Antibodies to an Interfering Epitope in Hepatitis C Virus E2 Can Mask Vaccine-Induced Neutralizing Activity

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Hepatitis C virus (HCV) neutralization occurring at the E2 region 412-426 (EP-I) could be enhanced when antibodies directed specifically to the E2 region 434-446 (EP-II) were removed from serum samples of persistently infected patients and vaccinated chimpanzees, a phenomenon of so-called antibody interference. Here, we show that this type of interference can be observed in individuals after immunization with recombinant E1E2 proteins. One hundred twelve blinded serum samples from a phase I, placebo-controlled, dose escalation trial using recombinant HCV E1E2 with MF59C.1 adjuvant in healthy HCV-negative adults were tested in enzyme-linked immunosorbent assay for binding reactivity to peptides representing the E2 regions 412-426 (EP-I) and 434-446 (EP-II). All samples were subsequently tested for neutralizing activity using cell-culture HCV 1a(H77)/2a chimera, HCV pseudotype particles (HCVpp) H77, and HCVpp HCV-1 after treatment to remove EP-II-specific antibodies or mock treatment with a control peptide. Among the 112 serum samples, we found 22 double positive (EP-I and EP-II), 6 EP-II positive only, 14 EP-I positive only, and 70 double negative. Depleting EP-II antibodies from double-positive serum samples increased 50% inhibitory dose (ID_{50}) neutralizing antibody titers (up to 4.9-fold) in up to 72% of samples (P ≤ 0.0005), contrasting with ID_{50} neutralization titer increases in 2 of 70 double-negative samples (2.9%; P > 0.5). In addition, EP-I-specific antibody levels in serum samples showed a significant correlation with ID_{50} neutralization titers when EP-II antibodies were removed (P < 0.0003). Conclusion: These data show that antibodies to the region 434-446 are induced during immunization of individuals with recombinant E1E2 proteins, and that these antibodies can mask effective neutralizing activity from EP-I-specific antibodies. Elicitation of EP-II-specific antibodies with interfering capacity should be avoided in producing an effective cross-neutralizing vaccine aimed at the HCV envelope proteins. (Hepatology 2015;62:1670-1682)

Hepatitis C virus (HCV) causes chronic infection in patients despite the presence of neutralizing antibodies. Overall, it has been determined that ~15%-20% of all individuals that become infected with HCV progress to serious end-stage liver disease, such as cirrhosis or hepatocellular carcinoma. Although current drug therapies can clear HCV infections, treatment success can be limited by numerous factors, including access to care, cost of therapy, patient adherence, relative efficacy of different regimens, side effects, viral genotype, and host factors. It is also unclear whether individuals are protected from...
reinfection after drug treatment.\textsuperscript{3-5} Antibody-based prophylaxis has not been successful in preventing HCV recurrence in liver transplant recipients,\textsuperscript{6,7} and an effective vaccine is yet to be developed.\textsuperscript{8}

The HCV envelope glycoproteins E1/E2, represent primary targets for vaccines designed to induce neutralizing antibody responses. It is clear that antibody to surface proteins of HCV can neutralize the virus \textit{in vitro} and \textit{in vivo},\textsuperscript{9,11} and a recent meta-analysis of chimpanzee vaccine studies showed that increased rates of clearance were achieved when structural proteins were included in the vaccine.\textsuperscript{12} Using \textit{in vitro} cell-culture systems, patient plasma samples and monoclonal antibodies (mAbs) have been identified that are capable of cross-neutralizing different genotypes of HCV.\textsuperscript{13-16} Despite this role for neutralizing antibodies in preventing HCV infection, mechanisms also exist for HCV evasion of the neutralizing response. These include genetic escape,\textsuperscript{17} polymorphisms in E2,\textsuperscript{18} cell-to-cell spread,\textsuperscript{19} association with lipoproteins,\textsuperscript{20} and glycan shielding of the surface proteins.\textsuperscript{21,22} Additionally, the presence of interfering antibodies recognizing the HCV E2 region (amino acids [aa] 434-446) have been demonstrated in sera from patients chronically infected with HCV.\textsuperscript{23,24} A role for interfering antibodies has been suggested for several viral agents in addition to HCV, including influenza, human immunodeficiency virus (HIV), and severe acute respiratory syndrome coronavirus (reviewed in a previous work).\textsuperscript{25} Interfering antibodies can bind to noncritical binding sites and prevent binding of neutralizing antibodies by steric hindrance, or non-neutralizing antibodies can cause conformational changes in an epitope and prevent binding of neutralizing antibodies.

Initially, the presence of interfering antibodies for HCV was shown in human immune globulin using pooled plasma generated from 198 anti-HCV-positive donors.\textsuperscript{25} Two epitopes, EP-I and EP-II, were studied in the E2 region. EP-I, spanning amino acids 413-423 of the E2 region, was previously shown to contain a neutralizing antibody epitope\textsuperscript{26,27} and be highly conserved among genotypes. Antibodies purified against EP-II (aa 432-446) were non-neutralizing and interfered with the neutralizing activity of antibodies directed to EP-I.\textsuperscript{23} Further data confirmed this interference in sera from chimpanzees immunized with recombinant E1E2 proteins.\textsuperscript{24} Depletion of interfering antibodies to EP-II increased the neutralization titer against 1a/2a chimeric cell-culture HCV (HCVcc) and revealed cross-neutralizing activities of the chimpanzee immune sera.

The nature of antibodies against EP-II is still under discussion. Neutralizing murine mAbs have been mapped to the region encompassing aa 432-477,\textsuperscript{14} and a recent study found that chronic patient immunoglobulin fraction affinity-purified on linear peptides did not interfere with EP-I neutralizing antibodies, but instead worked additively.\textsuperscript{28} We also recently characterized murine mAb mAb41 with high neutralizing activity against 1a/2a chimeric HCVcc, which recognized this region.\textsuperscript{29} It appears that the same region can induce neutralizing, non-neutralizing, and interfering antibodies. The function of these antibodies is associated with the critical residues that are recognized during binding.\textsuperscript{28,29} Recently, a bifurcated mode of mAb action at EP-II was described involving antibodies interacting with two primary anchor sites of the epitope.\textsuperscript{30} It was hypothesized that non-neutralizing antibodies bind preferably to the α-helical structure of the epitope whereas neutralizing antibodies recognize two additional amino acids in the region.

In this study, we further characterized the induction of neutralizing antibodies in vaccinated individuals and the role of interfering antibodies in masking neutralizing activities of immune sera. We used samples collected from a phase I clinical study (DMID 01-002; ClinicalTrials.gov identifier: NCT00500747) performed by the Saint Louis University Vaccine and Treatment Evaluation Trials.gov identifier: NCT00500747) performed by the Saint Louis University Vaccine and Treatment Evaluation Trials.gov identifier: NCT00500747) performed by the Saint Louis University Vaccine and Treatment Evaluation Trials.gov identifier: NCT00500747) performed by the Saint Louis University Vaccine and Treatment Evaluation Trials.gov identifier: NCT00500747) performed by the Saint Louis University Vaccine and Treatment Evaluation Trials.gov identifier: NCT00500747) performed by the Saint Louis University Vaccine and Treatment Evaluation Trials.gov identifier: NCT00500747) performed by the Saint Louis University Vaccine and Treatment Evaluation Trials.gov identifier: NCT00500747) performed by the Saint Louis University Vaccine and Treatment Evaluation Trials.gov identifier: NCT00500747) performed by 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Potential conflict of interest: Nothing to report.
Materials and Methods

Patient Samples. All samples were collected during a phase I randomized, double-blind, placebo-controlled study31 (study registration no.: NCT00500747). Sixty adults were enrolled into three groups of 20. Sixteen subjects per group received 4, 20, or 100 μg of HCV E1E2 with MF59C.1 as adjuvant, administered intramuscularly at 0, 4, 24, and 48 weeks; 4 subjects per group received saline as placebo.

Biotinylated Peptide Enzyme-Linked Immunosorbent Assay. Assays were performed using biotinylated peptides representing known linear epitopes EP-I (412-LNCNESLNTGWLAGLFYQHK-426) and EP-II (427-LNCNESLNTGWLALFQHK-446), as previously described.35 Sera were tested at 1:100 dilution. Cutoff was set as an optical density (OD) >2-fold the negative control (normal human serum). Values are expressed as positive/negative ratios (P/N ratios).

Depletion of Peptide-Specific Antibodies From Sera. Peptide-specific antibodies were depleted from sera, as previously described.36 A total of 750 ng of biotinylated peptide EP-II or unrelated peptide control (peptides representing the M2 protein from influenza virus) was mixed with 100 μL of streptavidin-coated magnetic Dynabeads (Invitrogen, Carlsbad, CA). After blocking of nonspecific binding sites with 5% milk/phosphate-buffered saline (PBS), beads were incubated with 100 μL of human serum for 60 minutes at room temperature. To remove peptide-specific antibodies, beads were captured with a magnet and supernatant was collected for further analysis.

HCVcc In Vitro Neutralization Assay. HCVcc 1a(H77)/2a chimeric virus was generated as previously described.36 The E1E2 region was based on the H77 consensus sequence (accession no.: AF009606). This region (aa 192-746 of the HCV polyprotein) shares 94.8% homology with the genotype 1a E1E2 sequence (accession no.: AF009606). Plasmids pNL4.3luc (3 μg/mL; obtained from the National Institutes of Health AIDS Reagent program), encoding the firefly luciferase reporter gene and pcDNAE1E2 (1 μg/mL) encoding either the H77 or the HCV-1 glycoproteins,32 were cotransfected into 293T cells. At 48 hours post-transfection, supernatants from cells were harvested and clarified at low-speed centrifugation (3,000 rpm/5 minutes). As negative control, pseudoparticles without envelope protein were generated. Neutralizing antibody titers were determined using Huh7.5 cells, as described above, by mixing 1:1 sera diluted 2-fold from 1:64 up to 1:512 with a set amount of HCVpp (5 x 10^4 relative luciferase units [RLU] per reaction). HCVpp infections were performed as previously described14 using samples in triplicate. After 72 hours, media were removed and cells were lysed by freezing and thawing in Reporter Lysis Buffer (Promega, Madison WI). Twenty microliters were used to determine luciferase activity using a Luciferase Assay System (Promega) and read using a MicroLumatPlusLB96V luminometer (Berthold Technologies, Oak Ridge, TN). Results for each well were obtained as RLU, and 50% inhibitory dose (ID_{50}) neutralization titers were calculated as described above. A titer of 64 was assigned to samples when <50% inhibition was obtained at 1:128 dilution.

Statistical Analyses. Mock- and EP-II-treated samples were compared using Wilcoxon's signed-rank test for paired data. Correlation analysis was performed using GraphPad software (version 5.0; GraphPad Software Inc., San Diego, CA). A P value of <0.05 was considered significant.

Results

EP-I- and EP-II-Specific Antibodies Are Induced After Vaccination. A total of 112 blinded samples were tested in enzyme-linked immunosorbent assay (ELISA) for reactivity to peptides representing aa 412-426 (EP-I) and aa 434-446 (EP-II). All 112 blinded samples were also tested for depletion of EP-II antibodies or mock treated with influenza-specific peptides, and tested in ELISA and neutralization assays. After completion of the studies, samples were unblinded and statistically analyzed based upon treatment groups or reactivity to EP-I and EP-II peptides. The blinded panel consisted of samples from 45 vaccinated subjects and 11 placebo subjects. Each subject was represented by two samples:
one prevaccination and one postvaccination. The distribution of single-positive, double-positive, and double-negative samples in each group (pre- and postplacebo or vaccine) is shown in Fig. 1. Within the placebo group, all pre-placebo samples tested negative for both epitopes (Fig. 1A) whereas two of the post-placebo samples demonstrated low reactivity to EP-I (Fig. 1B). Among the 45 prevaccine samples, six tested positive (P/N > 2.0) for EP-I only and four samples tested positive for both epitopes (Fig. 1C). After vaccination, reactivity against either or both epitopes increased significantly (P < 0.0002; Fig. 1D) with 30 of 45 (66.6%) samples testing positive for at least one epitope (Fig. 1D). The signal did not change for 4 of the 10 subjects that showed prevaccine reactivity to EP-I or EP-II, suggesting the presence of cross-reactive antibodies in these samples. Using the pre- and post-placebo and the prevaccine samples (n = 67), we calculated that ELISA specificity for EP-I and EP-II was 82% and 94%, respectively. The frequency of samples testing positive for EP-I or EP-II postvaccination correlated with the dose of vaccine given during the study (Fig. 2). The percentage of seropositive samples, based upon the EP-I and EP-II ELISA data, was found to be 40% (6 of 15) for subjects immunized with 4 μg of HCV E1E2 with MF59C.1, 71% (10 of 14) for subjects immunized with 20 μg of E1E2 with MF59C.1, and 87% (14 of 16) for subjects immunized with 100 μg of E1E2 with MF59C.1.

**Depletion of EP-II Antibodies From Serum Samples.** After ELISA testing of blinded samples, we classified 22 as positive for both EP-I and EP-II (double positive), six positive for EP-II only, 14 positive for EP-I only, and 70 negative for both epitopes (double negative). To address whether levels of EP-I and EP-II antibodies influence neutralization activity of sera, we treated all samples, regardless of the initial ELISA result,
with EP-II peptide to deplete specific antibodies. As controls, we treated all samples in parallel with influenza-specific (M2) peptides. Each sample was then tested as mock treated or EP-II treated in EP-II ELISA.

Figure 3 shows the EP-II ELISA signal for mock- and EP-II-treated samples based upon whether the samples tested double positive (+/++; n = 22), EP-II-only positive (−/+; n = 6), EP-I-only positive (+/−; n = 14), double negative (−/−; n = 70).
or double negative (−/−; n = 70) in the initial ELISA independent of the treatment group. When double-positive samples were treated, we observed significant decreases (P < 0.0001) in the EP-II-specific signals compared to the mock-treated samples (Fig. 3A). We also observed significant decreases in the EP-II ELISA signals (P < 0.03) for the six samples that tested positive for only EP-II (Fig. 3B). The majority of the samples negative for EP-II antibodies by ELISA (EP-I-only positive or double negative) also showed a decrease in EP-II signal (Fig. 3C,D), which was found to be significant (P < 0.005) for the EP-I-only-positive group. This could be owing to the presence of samples with low levels of EP-II antibodies that were not classed as positive in our original ELISA analysis or owing to removal from the samples of background antibodies that bound to EP-II with low specificity.

**Depletion of EP-II Antibodies Results in Increased Neutralization Titers Against 1aH77/2a HCVcc.** After depletion treatment, mock- and EP-II-treated samples were tested in neutralization assays using HCVcc 1a/2a chimera. Depleting EP-II antibodies from double-positive serum samples increased ID₅₀ titers in 14 of 22 samples (63.6%; P < 0.0001) 1.4- to 4.8-fold (Fig. 4A). When the EP-II-only-positive samples were tested for neutralization after mock and EP-II treatment, we observed slight increases in ID₅₀ titers (up to 2.9-fold) in three of six samples, although this increase was found to be nonsignificant (P > 0.25; Fig. 4B). For the EP-I-only-positive samples, we also observed increases in ID₅₀ titers (between 1.8- and 5.2-fold) for 5 of 14 samples (35.7%; P = 0.06; Fig. 4C). Although the P value was not significant for this analysis, it suggests a trend for these samples to display an increased neutralizing
activity, which could, again, indicate the presence of low levels of EP-II antibodies in these samples that were below the cutoff for our initial assay. All of the double-negative samples (Fig. 4D) were scored as non-neutralizing, with ID_{50} titers of 1:32, before treatment. After EP-II depletion, we observed increased titers for

![Fig. 5. ID_{50} titers for blinded serum samples after mock treatment or treatment with EP-II peptide using HCV-1 and H77 HCVpp. Signals from paired samples are shown. Data are presented based on EP-I and EP-II ELISA test results, regardless of treatment group. (A) HCV-1 HCVpp results for samples testing positive for both EP-I and EP-II (+/+). (B) H77 HCVpp results for samples testing positive for both EP-I and EP-II (+/+). (C) HCV-1 HCVpp results for samples testing positive for EP-II only (-/+). (D) H77 HCVpp results for samples testing positive for EP-II only (-/+). (E) HCV-1 HCVpp results for samples testing positive for EP-I only (+/-). (F) H77 HCVpp results for samples testing positive for EP-I only (+/-). Samples were tested in triplicate following 2-fold serial dilution starting at 1:128. ID_{50} titers represent the titer calculated to reduce the RLU signal by 50% compared to mock-treated controls. Samples displaying less than 50% reduction in RLU activity at a 1:128 dilution were considered non-neutralizing and were assigned a titer of 1:64 for analysis. Statistical analysis was performed using Wilcoxon’s matched pairs signed-rank test. A P value <0.05 was considered significant.

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Depletion of EP-II Antibodies Results in Increased Neutralization Titers Against H77 and HCV-1 HCVpp. The hypervariable region 1 (HVR1) region lies directly upstream from the EP-I epitope. To exclude a role for anti-HVR-1 antibodies in the interaction between EP-I- and EP-II-specific antibodies, we performed neutralization assays using HCVpp carrying HCV-1 E1E2 proteins (accession no.: M62321) and HCVpp carrying the H77 E1E2 proteins (accession no.: AF009606). All 112 samples were tested for anti-HVR1 antibodies by ELISA using a peptide specific for the HCV-1 sequence. The double-positive group demonstrated the highest reactivity to the HVR1 peptide (Supporting Fig. 2). In general, we observed higher ID50 titers using the HCVpp system than with the HCVcc system for both the HCV-1 and H77 HCVpp (Fig. 5). However, using the HCVpp system, we observed a similar impact on ID50 titers when EP-II antibodies were depleted from the sera regardless of whether HCV-1 or H77 HCVpp were used. Depleting EP-II antibodies from the double-positive serum samples increased ID50 titers in 16 of the 22 samples (72.7%; \( P = 0.0005 \)) for HCV-1 HCVpp (Fig. 5A) and in 12 of 22 samples (54.5%; \( P = 0.0002 \)) for H77 HCVpp (Fig. 5B). Similar to that observed for HCVcc, the ID50 titer increases were between 1.2- and 4.9-fold. We observed little or no change in ID50 titers for the EP-II-only-positive samples for both HCV-1 and H77 HCVpp (\( P = 0.63 \) and 0.5, respectively; Fig. 5C,D). For the EP-I-only-positive samples, we observed slight increases in ID50 titers in 5 of 14 treated samples (Fig. 5E,F). This change was found to be significant using the HCV-1 HCVpp (\( P = 0.03 \)), but was not significant using the H77 HCVpp (\( P = 0.06 \)). Consistent with the HCVcc data, we observed no significant changes to ID50 titers for the double-negative sera after EP-II treatment when tested with either HCVpp variants (data not shown).

Levels of EP-I, Not EP-II, Antibodies Correlate With ID50 Titers. mAbs to the EP-II region have been previously shown to be neutralizing,\(^{14,29}\) and samples from chronically infected patients have also been shown to contain neutralizing antibodies that target this epitope.\(^{28}\) We did not observe a decrease in ID50 titers in any of the samples tested after depletion with EP-II peptide, which suggests that antibodies recognizing this epitope do not contribute greatly to the overall neutralizing activity in these samples. To further assess whether levels of EP-I or EP-II antibodies are associated with neutralization, we analyzed the correlation between ID50 titers and antibody levels to both peptides after mock or EP-II treatment for all samples classified as antibody positive for either or both epitopes. We first
performed the analysis for H77-containing particle, both HCVcc and HCVpp. For clarity, data are shown for the HCVcc analysis; the same analyses performed for H77 HCVpp studies returned similar results. For mock-treated samples, levels of EP-I and EP-II did not show a significant correlation with ID50 titers (Fig. 6A,B); r2 values for both analyses were less than 0.1. However, after treatment with the EP-II peptide, levels of EP-I antibodies correlated significantly with ID50 titers (Fig. 6C; P = 0.0005; r2 = 0.442; Spearman’s correlation coefficient: r = 0.697) whereas reduced anti-EP-II levels in treated samples did not correlate with higher or lower ID50 titers, despite an increase in ID50 titers in many samples (Fig. 6D). When we performed the same analysis for HCV-1 HCVpp, we found a significant correlation between both EP-I (P = 0.01) and EP-II (P = 0.0005) antibody levels and ID50 titers in mock-treated samples (Fig. 7A,B). When EP-II antibodies were removed, we observed a similar increase in ID50 titer correlation for EP-I antibodies as that noted for H77 particles (Fig. 7C; P = 0.0002; r2 = 0.26; Spearman’s correlation coefficient: r = 0.594). Also, similar to that observed for H77 particles, reduced EP-II antibody levels in treated samples did not correlate significantly with higher or lower ID50 titers (P = 0.739) (Fig. 7D). These data suggest that levels of EP-I antibodies are a strong indicator of the neutralizing capacity of these samples only in the presence of low amounts of EP-II antibodies.

Subsequent to these findings, we asked whether the ratio between levels of EP-I and EP-II could be used as an indicator of the neutralizing ability of samples. When the EP-II/EP-I ratio was plotted against ID50 titer, we found, for both HCVcc and HCVpp, that the majority of samples positive for neutralizing activity had low EP-II/EP-I ratios (Fig. 7A-C). However, large numbers of samples with low ratios did not display any detectable neutralizing activity. This result reflects complex interactions between EP-I and EP-II antibody levels, such that direct comparisons of the titers cannot simply be used to predict the neutralizing capacity of samples and that a minimum EP-I antibody threshold must be met to achieve detectable neutralization.

Discussion

Development of prophylactic vaccines against HCV remains challenging. Current data suggest that it would be advantageous to include HCV structural proteins in vaccines. The circulating virus in infected individuals
exists as a quasi-species population with variability throughout the genome, but E1/E2 glycoproteins exhibit higher levels of variability both between and among genotypes and subtypes. Thus, generation of antibodies against the most conserved epitopes of the envelope proteins would be beneficial for development of effective vaccines designed to induce neutralizing antibodies. A mAb recognizing a conserved epitope located between aa 412-423 within E2 (EP-I) has been shown to inhibit E2 binding to CD81 and to be cross-neutralizing. Antibodies to this region have been shown to be induced during vaccination and natural infection, but, at the same time, antibodies to a downstream region, aa 434-446, are also induced and these can interfere with the neutralizing EP-I antibodies.

Our studies on 112 blinded serum samples derived from a phase I clinical trial in healthy volunteers support our hypothesis that antibodies to EP-II could interfere with the neutralizing activity of sera that also contain EP-I antibodies. This was demonstrated using both cellculture virus and pseudotype viruses carrying HCV envelopes from 1a genotypes H77 and HCV-1. Removal of EP-II-specific antibodies from double-positive sera resulted in up to a 4.9-fold increase in neutralizing activity of samples \(P < 0.0005;\) Figs. 4 and 5). This significant change in neutralization was not observed in samples positive for only EP-II or for samples negative for both epitopes. We observed increased neutralizing activity in a few EP-I-only-positive samples, which became significant \(P = 0.03\) when using HCV-1 HCVpp (Fig. 5E). This could have been owing to low amounts of EP-I- and EP-II-specific antibodies present in these samples. The juxtaposition of the HVR1 region to EP-I could potentially impact the EP-II interfering effects if anti-HVR1 antibodies are present. Our studies using the HCV-1 HCVpp indicate this is not the case. We observed similar effects of EP-II treatment on ID\(_{50}\) titers using HCV-1 HCVpp as we did when using H77 HCVcc and HCVpp.

Of the 22 double-positive samples treated in our studies, only eight did not display an increase in neutralizing activity after treatment; remaining negative for neutralizing antibodies. However, these samples contained some of the highest ratios of EP-II/EP-I antibodies, which could indicate that a threshold of antibody ratio is needed to achieve neutralization and/or that EP-I-specific antibodies are non-neutralizing in these samples. We investigated this concept of EP-II/EP-I antibody ratios, and although neutralizing activity in serum samples was associated with low ratios, it was not an absolute correlation (Fig. 8), suggesting that more-complex interactions exist between these antibody-antigen pairs that may determine neutralization in these serum samples.
Interference between EP-I and EP-II antibodies is further supported by the significant correlation of EP-I antibody titers with ID$_{50}$ neutralizing titers only when EP-II antibodies are removed from samples (Figs. 6C and 7C). When using the H77 HCVcc and HCVpp systems, we found a lack of correlation between levels of EP-II and neutralization (Fig. 6), which suggests further that antibodies to EP-II induced by vaccination do not contribute greatly to the overall neutralizing activity in the samples. We did find a significant correlation between EP-II antibody levels and neutralization using the HCV-1 HCVpp system (Fig. 7B). This could be owing to higher levels of HCV-1-specific antibodies, such as anti-HVR1 antibodies, being present in these samples with the EP-II signal acting as an indicator of a better immune response to the vaccine. This correlation was no longer observed after EP-II treatment (Fig. 7D). Treatment resulted in a greater number of samples with increased ID$_{50}$ titers and low EP-II signal, as opposed to samples with low EP-II signal losing neutralizing ability. Overall, we did not find a direct correlation between anti-EP-II antibody levels and neutralization in any of the systems used. Therefore, we conclude that the EP-II-specific antibodies induced by vaccination with recombinant E1E2 proteins bind distinct residues in the 434-446 region of E2, which could result in an interfering phenotype instead of a neutralizing phenotype. This is in contrast to a number of publications showing that antibodies directed at this region of E2 can inhibit viral infectivity and that residues within this region are involved in CD81 binding. However, these previous studies have also shown that the functional qualities of such antibodies differ depending upon the specific epitopes recognized or critical binding residues.

The majority of neutralizing activity detected in our assays appears to be associated with EP-I-specific antibodies. This is consistent with previous reports of cross-neutralization with samples from this phase I clinical trial, given that the EP-I region is highly conserved between genotypes. More recently, a subset of samples from this cohort was shown to bind competitively with cross-neutralizing mAbs recognizing residues within EP-I as well as residues located elsewhere in E1 and E2, which further supports the cross-neutralizing potential of these vaccine samples. Testing for cross-neutralizing activity to other genotypes was not possible in our studies using the treated sera owing to limited amounts of serum samples. The sequence of the E1E2 region used in our cell-culture chimeric virus differed slightly from that used for the recombinant vaccine (Supporting Fig. 1), with the greatest variations located in HVR1. At least part of the neutralizing activity in these serum samples has previously been shown to be directed against HVR1. We did not observe significantly higher ID$_{50}$ titers using the HCV-1 HCVpp compared to the H77 HCVpp. We cannot exclude an interaction between HVR1 antibodies and EP-I antibodies during HCV-1 neutralization. It is possible that antibodies recognizing both regions do not act synergistically or additively and could compete for binding or neutralization given the position of the epitopes. Such analyses are complex, and, although important to understand the mechanism of HCV neutralization by polyclonal sera, they are beyond the scope of these studies.

The mechanism of EP-II antibody interference is still to be elucidated. Given that EP-I and EP-II are adjacent to each other on the HCV E2 protein, the interference could therefore occur through steric hindrance that prevents binding of the EP-I-specific antibody or through conformational changes that are induced through binding to EP-II. Interestingly, structural studies have shown that both EP-II and EP-I could adopt different conformations, indicating a structural flexibility of these sites on the E2 protein. This flexibility has been confirmed from crystal structures of the HCV E2, with complex structural interactions occurring between regions downstream of the E2 N-terminus encompassing the EP-I and EP-II regions. Part of the EP-II region has been shown to form part of a conformational epitope recognized by broadly neutralizing antibodies, and structural studies confirm that the epitope includes a portion of the CD81 receptor-binding loop, which could account for some EP-II-specific antibodies exhibiting neutralizing activity. The presence of alternative conformations on the virion could change the specificity and/or affinity of antibody recognition at these two epitopes, thus constituting a strategy for HCV to evade humoral immune response.

Alternatively, EP-II-specific antibodies could enhance infection and this could compete with the neutralizing effects of other antibodies, including those to EP-I. Our data do not seem to support this mechanism given that there is no inverse correlation between levels of EP-II antibody and neutralization (Figs. 6 and 7) and removal of EP-II antibodies from the EP-II-only-positive group of samples did not result in significant increases in neutralizing activity (P > 0.25; Figs. 4B and 5C,D); however, sample size was too small (n = 6) to provide sufficient statistical power. Removal of EP-II antibodies did result in increased ID$_{50}$ titers for a subset of samples, but when foci numbers or RLU signal were analyzed with respect to EP-II-antibody levels, we did not observe increased foci or RLU associated with increasing
amounts of EP-II (data not shown), further indicating that EP-II-specific antibodies induced by vaccination do not enhance viral infection.

Overall, these studies have shown that antibodies to both epitopes 412-426 (EP-I) and 434-446 (EP-II) are induced during vaccination with recombinant E1E2 protein. Induction of these antibodies was dose dependent, in that the group of patients receiving the highest dose of vaccine (100 μg) contained the highest number of double-positive samples (62.5%), compared to the lowest-dose group receiving 4 μg, in which only 26.7% were double positive. Through a number of analyses using samples depleted of EP-II-specific antibodies, we show a significant effect of these antibodies on the neutralizing activity of EP-I-specific antibodies in both HCVcc and HCVpp systems, supporting our previous hypothesis that antibodies to EP-II can interfere with the function of antibodies to EP-I. Given the cross-neutralizing potential of antibodies to EP-I, preventing induction of antibodies to EP-II that interfere with this activity may lead to improvements in the quality and efficacy of future HCV vaccines.

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