experiments, in which all autologous HCVpp were assayed concurrently with the full array of plasma samples. The dotted line represents the limit of detection for the HCV RNA assay (50 IU/mL), and negative values were plotted as one half this value (25 IU/mL). The cross-hatched areas for s11 and s26 represent the time period between detection of

phylogenetically distinct virus strains. *Only a single infectious HCVpp could be generated for s13.

Figure 4.

Heterologous versus autologous HCVpp neutralization results. Autologous neutralization of HCVpp expressing the earliest envelope clone for each subject was compared to heterologous neutralization of HCVpp expressing an H77 envelope sequence. HCVpp were incubated with two-fold dilutions of plasma, and results are reported as ID₅₀ titers. If 50% neutralization was not detected at the starting plasma dilution of 1:50, the result was recorded as one half this value, a titer of 1:25. Results for each subject represent a single experiment or median values from two experiments, in which each HCVpp was assayed with the full array of plasma samples. The dotted line represents the limit of detection for the HCV RNA assay (50 IU/mL), and negative values were plotted as one half this value (25 IU/mL). The cross-hatched areas

for s11 and s26 represent the time period between detection of phylogenetically distinct virus strains.

Figure 5.

Heterologous neutralization sensitivity decreases as the autologous E1E2 sequence diverges from H77. For each subject, over the entire time of follow-up, we calculated geometric mean titers (GMT) for heterologous (H77) and autologous (early variant) HCVpp neutralization results, including titers from all time points where both HCVpp variants were tested and neutralization was detectable against at least one of the two. The difference in GMTs [$\log_{10}(\text{GMT}_{\text{autologous}}) - \log_{10}(\text{GMT}_{\text{heterologous}})$] was plotted against the % amino acid similarity of the subject-specific E1E2 sequence to the heterologous H77 E1E2 sequence. Boxed points represent s28, s29, and s110, who displayed only modest differences in heterologous versus autologous neutralization sensitivity and represent the autologous sequences most closely related to H77. The open circle denotes s11 (infected with a genotype 1b virus), representing the only non-genotype 1a sequence.

Figure 6.

Hierarchy of neutralization against autologous and heterologous HCVpp. Combining results shown in Figures 4 and 5, neutralization titers against autologous (early and late variants) and heterologous HCVpp are shown for s110. Taken together, the results demonstrate a hierarchy of neutralization, such that neutralization of autologous (2.9 month) > heterologous (H77) > autologous (9.2 month) HCVpp.

A

B

Figure 7. Mapping neutralization determinants using autologous HCVpp. A) For s110, neutralization of 2.9 month pp (early variant) compared to 9.2 month pp (late variant) by plasma from 9.2 months into infection showed a significant decrease in ID₅₀ titer, as denoted by the arrow. B) Amino acid substitutions in the 9.2 month pp sequence were back-mutated to express amino acids

present in the 2.9 month pp. Mutant and wild-type HCVpp were assessed for neutralization by 9.2 month plasma. Results of 1–4 experiments are reported as both % neutralization (1:50 dilution of serum, shown as mean + SD), and as median ID₅₀ titers. When 50% neutralization was not detected at the starting plasma dilution of 1:50, ID₅₀s were reported as 1:25, represented by the dotted line. Numbers refer to the HCV polyprotein location according to the reference strain H77.

Table 1

Characteristics of eight study subjects during acute HCV infection.

ID	Gender	Age	Race	Peak Viremia (IU/mL)	Peak ALT (IU/L)	Duration of Viremia (months)	Length of Follow-up (months)
18	Male	23	White	1 150 000	99	19.0	37.0
110	Male	28	White	3 600 000	ND	11.9	32.3
117	Female	31	White	23 800 000	ND	15.3	32.0
13	Female	25	White	2 440 000	870	Persistent	24.4
28	Male	18	White	302 000	406	Persistent	30.3
29	Female	25	White	2 390 000	270	Persistent	37.6
11 ^a	Male	25	Black	281 000	ND	15.6	42.1
26 ^a	Male	23	White	16 700 000	ND	3.0	27.0

NOTE. All subjects declined treatment. Age refers to age at seroconversion. IU, international units; ALT, serum alanine aminotransferase; ND, not determined.

^a As noted in the text, s11 and s26 demonstrated evidence of secondary infections. The reported peak viremia and duration of viremia refer to characteristics of the primary infection only.