Determination of the Human Antibody Response to the Neutralization Epitopes Encompassing Amino Acids 313–327 and 432–443 of Hepatitis C Virus E1E2 Glycoproteins

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Abstract
It has been reported that monoclonal antibodies (MAbs) to the E1E2 glycoproteins may have the potential to prevent hepatitis C virus (HCV) infection. The protective epitopes targeted by these MAbs have been mapped to the regions encompassing amino acids 313–327 and 432–443. In this study, we synthesized these two peptides and tested the reactivity of serum samples from 336 patients, 210 of whom were from Chronic Hepatitis C (CHC) patients infected with diverse HCV genotypes. The remaining 126 samples were isolated from patients who had spontaneously cleared HCV infection. In the chronic HCV-infected group (CHC group), the prevalence of human serum antibodies reactive to epitopes 313–327 and 432–443 was 24.29% (51 of 210) and 44.76% (10 of 210), respectively. In the spontaneous clearance group (SC group), the prevalence was 0.79% (1 of 126) and 12.70% (16 of 126), respectively. The positive serum samples that contained antibodies reactive to epitope 313–327 neutralized HCV pseudoparticles (HCVpp) bearing the envelope glycoproteins of genotypes 1a or 1b and/or 4, but genotypes 2a, 3a, 5 and 6 were not neutralized. The neutralizing activity of these serum samples could not be inhibited by peptide 313–327. Six samples (SC17, SC38, SC66, SC92, CHC75 and CHC198) containing antibodies reactive to epitope 432–443 had cross-genotype neutralizing activities. The neutralizing activity of SC38, SC66, SC92 and CHC75 was not inhibited by peptide 432–443. However, the neutralizing activity of sample SC17 for genotype 4 HCVpp and sample CHC198 for genotype 1b HCVpp were not inhibited by the peptide. This study identifies the neutralizing ability of endogenous anti-HCV antibodies and warrants the exploration of antibodies reactive to epitope 432–443 as sources for future antibody therapies.

Introduction
Worldwide, an estimated 130–200 million people are infected with HCV[1–4]. Among these individuals, approximately 80% of the infections will progress to chronic hepatitis C, which can lead to liver cirrhosis and hepatocellular carcinoma [5,6]. Currently, there is no available vaccine to prevent HCV infection, and polylethylene glycol interferon-α-based standard anti-virus treatment is less efficacious against the most common genotypes 1 and 4 [7]. Thus, there is an urgent need for the development of an effective vaccine and new therapeutic regimens.

HCV variants are classified into 6 genotypes and more than 90 subtypes [8,9]. Adding to the complexity, the virus of an infected individual may have extensive heterogeneity and exist as a quasispecies, which enables the virus to effectively evade host immunity. When viral clearance is successful, some reports have shown this process to be associated with host genetic backgrounds including host HLA types, cytokine and chemokine expression (e.g., IL-10, IL-28B, and CCR5) [10–15]. Moreover, several studies indicate that a strong, multi-specific, and long-lasting cellular immune response is important for the control of viral infection in acute hepatitis C[16–18].

Neutralizing antibodies also play an important role in controlling HCV infection. Studies have suggested that viral clearance is associated with a rapid induction of neutralizing antibodies in the early phase of infection [19,20], and a large collection of antibodies has been reported to prevent HCV pseudoparticles (HCVpp) or Cell culture-produced HCV (HCVcc) infection [9,21–29]. One other antibody, named D32.10, plays a protective role by inhibiting the interaction between serum-derived envelope HCV particles and hepatocytes [30,31].

Among these protective antibodies, two monoclonal antibodies (MAbs), which recognize an epitope including amino acid residues 313 to 327 of glycoprotein E1, were recently reported to strongly neutralize diverse genotypes of HCVpp (1a, 1b, 4, 5 and 6) and to a lesser extent genotype 2a HCVpp [24]. The report suggests that MAbs to the 313–327 region of glycoprotein E1 may have the potential to prevent HCV infection. MAbs specific amino acids 432–443 of glycoprotein E2 can also neutralize genotypes 1a and 1b [32,33]. The MAbs to an overlapping epitope 434–446 can...
neutralize 1a, 2a, 4, 5 and 6 HCVcc [28]. The ability of anti-sera specific for the epitope spanning 432–443 to inhibit entry of HCVpp into Huh-7 cells was tested. Study shows that these anti-sera can prevent HCVpp bearing the envelope glycoprotein H77c from entering the cell [34]. These findings may be useful for the development of novel immunotherapeutic strategies and prophylactic vaccines against HCV. However, the described antibodies or anti-sera were discovered either in animal models [34,35] or in one single HCV infected patient [24]. Thus, confirming their neutralizing activities using large size human serum samples of HCV-infected individuals is necessary.

In this study, the reactivity of serum samples from 336 HCV-infected individuals was tested against peptide 313–443. HCVpp and HCVcc neutralization and peptide-blocking assays were then used to test the neutralizing activity of the positive serum samples. Finally, we determined the prevalence of these two epitope-reactive antibodies and their cross-genotype neutralizing activities. This study confirmed that epitope 432–443 reactive antibodies have cross-genotype neutralizing activities.

**Materials and Methods**

**Patient Samples**

Serum samples were obtained from 336 HCV antibody-positive subjects (Table 1), and tested by Anti-HCV VITROS Immunodiagnostic Products (Ortho, Wales, UK). Chronic Hepatitis C patients represented 210 of these serum samples (group 1, CHC group). The remaining 126 samples were from individuals who had spontaneously cleared the HCV infection (anti-HCV positive, RNA-negative) (group 2, spontaneous clearance group, SC group). The Ethical Committee of Human Experimentation in Peking University People’s Hospital approved the study. Informed consent for the experimental use of serum samples was obtained from all patients in written form according to the hospital’s ethical guidelines. Sera complement was inactivated by heating to 56°C for 30 min. All serum samples were stored at −80°C upon collection. The control group was composed of 60 normal human serum (NHS) samples from blood donors, which were negative for HCV, human immunodeficiency virus (HIV), and hepatitis B virus (HBV).

**Determination of the Infecting HCV Genotypes**

A total of 210 serum samples from chronically infected individuals were tested using the VERSANT HCV Genotype 2.0 Assay (Siemens Healthcare, Belgium) according to the manufacturer’s instructions.

**Table 1.** Demographic characteristics of the 336 subjects.

| State | Chronic infection | Self-limited infection |
|-------|------------------|-----------------------|
| Age (M ± SD) | 50.87 ± 5.77 | 52.31 ± 6.6 |
| Sex (Male/Female) | 104/106 | 60/66 |
| Genotypes (1/2/1 mixed) | 126/67/17 | ND |
| Anti-HCV | 29.62 ± 3.40 | 22.65 ± 8.11 |
| HCV RNA M ± SD | 5.24E+06 ± 9.29E+05 | Target not detected |

*HCV genotype determined by Lipa (Siemens Healthcare).

**Peptides**

The biotinylated peptides 313–327 (representative of the region encompassing aa 313–327 of the HCV H77 polypeptide) of the E1 glycoprotein and 432–443 of E2 were synthesized as follows: BioITGHRMAWDMMNWS-amide(313–327), BioSLINTGWLAGFLY-amide(432–443) [In vitro, Shanghai, China], and were resuspended in dimethyl sulfoxide (2.5% final), diluted with phosphate-buffered saline (PBS) to 1 mg/ml, and stored at −20°C. We also synthesized two peptides as positive and negative controls as previously described by Tarr et al. [9]. The positive control peptide contained a non-structural protein 4 (NS4) immunogenic epitope (Bio-KPAIPDREVLYREFDEM-amide; aa 1691–1708) [36] and the negative control peptide corresponds to a sero-reactive region of the rabies virus glycoprotein (BioVNLHDFRSDEIE-amide) [37].

**Epitope-reactive Antibody detection**

Anti-peptide 313–327 and 432–443 antibodies were detected using indirect ELISA as described previously [31]. Briefly, 100 µl of streptavidin (Pierce, Madison, WI, USA) wascoated onto 96-well Maxisorp microtiter plates (Nunc, Roskilde, Denmark) by incubation (1 mg/ml stored solution diluted 1:100 in 0.05 M carbonate buffer [pH 9.6, Sigma, Louis, Mo, USA]), i.e., 10 µg/ml final concentration) in each well (1 µg/well) overnight at 4°C. Plates were washed three times with 300 µl of PBS per well. The wells were blocked with 200 µl of PBS containing 10% goat serum (Gibco/Invitrogen, Grand Island, NY, USA) for 1 hour at 37°C. Plates were washed three times with PBS, and 100 µl of the biotinylated peptide solution (10 µg/ml) was added to each well for 2 hours at 37°C. After another wash with PBS, 100 µl of human serum, diluted 1/250 in PBSTG (PBS containing 0.05% Tween 20 and 10% goat serum), was added to the wells and incubated for 2 hours at 37°C. The plates were washed four times with 300 µl of PBSTG per well. Peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (Sigma, Louis, Mo, USA) was diluted 1/5000 in PBSTG and added to each well for 1 hour at 37°C. The plates were washed four times with 300 µl of PBSTG per well. Then the substrate (o-phenylenediaminedihydrochloride and H2O2[Sigma, Louis, Mo, USA]) was added, and after 30 minutes, 100 µl of 2 N HCl was added to each well to stop the reaction. Optical density (OD) values were measured at 490nm using an ELISA plate reader. Each plate contained 10 NHS control wells. The cutoff value for the peptide was calculated as the mean value obtained from at least 10 NHS+3SD.

**HCVpp Production**

HCVpp were produced by co-transfection of 293T cells with an HCV envelope protein expression vector and a packaged plasmid based on the HIV-1 strain NL4–3 (Invitrogen) as described previously [32,38]. Briefly, 293T cells were co-transfected with expression plasmids encoding the HCV envelope glycoproteins, HIV gag/pol (pLP1), HIV rev (pLP2), and pLenti7 encoding Emerald Green Fluorescent Protein (EmGFP) [39]. HCV envelope expression plasmids used here include genotype 1a strain H77 (provided by F. L. Cosset, INSERM U758, Lyon, France), genotype 1b strain Con-1 (provided by C. Rice, Rockefeller University, New York, NY), and genotypes 2a (clone UKN2A1.2), 3a (clone UKN3A1.29C), 4 (clone UKN4:21.16,5(UKN5.14.4) and 6(UKN6.5.340)[provided by J. K. Ball, The University of Nottingham, United Kingdom]. After 48 hours of co-transfection, the virus-containing supernatants were harvested, filtered through 0.45 µm membranes; concentrated 200-fold ( Pall, Macrosep Advance Centrifugal Device, 100K, USA) and used to infect Huh7.5 cells.

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**HCVpp Neutralization Assays**

Huh7.5 cells were pre-seeded into 96-wells plates at a density of $1 \times 10^4$ per well. The next day, the HCVpp supernatants (20 μl/well) were incubated with each positive sera and control sera at various concentrations, plus 4 μg/ml polybrene at 37°C for 1 hour. The mixtures were then added to each well. After incubation at 37°C for 5 hours, the supernatants were replaced with fresh complete medium and incubated for 72 hours at 37°C. HCV entry was determined as the percentage of GFP-positive cells measured by flow cytometric analysis. Serum-mediated neutralization was described as the concentration that inhibited infection of HCVpp derived from diverse genotypes by 50%.

**HCVpp Neutralization Peptide-blocking Assays**

Huh7.5 cells were pre-seeded into 96-well plates at a density of $1 \times 10^4$ per well. The next day, virus-containing supernatants were

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*Figure 1. Determination of the cut-off value of 60 NHS samples.* The cutoff values of peptide 313–327 and 432–443 for 1/250 dilution were calculated from 60 NHS samples and corresponded to the mean OD values +3SD. (A) The cutoff value of peptide 313–327 was 0.505. (B) The cutoff value of peptide 432–443 was 0.507. doi:10.1371/journal.pone.0066872.g001
incubated with each positive serum and control serum sample at a
dilution that was approximately the 50% inhibiting concentration
(IC50) value. The peptides 313–327/432–443 or an irrelevant
negative control peptide plus 4 μg/ml polybrene were added
and incubated at 37°C for 1 hour. A peptide concentration of 10 μg/
ml was used for inhibition. The peptide mixtures were added to
each well. After incubation at 37°C for 5 hours, the supernatants
were replaced with fresh complete medium and incubated for 72
hours at 37°C. HCV entry was determined as the percentage of
GFP-positive cells measured by flow cytometric analysis.

**Neutralization Epitopes 313–327 and 432–443**

HCVcc Neutralization Assays

Huh7.5 cells were seeded in 96-well plates 1 day before
infection at a density of 7 × 10^4 per well. HCVcc was incubated
with each positive sera and control sera at various concentrations-
for 1 hour at 37°C. The mixture was then incubated with Huh7.5
cells for 5 hours at 37°C, and cultured for 72 hours after the
addition of fresh medium. Each test was performed in triplicate.
Neutralization activity of the sera was evaluated by counting of
HCV NS3-positive foci. Serum-mediated neutralization was de-
scribed as the diminution that inhibited infection of HCVcc derived
from JFH-1 and J6/JFH-1. The percentage of neutralization was
estimated by comparison with the mean neutralization for
Huh7.5 infected cells for 5 hours at 37°C. The mixture was then
incubated with Huh7.5 cells grown in T25 flasks at a multiplicity of
infection (MOI) of 0.01. The infected cells were passaged at 3-day intervals
with 1:3 split ratios into progressively larger culture vessels. At
12 days post-infection, viral stocks were obtained by harvesting
cell culture supernatants clarified by centrifugation (5 min at
4000 rpm), and stored in aliquots at −80°C.

HCVcc Neutralization Peptide-blocking Assays

Huh7.5 cells were pre-seeded into 96-well plates 1 day before
infection at a density of 7 × 10^4 per well. HCVcc was incubated
with each positive and control serum sample at a dilution that
was approximately the 50% inhibiting concentration (IC50) value. The
peptides 313–327/432–443 or an irrelevant negative control peptide
were added and incubated at 37°C for 1 hour. A peptide concentration of 10 μg/ml was used for inhibition. The peptide mixtures
were added to each well. After incubation at 37°C for 5 hours, the supernatants were replaced with fresh complete medium
and incubated for 72 hours at 37°C. Each test was performed in triplicate. Neutralization activity of the sera was evaluated by
counting of HCV NS3-positive foci. The percentage of neutralization was evaluated by comparison with the mean neutralization for
triple HCVcc incubations with an irrelevant-antibody control.


direct Immunofluorescence Staining

Indirect immunofluorescence staining was performed as previously
described [40]. Briefly, Huh7.5 cells were seeded on 96 well
plates (7000 cells/well). After 5 hours of incubation at 37°C, the
supernatants were replaced with fresh complete medium. Follow-
ing an additional 72-hour incubation, the cells were fixed in 4%
paraformaldehyde for 30 min at room temperature (RT), and then
blocked for 60 min in a blocking buffer (3% BSA, 0.3% Triton
X-100, 10% FBS in PBS). Following this, the cells were incubated
with an anti-hepatitis C virus NS3 antibody (Abcam, ab13930) at a
1:200 dilution. After 2 hours of incubation at RT, cells were
washed extensively with PBS and then incubated with Alexa Fluor
488 rabbit anti-mouse IgG (Invitrogen, A11059) at a 1:200
dilution for 1 hour. Following PBS washes, the numbers of
fluorescent foci (defined as a cluster of infected cells immunostained
positive for NS3 antigen) per well were counted.

**Statistical Analysis**

Statistical comparison of the prevalence of epitope-reactive antibodies between two groups of patients was performed with a χ²
test. Statistical comparison of HCVpp and HCVcc neutralization
assay results and peptide-blocking assay results between groups
was performed with One-Way ANOVA analysis, and P values
were calculated using the SPSS 16.0 software. P values corresponding to
<0.01 and, 0.01 < P < 0.05 are represented by ** and * respec-
tively. P values > 0.05 were not considered significant and left un-
denoted.

**Results**

Determination of the cutoff value for epitope-
reactive antibody

Determining the cutoff value for epitope-reactive antibodies was
performed as previously described using 60 NHS samples [31].
The cutoff value using a standard dilution of 1/250 (HCV, HIV,
HBV negative; Fig. 1A, B) was calculated as the mean
value±3 SD. Each serum sample was tested in triplicate for each
peptide. If the mean value was greater than or equal to the cutoff
for a fixed dilution, the sample was defined as either positive. If it
was under the cutoff, the sample was considered negative. At least
five NHS results were systematically included in each assay, and
the cutoff was recalculated for each type of experiment. Positive
sera contain 313–327 or 432–443 peptide-reactive antibodies, but
negative sera do not contain any of these antibodies.

**Table 2. Serum reactivity to the 313–327, 432–443 and NS4 peptides differs between infecting HCV genotypes.**

| Infection type | genotype | 313–327 peptide** | 432–443 peptide* | NS4 peptide |
|---------------|----------|-------------------|-----------------|-------------|
|               |          | n                 | No. positive (%)| n            | No. positive (%)| n | No. positive (%)|
| Chronic       | 1        | 126               | 35(27.78)       | 126          | 6(4.76)         | 53 | 20(37.74)      |
|               | 2        | 67                | 10(14.92)       | 67           | 3(4.48)         | 25 | 5(20)          |
|               | 1 mixed 2| 17                | 6(35.29)        | 17           | 1(5.88)         | 6  | 2(33.33)       |
| Sp. Cleared   | ND       | 126               | 1(0.79)         | 126          | 16(12.7)        | 30 | 1(3.33)        |

**Values for the CHC group are significantly higher than those of the SP group (P<0.01).**

*Values for the spontaneous clearance group are significantly higher than those of the CHC group (P<0.05).
### A

| Genotype | Strain/Isolate | Genbank Accession | Sequence |
|----------|----------------|-------------------|----------|
| 1a       | H77            | GI:22129792       | P G H I T G H R M A W D M M M N W S P T A |
| 1a       | H77            | GI:130461         |          |
| 1b       | HC-C2          | GI:471116         |          |
| 1b       | Con1           | GI:5420377        |          |
| 2a       | WYHCV315       | GI:339647237      |          |
| 2a       | JFH-1          | GI:116078059      |          |
| 2a       | J6             | GI:221651         |          |
| 2        | HC-J6CH        | GI:157781212      |          |
| 3a       | UKN3a.1.28     | GI:58198337       |          |
| 4        | UKN4.21.16     | GI:109259767      |          |
| 5        | UKN5.14.4      | GI:58220848       |          |
| 6        | UKN6.5.340     | GI:58220846       |          |

### B

| Genotype | Strain/Isolate | Genbank Accession | Sequence |
|----------|----------------|-------------------|----------|
| 1a       | H77            | GI:22129792       | C N E S L N T G W L A G L F Y Q H K |
| 1a       | H77            | GI:130461         |          |
| 1b       | HC-C2          | GI:471116         |          |
| 1b       | Con1           | GI:5420377        |          |
| 2a       | WYHCV315       | GI:339647237      |          |
| 2a       | JFH-1          | GI:116078059      |          |
| 2a       | J6             | GI:221651         |          |
| 2        | HC-J6CH        | GI:157781212      |          |
| 3a       | UKN3a.1.28     | GI:58198337       |          |
| 4        | UKN4.21.16     | GI:109259767      |          |
| 5        | UKN5.14.4      | GI:58220848       |          |
| 6        | UKN6.5.340     | GI:58220846       |          |
Antibody Reactivity to Epitopes 313–327 and 432–443
Diffs between Chronically Infected and Spontaneously Cleared Samples

In an effort to better understand the reactivity of the immune system during HCV infection, serum samples from 210 chronically infected individuals (CHC) and 126 individuals who spontaneously clear an HCV infection were tested using the VERSANT HCV Genotype 2.0 Assay (Siemens Healthcare, Belgium). Among CHC samples, 125 were genotype 1b, 1 was genotype 1, 67 were genotype 2a, and the remaining 17 patients were infected with a mixture of genotype 1 and 2. The percentages of the total sample size were 59.52%, 0.48%, 31.9% and 8.1% respectively. Other HCV genotypes were not found in these serum samples.

Within the 210 CHC serum samples, 51 had antibodies reactive to epitope 313–327 (24.29%) while antibodies reactive to epitope 432–443 were found in 10 samples (4.76%). In the spontaneous clearance group, 1 (0.79%) sample was positive for antibodies reactive to epitope 313–327 and 16 (12.70%) were positive for antibodies reactive to epitope 432–443 (Table 2). Two samples, named CHC65 and CHC123, were displayed positive reactivity against both epitopes. The percentage of samples reactive to epitope 313–327 was significantly higher than those reactive to epitope 432–443 (P<0.01). Moreover, there was a significantly higher percentage of samples reactive to epitope 313–327 in the CHC group compared to the spontaneous clearance group (P<0.05). However, the percentage of samples reactive to epitope 432–443 was more prominent in the spontaneous clearance group.

Human Antibody Responses Vary Depending on HCV Genotype

Among the 51 CHC samples that were reactive to epitope 313–327 (24.29%), 34 (66.67%) were against genotype 1b, 1 (1.96%) was against genotype 1, 10 (19.61%) were against genotype 2a, and 6 (11.76%) were against a mixture of genotype 1 and 2. Compared with samples reactive to genotype 2a, samples reactive to genotype 1b were better equipped to produce antibodies reactive to epitope 313–327; however, this difference was not statistically significant (P>0.05). Ten samples in the CHC group were reactive to epitope 432–443. Within these positive samples, 6 (60%) were against genotype 1b, 3 (30%) were against genotype 2a, and 1 (10%) was against a mixture of infection by genotypes 1b and 2; however, no significant difference was found between the reactivity to these genotypes.

The amino acid sequences of these two regions of several HCV strains that belonged to genotype 1a, 1b, 2a and 2 were aligned to show if the variability in these two regions affected ELISA results (Fig. 2A, B). The region corresponding to 313–327 of H77 (GI: 130461) and Con1 strains harbored the D321N and I313V substitutions, respectively. The corresponding regions of other HCV strains were the same with the peptide used for ELISA and peptide blocking assay (Fig. 2A). The region corresponding to 432–443 of H77 (GI: 130461), HC-C2, Con1, WYHCV315, JFH-1, J6 and HC-J6CH harbored the L433F, N434H, W437F, G440A and G440S substitutions, respectively (Fig. 2B). Thus, the differences of the reactivity between genotypes in CHC group might be affected by the variability of these sequences corresponding to epitopes 313–327 and 432–443.

Antibody Reactivity is Epitope Specific

To strengthen the specificity of the reactivity test, a subset of serum samples was analyzed in the absence of peptide (no peptide). As shown in Fig. 3, 19 CHC samples with reactivity to epitope 313–327 were tested at a 1/250 dilution. All wells without peptide were negative, whereas wells coated with peptide 313–327 were positive. These data suggest that epitope-reactive antibody detection test was highly specific.

Human Samples Reactive to Epitope 432–443 have Neutralizing Activity Against Multiple Genotypes of HCVpp

In order to test the neutralizing activity of the epitope reactive samples, we tested the ability of these samples to neutralize HCVpp of multiple genotypes. All 26 positive serum samples from spontaneous clearance group and CHC group were tested for their ability to neutralize HCVpp bearing the envelope glycoproteins of genotypes 1a, 1b, 2a, 2a, 3a, 4, 5 and 6. Six serum samples were found to have cross-genotype neutralizing capacity. Among these samples, SC38 efficiently neutralized genotypes 1a, 1b, 2a and 4HCVpp (Fig. 4A, B, C, E). SC17, SC36 and CHC75 efficiently neutralized genotypes 1a, 2a and 4HCVpp and slightly inhibited the infection activity of genotype 1b HCVpp (Fig. 4A, C, D, E). SC92 efficiently neutralized genotypes 1a, 2a and 4HCVpp and slightly inhibited the infection activity of genotypes 1b and 6 HCVpp, while CHC198 efficiently neutralized genotype 2a HCVpp and inhibited the infection activity of genotypes 1a, 1b and 4HCVpp (Fig. 4A, B, C, E). However, none of these 6 samples neutralized genotypes 3a and 5HCVpp (Fig. 4D, F). Re-examination of the sequence data in Fig. 2, revealed that variability may affect the neutralizing activity of these 6 samples. Compared with genotypes 1a, 1b, 2a and 4 HCVpp (Fig. 2B, GI: 130461,
Figure 4. Cross-genotype neutralizing capacity of 432–443 epitope-reactive human serum samples at the dilution 1/100. Six samples reactive to epitope 432–443 have cross-genotype neutralizing activity. Serum was assessed using neutralizing assays to genotype 1a HCVpp (A), genotype 1b HCVpp (B), genotype 2a HCVpp (C), genotype 3a HCVpp (D), genotype 4 HCVpp (E), genotype 5 HCVpp (F) and genotype 6 HCVpp (G). doi:10.1371/journal.pone.0066872.g004
GI:5420377, GI:116078059, GI:109259767), the L438I substitution occurred only in E2 glycoprotein of genotypes 3a (Fig. 2B, GI:58198337), 5(Fig. 2B, GI:58220848) and 6 (Fig. 2B, GI:58220846). The infection activity of genotypes 3a and 5 HCVpp cannot be inhibited by any of the six sera, while genotype 6 HCVpp may only be slightly inhibited by sample SC92, which suggest that L438 may be critical for the neutralizing activity of 432–443 reactive antibodies. Among these 6 samples, 4 came from the spontaneous clearance group and 2 from the CHC group. Other samples could only neutralize homologous genotype HCVpp or could not neutralize any HCVpp genotype mentioned above (data not shown).

Human Samples Reactive to Epitope 313–327 can Neutralize Homologous Genotypes of HCVpp

In order to test the functionality of the anti-HCV antibodies generated during HCV infection, 52 samples reactive to epitope 313–327 were also tested for their ability to inhibit HCVpp entry. Among these samples only 1 was from the spontaneous clearance group. We found that 47 of 52 serum samples neutralized their homologous genotype HCVpp (data not shown). Among these 47 samples, CHC30 (genotype 1) and CHC42 (mixed genotype 1 and 2 infection) also neutralized genotype 4HCVpp (Fig. 4E). CHC30 neutralized genotypes 1a and 4HCVpp (Fig. 4A, E), while CHC42 neutralized genotypes 1b and 4HCVpp (Fig. 4B, E). However, other genotypes of HCVpp were not neutralized. The sample from the spontaneously clearance group did not neutralize any genotype besides HCVpp used here (data not shown). Together these data suggest that serum samples reactive to epitope 313–327 may not have broad neutralizing activities.

Neutralization Activity can be Blocked by Peptide 432–443 but not Peptide 313–327

To determine the specificity of the neutralizing activity of antibodies reactive to epitopes 432–443 and 313–327, six samples reactive to epitope 432–443, sample CHC30, and sample CHC42 were used in a competition experiment with saturating amounts of peptides 432–443 and 313–327, respectively. At the IC50 dilution, neutralizing activities of SC38, SC86, SC92 and CHC75 for genotypes 1a, 1b, 2a and 4HCVpp were partially blocked by the addition of exogenous peptide 432–443 (Fig. 5A, B, C, D). The neutralizing activity of SC92 for genotype 6 HCVpp was also partially blocked by peptide 432–443 (Fig. 6D). The neutralizing activity of SC17 for genotypes 1a, 1b and 2a HCVpp were partially blocked by peptide 432–443 (Fig. 5A, B, C), while this peptide had no effect on the infection activity of genotype 4HCVpp (Fig. 5D). Peptide 313–327 had no effect on the neutralizing activity of its binding antibody (Fig. 6A, B, and C). Taken together, these data suggest that antibodies reactive to epitope 432–443 may have cross-genotype neutralizing activities.

Six 432–443 Epitope-reactive Human Antibody-positive Samples have Neutralizing Activity Against Cell Culture-produced HCV Strains of Genotype 2a

If the neutralization of HCVpp infectivity demonstrated for these sera was biologically relevant, we expected that these sera samples would also neutralize cell-cultured HCV strains. Two cell-cultured strains of genotype 2a, JFH-1 and chimeric J6/JFH-1, were used in the analysis. Therefore, neutralization tests were

Figure 5. Neutralizing activity of 432–443 epitope-reactive antibodies can be partially inhibited. Exogenous peptide in the presence of sera at a dilution resulting in approximately 50% inhibition of HCVpp infection (dilutions varied between 1:50 and 1:600) was used to test inhibition of genotype 1a HCVpp (A), genotype 1b HCVpp (B), genotype 2a HCVpp (C) and genotype 4 HCVpp (D). Shaded bars and filled bars represent control peptide and peptide 432–443, respectively. doi:10.1371/journal.pone.0066872.g005
repeated with HCVcc of these strains. Prior to incubation with Huh7.5 cells, a cell culture supernatant containing HCVcc particles (200 focus-forming units/well) was mixed with each positive sera and control sera at various concentrations. Quantification of foci showed that SC17, SC86, SC92 and CHC75 strongly neutralized JFH-1 and J6/JFH-1 infectivity at the dilution 1/100 (Fig. 7). SC38 and CHC198 also efficiently neutralized JFH-1 and J6/JFH-1 at the same dilution (Fig. 7). These data suggest that the neutralization demonstrated by these human samples is biologically relevant.

Neutralizing Activity of 432–443 Reactive Human Serum Samples can be Partially Blocked by the Corresponding Peptide While 313–327 Reactive Samples cannot be Blocked

Given that six serum samples from two groups containing antibodies recognizing epitope 432–443 are likely to contribute to the sera’s ability to neutralize cell-cultured strains of genotype 2a, we started by assessing the neutralizing potential of these sera. Cell-cultured JFH-1 and chimeric J6/JFH-1 virus (200 focus-forming units/well) were mixed with sera at a dilution that was approximately the IC50 value, in the presence of the 432–443 peptide or an irrelevant negative control peptide, and the resulting HCVcc infectivity was determined (Fig. 8). At the IC50 dilution, neutralizing activities of these six sera were also partially blocked by peptide 432–443. Increasing peptide concentration did not affect the inhibition of HCVcc neutralization (data not shown). These data suggest that genotype 2a HCVcc stocks can be neutralized by 432–443 epitope-reactive antibodies.

Fifty-two samples reactive to epitope 313–327 were also tested for an ability to neutralize genotype 2a HCVcc (JFH-1 and chimeric J6/JFH-1). Prior to incubation with Huh7.5 cells, a cell culture supernatant containing HCVcc particles (200 focus-forming units/well) was mixed with each positive sera and control sera at various concentrations. Ten serum samples named CHC61,
CHC65, CHC117, CHC150, CHC159, CHC165, CHC181, CHC203 (genotype 2), CHC25 and CHC133 (mixed genotype 1 and 2 infection) had neutralizing activity (Fig. 9). One sample came from the spontaneous clearance group, however, it did not neutralize genotype 2a HCVcc (data not shown). We next assessed the contribution of antibodies reactive to epitope 313–327 to the neutralizing potential of these sera. Cell-cultured JFH-1 and chimeric J6/JFH-1 virus (200 focus-forming units/well) were mixed with sera at a dilution that was approximately the IC50 value (dilutions varied between 1:200 and 1:1600), in the presence of the 313–327 peptide or an irrelevant negative control peptide, and the resulting HCVcc infectivity was determined (Fig. 10). At the IC50 dilution, neutralizing activities of these 10 sera could not be blocked by exogenous peptide 313–327. These ten serum samples could also neutralize genotype 2a HCVpp, while neutralizing activities cannot be blocked by corresponding peptide (data not shown).

**Discussion**

It has been reported that MAbs against HCV may have the potential to neutralize viral entry [24,33]. The protective epitopes targeted by these MAbs have been mapped to the regions encompassing amino acids 313–327 and 432–443. In this study, we synthesized these two peptides and tested the reactivity of serum samples from 336 patients, 210 of which were from Chronic Hepatitis C (CHC) patients infected with diverse HCV genotypes. Our data revealed that sera reactive to epitope 313–327 could only neutralize homologous genotype HCVpp and/or HCVcc. Two exceptions being samples CHC30 and CHC42 that could also neutralize genotype 4 HCVpp. Neutralizing activity was not blocked using exogenous peptide 313–327 in any of the samples tested. This data suggests that 313–327 epitope-reactive antibodies in these samples may not have neutralizing activities.

One study has reported that serum antibodies reactive to epitope 313–327 can strongly neutralize HCVpp bearing the envelope glycoproteins of genotypes 1a, 1b, 2a, 4, 5 and 6. In addition, the antibodies can also neutralize HCVpp bearing the envelope glycoproteins of genotype 2a, but to a lesser extent. Genotype 3a was not neutralized [24]. The data reported here draws different conclusions. First, in their study the authors argue that region 313–327 contains multiple but mostly conformational epitopes; however, the antibodies recognizing conformational epitopes might not be detected by assays based on synthetic peptides used in our study. Second, we hypothesize that these antibodies recognize overlapping but distinct epitopes, and that the protective epitopes within peptide 313–327 might not be recognized in samples collected from individuals naturally infected with HCV. Therefore, the neutralizing activities reported by Meunier et al. are quite different [24]. Finally, studies have shown that binding of non-neutralizing antibodies to a virus can interfere with neutralizing antibody capability [33,41]. In our study, polyclonal sera containing all the antibodies were used, and non-neutralizing antibodies may have interfered with the activity of neutralizing antibodies, and may be another reason for the narrow reactivity of serum reactive to epitope 313–327.

Differences in reactivity were also observed to epitope 432–443. Our data revealed that 6 serum samples reactive to epitope 432–443 could effectively neutralize genotypes 1a, 2a, 4 HCVpp, genotype 2a HCVcc and weakly neutralize or inhibit the infection activity of genotypes 1b and/or 6 HCVpp. However, no samples were able to neutralize genotype 3a and 5 HCVpp. This may be due to the fact that amino acid substitution in some sites of the region 432–443 of these two genotypes HCVpp may provide resistance to the sera’s neutralizing activity.

We also examined the specificity of the neutralizing antibodies. Among twenty-six 432–443 epitope-reactive human antibody-positive samples, six samples (SC17, SC38, SC86, SC92, CHC75 and CHC198) have neutralizing activity against multiple genotypes of HCVpp and genotype 2a HCVcc. Neutralizing activities of samples SC38, SC86, SC92 and CHC75 were partially blocked by addition of exogenous peptide 432–443. However, the neutralizing activity of sample SC17 for genotype 4 and sample CHC198 for genotype 1b HCVpp were not inhibited by addition of this peptide. Thus, these data suggest that antibodies reactive to epitope 432–443 may have the ability to prevent infection by genotypes 1a, 1b, 2a and 4 HCVpp and genotype 2a HCVcc, which is consistent with previous studies [32,33]. However, neutralizing activities of 432–443 epitope-reactive antibodies were different and were not absolutely blocked by exogenous peptide. There are several, non-mutually exclusive, possibilities for this observation. First, antibodies may...
recognize overlapping but distinct epitopes. In our study epitope samples reactive to epitope 432–443 had cross-genotype neutralizing activity, which is similar to antibodies targeting the region encompassing amino acids 434–446 reported by Tarr et al., in their study the authors argue that neutralizing activities of 434–446 epitope-reactive antibodies are different [29]. Consistent with this idea, our data revealed that serum samples reactive to epitope 432–443 have different neutralizing activities. Thus, amino acid region 432–443 may contain multiple epitopes, and antibodies directed to the peptide may recognize overlapping yet distinct epitopes. Second, antibodies targeting other regions may also be involved. In our study, some samples were able to neutralize other genotypes of HCVpp, but were not inhibited by exogenous peptide 432–443. These data suggest that other anti-HCV antibodies may be responsible for the observed neutralizing activity. Finally, studies have shown that binding of non-neutralizing antibodies to the virus can interfere with neutralizing antibody capability [33, 41]. In our study, polyclonal sera containing all the antibodies were used, and non-neutralizing antibodies may have interfered with the activity of neutralizing antibodies.

Because the serum used in this study included both chronic infection and spontaneously cleared samples, we were able to broadly assess the immunogenicity of epitopes 313–327 and 432–443 in natural HCV infection. The prevalence of serum reactive to epitope 313–327 in the CHC and spontaneously cleared group was 24.29% and 0.79%, respectively. On the other hand, serum samples reactive to epitope 432–443 for these two groups was 4.76% and 12.70%, respectively. An immunogenic epitope (KPAIPDREVLYREFDEM; aa 1691–1708) [36] in the NS4 protein-binding antibodies and a control epitope peptide corresponding to a sero-reactive region of the rabies virus glycoprotein (VNLHDFRSDEIE) [37] were also tested to ensure that the peptide
The capture assay used here was reliable. Reactivity to peptide 1691–1708 was observed in 32.14% of the 84 CHC serum samples tested here, similar to results reported by Tarr et al. [9], but slightly lower than earlier findings [36]. No serum sample was reactive to the rabies virus control peptide. The percentage of positive serum samples against epitope 313–327 in the CHC group was 24.29%, showing that this epitope has potent immunogenicity. However, these antibodies did not neutralize any genotypes of HCVpp, suggesting that they were different from MAbs previously reported [24]. If this epitope is used to develop a vaccine in the future, the immunogen must be designed to elicit a neutralizing antibody response and to enhance immunogenicity.

Our data do, however, provide evidence that antibodies reactive to epitope 432–443 may be generated in humans by immunization, and some of these antibodies have cross-genotype neutralizing capability. Therefore, epitope 432–443 may be useful in the development of antibody therapies. One study reported that HCV1, a cross-genotype neutralizing antibody, may be an effective therapy for the prevention of graft infection in HCV-infected patients undergoing liver transplantation [33]. However, the mutation of N415K/D and N417S of E2 of HCV virus conferred resistance to HCV1 neutralization, while epitope 432–443-specific antibody, 96–2, could potently neutralize H77N417S/Q444R–HCVpp. This report, together with the data presented here, suggest that antibodies reactive to epitope 432–443 may be another potentially effective therapy for the prevention of graft infection. Our data confirm that 432–443-reactive antibodies have different neutralizing activities, which implies that generating antibodies specific for the 432–443 epitope requires alternative approaches for developing an appropriate immunogen. Above all,
this study identifies the neutralizing ability of endogenous anti-HCV antibodies and warrants the exploration of antibodies reactive to epitope 432–443 as sources for future antibody therapies.

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References

1. Shepard CW, Finelli L, Alter MJ (2005) Global epidemiology of hepatitis C virus infection. Lancet Infectious Diseases 5: 558–567.

2. Li YP, Ramirez S, Jensen SB, Purcell RH, Gottwein JM, et al. (2012) Highly efficient full-length hepatitis C virus genotype 1 (strain TN) infectious culture system. Proc Natl Acad Sci U S A.

Figure 10. Neutralizing activity of serum samples reactive to epitope 313–327 cannot be blocked by corresponding exogenous peptide. Serum samples were cultured in the presence of sera at a dilution resulting in approximately 50% inhibition of HCVcc infection (dilutions varied between 1:200 and 1:1600). (A) The neutralizing activity of ten samples reactive to epitope 313–327 to JFH-1 virus stock cannot be blocked by peptide 313–327. (B) The neutralizing activity of ten samples reactive to epitope 313–327 to J6/JFH-1 virus stock cannot be blocked by peptide 313–327. Shaded bars and filled bars represent control peptide and peptide 313–327, respectively.

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Author Contributions

Conceived and designed the experiments: LW RL. Performed the experiments: RL HR. Analyzed the data: RL JW XX DJ XP. Contributed reagents/materials/analysis tools: XP PZ HZ. Wrote the paper: RL HR LW.
3. Rosmohle C, Fritzinger B, Legrand D, Laumay H, Wierzuskezi JM, et al. (2012) Hepatitis C Virus NS5B and Host Cyclophilin A share a common binding site on NS5A. Journal of Biological Chemistry.

4. Watanabe T, Sugachi F, Tanaka Y, Matsuura K, Yasuhashi H, et al. (2012) Hepatitis C virus kinetics by administration of pegylated interferon-alpha in human and chimeric mice carrying human hepatocytes with variants of the IL28B gene. Gut.

5. Kopp M, Murray CL, Jones CT, Rice CM (2009) Genetic Analysis of the Carbonyl-Terminal Region of the Hepatitis C Virus Core Protein. Journal of Virology 84: 1666-1673.

6. Osweianka A, Tarr AW, Justla VS, Lavillette D, Bartosch B, et al. (2005) Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. J Virol 79: 11095–11104.

7. Zeuzem S, Berg T, Moeller B, Hinrichsen H, Mauss S, et al. (2009) Expert opinion on the treatment of patients with chronic hepatitis C. J Viral Hepat 16: 73–90.

8. Simmonds P (2001) The origin and evolution of hepatitis viruses in humans. J Gen Virol 82: 693–712.

9. Tarr AW, Osweianka AM, Jayaraj D, Brown RJP, Hickling TP, et al. (2007) Determination of the human antibody response to the epitope defined by the hepatitis C virus neutralizing monoclonal antibody AP33. Journal of General Virology 88: 2991–3001.

10. Knapp S, Hemig BJ, Fordham AJ, Zhang J, Heller S, et al. (2003) Interleukin-10 promoter polymorphisms and the outcome of hepatitis C virus infection. Immunogenetics 55: 362–369.

11. Ge D, Fellay J, Thompson AJ, Simon JS, Shiu KV, et al. (2009) Genetic variation in IL28B predicts hepatitis C virus treatment-induced viral clearance. Nature 461: 399–401.

12. Suppiah V, Moldovan M, Annette M, Tegtberg B, Weltman M, et al. (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nature Genetics 41: 1100–U1174.

13. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, et al. (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nature Genetics 41: 1105-U1181.

14. Goulding C, McManus R, Murphy A, MacDonald G, Barrett S, et al. (2005) The CCR5-delta32 mutation: impact on disease outcome in individuals with hepatitis C infection from a single source. Gut 54: 1157–1161.

15. Neumann-Haefelin C, McKiernan S, Ward S, Viazov S, Spangenberg HC, et al. (2006) Dominant influence of an HLA-B27 restricted CD8+ T cell response in mediating HCV clearance and evolution. Hepatology 43: 563–572.

16. Rehermann B, Nascimbeni M (2005) Immunology of hepatitis B virus and hepatitis C virus. Nat Med 11: 169–173.

17. Bowen DG, Walker CM (2005) Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature 436: 946–952.

18. Thimme R, Oldlach D, Chang KM, Steiger C, Ray SC, et al. (2003) Interferon-alpha and ribavirin therapy for chronic hepatitis C. Nature Genetics 41: 1105-U1181.

19. Torresi J, Fischer A, Grollo L, Zeng W, Drummer H, et al. (2007) Induction of neutralizing antibody responses to hepatitis C virus with synthetic peptide constructs incorporating both antibody and T-helper epitopes. Immunology and Cell Biology 85: 169–173.

20. Osweianka A, Clayton RF, Loomis-Price JD, McKeating JA, Patel AH (2001) Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. J Gen Virol 82: 1877–1883.

21. Simmonds P, Rose KA, Graham S, Chan SW, McMounih F, et al. (1993) Mapping of serotype-specific, immunodominant epitopes in the NS4 region of hepatitis C virus (HCV): use of type-specific peptides to serologically differentiate infections with HCV types 1, 2, and 3. Journal of Clinical Microbiology 31: 1493–1503.

22. Simmonds P, Rose KA, Graham S, Chan SW, McMounih F, et al. (1993) Mapping of serotype-specific, immunodominant epitopes in the NS4 region of hepatitis C virus (HCV): use of type-specific peptides to serologically differentiate infections with HCV types 1, 2, and 3. Journal of Clinical Microbiology 31: 1493–1503.

23. Dietzschold B, Gorse M, Marchand D, Niu H, Bunschoten HM, et al. (1990) Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. Proc Natl Acad Sci U S A 87: 8293–8297.

24. Dietzschold B, Gorse M, Marchand D, Niu H, Bunschoten HM, et al. (1990) Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. Proc Natl Acad Sci U S A 87: 8293–8297.

25. Dietzschold B, Gorse M, Marchand D, Niu H, Bunschoten HM, et al. (1990) Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. Proc Natl Acad Sci U S A 87: 8293–8297.

26. Dietzschold B, Gorse M, Marchand D, Niu H, Bunschoten HM, et al. (1990) Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. Proc Natl Acad Sci U S A 87: 8293–8297.

27. Dietzschold B, Gorse M, Marchand D, Niu H, Bunschoten HM, et al. (1990) Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. Proc Natl Acad Sci U S A 87: 8293–8297.

28. Dietzschold B, Gorse M, Marchand D, Niu H, Bunschoten HM, et al. (1990) Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. Proc Natl Acad Sci U S A 87: 8293–8297.

29. Dietzschold B, Gorse M, Marchand D, Niu H, Bunschoten HM, et al. (1990) Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. Proc Natl Acad Sci U S A 87: 8293–8297.

30. Dietzschold B, Gorse M, Marchand D, Niu H, Bunschoten HM, et al. (1990) Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. Proc Natl Acad Sci U S A 87: 8293–8297.

31. Dietzschold B, Gorse M, Marchand D, Niu H, Bunschoten HM, et al. (1990) Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. Proc Natl Acad Sci U S A 87: 8293–8297.