Humoral immune system targets clonotypic antibody-associated hepatitis C virus

Amruta S. Naik,1 Brendan A. Palmer,1 Orla Crosbie,2 Elizabeth Kenny-Walsh2 and Liam J. Fanning1,*

Abstract

Hypervariable region 1 (HVR1) is one of the potential neutralization domains in the E2 glycoprotein of hepatitis C virus (HCV). Point mutations of the HVR1 can lead to humoral immune escape in HCV-infected patients. In this study, we segregated the chronically infected viraemic sera from HCV-infected patients into populations of antibody-free virus and antibody-associated virus (AAV) and mapped potential epitopes within the E1E2 gene junction of AAV sequences (residues 364–430). Furthermore, we generated HCV pseudoparticles (HCVpp) derived from AAV sequences to assess their infectivity. We studied the neutralization potential of virus-free Fab obtained from antibody–virus complexes, in the HCVpp system. We observed selective targeting of clonotypic HCV variants from the quasispecies pool. Moreover, we identified potential neutralizing epitopes within the HVR1 and an additional epitope that overlapped with a broadly neutralizing AP33 epitope (amino acid 412–423 in E2). We observed a marked difference in the infectivity of HCVpp generated using E1E2 sequences isolated from AAV. We document reduction in the infectivity of HCVpp-H77 and HCVpp derived from AAV sequences when challenged with virus-free Fab. Our results provide novel insights into the complexities of engagement between HCV and the humoral immune system.

INTRODUCTION

Globally, approximately 130–150 million people are chronically infected with hepatitis C virus (HCV) [1]. The quasi-species nature of HCV helps the virus to establish persistent infection [2–4]. The current focus in HCV management is to achieve an IFN-free regime. Direct-acting antivirals have emerged as an effective HCV treatment. However, prohibitive costs limit direct-acting antiviral access to discrete patient cohorts with cirrhosis and end-stage liver disease [5]. Vaccination is the most effective means of controlling an infectious disease, yet there is no prophylactic or therapeutic vaccine available to treat HCV [6].

A minority of infected individuals clear HCV infection spontaneously [7]. This requires a rapid, rigorous and multi-specific antiviral response by the host immune system [7]. Numerous studies have shown that broadly specific neutralizing antibodies (nAbs) are elicited early in infection [8–10]. Several experiments using HCV pseudoparticles (HCVpp) and cell-culture-derived HCV (HCVcc) have revealed that point mutations of immune dominant epitopes within viral E2 envelope protein aid humoral immune escape [11]. Hypervariable region 1 (HVR1) is one target of nAbs [2, 7, 12–14]. HVR1 is located in a stretch of 27 residues at the amino-terminus of E2 envelope glycoprotein (comprising amino acids 384–410), and is an immuno-dominant epitope with multiple linear epitopes [15]. Mutations within HVR1 are associated with humoral immune escape.

Potent and broadly nAbs are widely being considered as a potential therapy to treat viral infections [16–18]. However, over time, immune pressure drives replication of HCV variants to escape targeting by nAbs raised against dominant variants, even in cohorts infected with the same inoculum [13, 19]. Conversely, a note of caution needs to be applied here as cross-reactive nAbs may lead to antibody-dependant enhancement of infection [20]. It has been observed that selection pressure from nAb responses shapes the evolution of viral envelope protein [21–24].

To date, the neutralization potential of anti-HCV antibodies has been assessed in cell culture using HCVpp and HCVcc
bearing glycoproteins of prototype laboratory strains. These clonotypic systems lack the diversity of the virus population within the serum [25–28]. In the current study, we fractionated viraemic HCV sera into antibody-associated virus (AAV) and antibody-free virus (AFV) subpopulations to investigate the viral variants targeted by the humoral immune system [29]. The presence of AAV is a key signature of active immune response to the antigenic epitopes. Virus-free Fab (VF-Fab) was obtained from the AAV complexes and used to challenge homologous sera where AAV was not detected. We mapped prospective epitopes within amino acid position 364–430, which includes HVR1 and AP33 epitopes using VF-Fab. Owsianka et al. [30] have shown that the mouse mAb AP33 recognizes a broadly neutralizing linear epitope in E2 (412–423) [30].

We generated HCVpp using sequence information from the AAV population. We further assessed the ability of VF-Fab to target viral variants from unrelated patients both in the HCVpp and serum-derived HCV system. Notably, we demonstrate that VF-Fab has distinct binding activity in the context of a homogeneous pseudoparticle system when compared to the complex heterogeneous serum environment. Our results provide an insight into the humoral immune response in chronic HCV infection.

RESULTS

Antibodies from AAV-positive sera capture viral variants from unrelated patients

Viraemic serum samples were obtained from 16 unrelated patients (Table 1). Out of 18 specimens, n=3/3 (1a), n=5/12 (1b) and n=2/3 (3a) genotypes were positive for AAV (Table 1). We further selected these AAV-positive sera for our antibody challenge experiment.

We postulated that antibodies that have targeted discrete viral variants in AAV-positive sera are isolate specific in the context of a complex mix of variants [23, 31–33]. In order to test this hypothesis, we challenged sera identified as negative for detectable AAV (Table 2, Fig. 1a) with total IgG purified from sera, which were classified as AAV positive (referred to as 1’AAV). We discovered the presence of a newly formed AAV (referred to as 2’AAV) in addition to the pre-existing parental 1’AAV (schematic representation in Fig. 1a). We observed that total IgG from 1b-5 targeted a viral variant from 1b-4 [KT873176–77, KT873180–81] and 1b-6 [KT873186, KT873194], total IgG from 1b-10 captured a variant from 1b-7 [KT873195, KT873197–204] and antibodies from 3a-2 were bound to viral variant in 3a-3 [KT873229]. However, we were not able to detect 2’AAV for 1b-8 when mixed with the total IgG from 1b-5. 1’AAV and 2’AAV sequences are unrelated and non-identical (Fig. 1b–d). Similarly, total IgG purified from 1b-4, 1b-6, 1b-7, 1b-8 and 1b-9, which were initially classified as AAV negative, were mixed from these experiments in a cross-panel challenge (e.g. total IgG from 1b-4 were mixed with sera 1b-6, 1b-7, 1b-8 and 1b-9). We observed that total IgG purified from AAV-negative sera did not capture any viral variant.

Separately, our sequence analysis observed that 1b-5 has a 28 amino acid HVR1 domain with an in-frame 3 bp insertion at nucleotide 1492–94 [ref: AF011751] at the 5’ end of E2 (Fig. 1b, Table 2). We observed an atypical 30 amino acid HVR1 sequence in both the 2’AAV and AFV fractions of serum 1b-4 (Fig. 1b, Table 2), as a result of 9 bp in-frame insertion at the 5’ end from nucleotide - 1492-1500 [ref: AF011751]. We also identified a 26 amino acid HVR1 profile from specimen 1b-7 (Fig. 1c) as a consequence of an in-frame deletion at the 5’ end from nucleotide - 1491-93 [ref: AF011751]. The rest of the targeted 2’AAV sequences harboured a classic 27 amino acid HVR1 (Fig. 1d).

Analysis of proteinase-K-treated sera

We obtained virus-free antibody fraction by treating AAV-positive sera with proteinase K. Absence of E1E2-junction-specific PCR product confirmed the virus-free status of the post-proteinase-K-treated samples. We further analysed the products of proteinase-K-treated sera eluted from Ab Spin Trap, LambdaFabSelect and Kappa Select on 4–12 % Bis-Tris gradient gel. Analysis revealed that, in the process of dissociating the antibody–virus complex, the intact antibody was fractionated into several peptides. An intact Fab fragment was identified at ~50 kDa (Figs 2 and S1, available in the online Supplementary Material) from all three fractionation procedures, hence, VF-Fab (Ab Spin Trap), λ-VF-Fab (LambdaFabSelect) and κ-VF-Fab (KappaSelect). It has been previously shown that the CH1 domain of the Fab arm has a binding site for streptococcal protein G [34]. This is a likely explanation as to how we obtained VF-Fab from proteinase-K-treated AAV-positive serum samples.

Patient-derived VF-Fab selectively targets homologous genotypes

We used Fab fragments obtained from three different columns to challenge the AAV-negative sera. We observed that VF-Fabs were able to capture viral variants (2’AAV) in all instances in comparison to λ-VF-Fab and κ-VF-Fab (Table 2). Clonal analysis of 2’AAV variants revealed that 2’AAV variants were clonotypic in nature. Both intact mAb AP33 and proteinase-K-treated AP33 retained identical viral variants from 1b-9 and 3a-3 only (Table 2). The AAV fraction obtained from AP33-challenged 1b-9 and 3a-3 sera yielded a homogenous virus population of [KT873217] and [KT873229], respectively. Significantly, this mirrors the viral variants captured by VF-Fab1b-2 and VF-Fab3a-2.

Both VF-Fab and λ-VF-Fab derived from respective homologous genotypes captured identical viral variants from 1b-4, 1b-7 and 3a-3 forming a 2’ AA V (Table 2), κ-VF-Fab did not capture viral variant from any of the challenge sera.

Source of VF-Fab does not affect the selective binding to viral variants

VF-Fab1b-2 was obtained from a serum that belongs to an anti-D cohort [35]. Patients in an anti-D cohort were heterogeneically infected with the same source of HCV genotype 1b [35]. Three of four HCV 1b sera which were challenged with
VF-Fab1b-2 were from an anti-D cohort (1b-1-1, 1b-8 and 1b-9) (Table 2). However, viral variants from only two of viraemic sera (1b-1-1, 1b-9) were captured by the VF-Fab1b-2. Interestingly, the serum sample from which the VF-Fab1b-5 was obtained does not belong to an anti-D cohort yet successfully retained virus from anti-D sera 1b-4, 1b-6 and 1b-8. On the other hand, 1b-7 was targeted by VF-Fab1b-10 obtained from another anti-D serum. Of note, no shared reactivity with respect to capture viral variants (2/C14 AAV) for VF-Fab1b-5 or VF-Fab1b-10 was observed.

### Potential epitopes targeted by VF-Fab1b-5 and VF-Fab1b-10

Based on previous research by Guan et al. [4], we explored the 364–430 region for probable epitopes targeted by the humoral immune system [4]. Five different potential binding motifs were observed for included sequences. All the peptides in this study were designed as 15 mer with overlapping 14 mer peptides. The candidate epitopes are proposed by subtracting positive peptides and by aligning them to extract overlapping residues. VF-Fab1b-5 targeted a motif that shares amino acid residues 412, 413 and 415–423 with the AP33 epitope (QLINTNGSWHIN) in the 1/C14 AAV fraction of 1b-5 and the 2/C14 AAV fraction of 1b-8 (Table 3) [30]. VF-Fab1b-10 targeted a motif within the HVR1 domain of the 2/C14 AAV fraction of 1b-4 [KU888835] and 1b-8 [KU888836] and the 1/C14 AAV fraction of 1b-5 [KU888834]; it is the same antigenic domain targeted by mAb 3 C7 and 9/27 (396–407) (reviewed by Helle et al. [36]) (Table 3). Heatmap representation of scaled and centred data for each peptide recorded from both VF-Fabs is shown in Figs 3 and S3.

VF-Fab1b-5 efficiently neutralizes HCVpp-H77 and HCVpp1b-4

We generated five pseudotyped HCV particles. HCVpp-H77 was used as a reference clone. However, not all the expressed clones were infectious in Huh7 (Fig. 4a). Out of four AAV-E1E2 pseudotyped viruses, HCVpp1b-4 was infectious, yielding a 10-fold greater relative luminescence value [relative light unit (RLU)] than no envelope control. We observed that VF-Fab1b-5 at a concentration of 0.167 mg ml⁻¹ was highly neutralizing, reducing the HCVpp-H77 infection by 85 % (Fig. 4b). On the other hand, VF-Fab1b-10 reduced HCVpp-H77 infection by 75 % at 0.400 mg ml⁻¹ (Fig. 4b). We used the highest neutralizing concentration of VF-Fab1b-5 and VF-Fab1b-10 in the neutralization assay for HCVpp1b-4. VF-Fab1b-5 reduced HCVpp1b-4 infection by 88 % at 0.167 mg ml⁻¹ (Fig. 4c). VF-Fab1b-10 showed 72 % of inhibition of infection of HCVpp1b-4 at 0.400 mg ml⁻¹ (Fig. 4c). In both neutralization assays, we found VF-Fab1b-5 to be highly neutralizing.

### Table 1. Sample characteristics used in the current study

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of samples</th>
<th>1/C14 AAV</th>
<th>Sample identifier*</th>
<th>Accession no. AFV†</th>
<th>Accession no. 1/C14 AAV‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>3</td>
<td>+</td>
<td>1a-1</td>
<td>KT873141</td>
<td>KT873142</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1a-2</td>
<td>KT873143</td>
<td>KT873144</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1a-3</td>
<td>KT873145</td>
<td>KT873146</td>
</tr>
<tr>
<td>1b</td>
<td>12</td>
<td>–</td>
<td>1b-1–16,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1b-1–25,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1b-1–36,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1b-2§</td>
<td>KT873162</td>
<td>KT873163</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>1b-3</td>
<td>KT873164–71</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>1b-4§</td>
<td>KT873173–81</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1b-5</td>
<td>KT873182</td>
<td>KT873183</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>1b-6§</td>
<td>KT873184–94</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>1b-7§</td>
<td>KT873195–04</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>1b-8§</td>
<td>KT873205–11</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>1b-9§</td>
<td>KT873212–17</td>
<td>–</td>
</tr>
<tr>
<td>3a</td>
<td>3</td>
<td>+</td>
<td>1b-10§</td>
<td>KT873218</td>
<td>KT873219</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>3a-1</td>
<td>KT873220</td>
<td>KT873221</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>3a-2</td>
<td>KT873234</td>
<td>KT873233</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>3a-3</td>
<td>KT873222–32</td>
<td>–</td>
</tr>
</tbody>
</table>

*Genotype/subtype patient identifier.
†Samples positive for 1/C14 AAV; both AFV and 1/C14 AAV sequences were analysed by direct sequencing only.
‡Samples without accession numbers had no detectable levels of AAV.
§Source of infection: contaminated anti-D immunoglobulin [35].
||Obtained from the same patient at three different time points (2002, 2013 and 2014, respectively). Genotype/subtype patient identifier-sample number.
†Positive sera (as per Table 1).
‡Insufficient amounts of AAV-negative sera and/or 1
§Insufficient amounts of AAV-negative sera and/or 1

DISCUSSION

This is the first successful attempt to capture inter-patient viral variants from viraemic HCV sera using antibodies from homologous AAV-positive sera. It has been shown in HCVpp and HCVcc in vitro infection systems that antibodies obtained from patient sera are broadly reactive (reviewed in Ball et al. [37]). However, in our experiments, we observed that in the complex serum environment, antibodies target unique viral variants (from the quasispecies pool) from unrelated patient sera. We made similar observations for VF-Fab. From our results, we were unable to determine whether (i) the antibodies that targeted 1' AAV are the same antibodies that targeted 2' AAV and/or (ii) the antibodies were not saturated with antigen/virus. Additionally, we have shown that total IgG purified from AAV-negative sera were not capable of capturing viral variants from other AAV-negative sera. In this context, absence of AAV might represent a period when antibody-sensitive viral variants were removed (from a quasispecies pool), leaving behind the humoral immune escape mutants. Nonetheless, we acknowledge that these AAV-negative sera might have nAbs against previously culled viral variants. Analysis of the relative distribution of 2' AAV (Fig. S2) suggests that the viral variant targeted by VF-Fab need not dominate the heterogeneous virus population. Immunogenicity, accessibility and antibody–epitope binding kinetics might play a crucial role in selecting the clonotypic population out of the diverse mixture of variants in patient sera.

Extra-long HVR1 is a feature of the biology of HCV [23, 29, 38]. Guan et al. [4] in their experiments have shown that the first 13 amino acids do not affect infectivity in the HCVpp system. We are the first to report the capture of a non-classic 30 amino acid HVR1 (from sample 1b-4, Fig. 1b) using VF-Fab1b-5 from an unrelated sample. This latter variant harboured a non-classical 28 amino acid HVR1 (sample 1b-5, Fig. 1b). Our data indicate that this three amino acid insertion at the N-terminus of HVR1 did not interfere with the binding capacity of the aforementioned VF-Fab from sample 1b-5. This is likely because nAbs target the C-terminus of HVR1, and hence, deletion or insertion at the N-terminus doesn’t affect this phenomenon [39].

In this study, we used human anti-HCV VF-Fabs for epitope mapping of viral sequences that were previously targeted by host humoral immune system. Our epitope mapping data showed that VF-Fab1b-5-targeted motifs in the 1'AAV fraction of 1b-5 [KU888834] and the 2'AAV fraction of 1b-8 [KU888836] overlap with the well-characterized AP33 epitope. It has been shown that the linear AP33 epitope is highly conserved across different HCV genotypes and is broadly neutralizing [30]. Importantly, mAb AP33 (either intact or proteinase-K-treated) was able to retain epitope-positive viral variant(s) from only 1b-9 and 3a-3 (Table 2). Deng et al. [40] in their research found no detectable antibody response to a peptide (PUHI 19) harbouring AP33 epitope (409–423), suggesting weak immunogenicity of the epitope, which might explain selective targeting of AP33-epitope-positive variants in our antibody-serum binding experiments [40].

Studies have shown that HCVpp and HCVcc lacking HVR1 are sensitive to neutralization by patient-derived

Table 2. Antibody challenge of serum samples without detectable AAV following initial fractionation

<table>
<thead>
<tr>
<th>AAV-negative sera*</th>
<th>1' AAV-positive sera</th>
<th>Antibody challenge†</th>
<th>Unique HVR1</th>
<th>Accession nos. of 2' AAV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Untreated</td>
<td>Proteinase-K-treated</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total IgG AP33 VF-Fab λ-VF-Fab κ-VF-Fab AP33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b–1†§</td>
<td>1b–2§</td>
<td>–</td>
<td>ND</td>
<td>+ – ND – 1</td>
</tr>
<tr>
<td>1b–3§</td>
<td>1b–2§</td>
<td>ND</td>
<td>ND</td>
<td>+ ND – ND – 1</td>
</tr>
<tr>
<td>1b–4</td>
<td>1b–5</td>
<td>+ –</td>
<td>+ –</td>
<td>– – 1</td>
</tr>
<tr>
<td>1b–6</td>
<td>1b–5</td>
<td>+ –</td>
<td>+ –</td>
<td>– – 1</td>
</tr>
<tr>
<td>1b–7</td>
<td>1b–5</td>
<td>– –</td>
<td>+ –</td>
<td>– – 1</td>
</tr>
<tr>
<td>1b–8</td>
<td>1b–5</td>
<td>+ –</td>
<td>+ –</td>
<td>– – 1</td>
</tr>
<tr>
<td>1b–9§</td>
<td>1b–2§</td>
<td>+ +</td>
<td>+ –</td>
<td>ND + 1</td>
</tr>
<tr>
<td>3a–3</td>
<td>3a–2</td>
<td>+ +</td>
<td>+ +</td>
<td>+ + 1</td>
</tr>
</tbody>
</table>

ND: Not done.
*Patient sera without detectable AAV following initial fractionation were subsequently challenged with genotype/subgenotype-matched 1' AAV-positive sera (as per Table 1).
†Individual antibody preparations originating from 1' AAV-positive serum or AP33 are described in Methods.
‡Cumulative number of unique HVR1 amino acid sequences identified in 2' AAV-positive samples.
§Insufficient amounts of AAV-negative sera and/or 1' AAV-positive sera limited the number of possible experimental combinations.
antibodies, which again indicates an important role of HVR1 in overall viral fitness [41–43]. We observed fewer changes outside the HVR1 in all the isolates. VF-Fab1b-10 targeted a motif within HVR1 of the 2°AAV fraction of 1b-4 and 1b-8 and in the 1°AAV fraction of 1b-5 upon epitope mapping (Table 3, Fig. 3). Notably, these results suggest the existence of an immunodominant epitope on the C-terminal region of HVR1 recognized by patient-derived VF-Fabs. Our data are in strong agreement with that of previously published data, supporting that HVR1 is one of the potential antigenic epitopes under immune selection pressure [24, 32, 36, 44, 45].

In our study, we observed that both VF-Fab1b-5 and VF-Fab1b-10 neutralize HCVpp with different efficiencies, VF-Fab1b-5 being highly neutralizing (Fig. 4b, c). A likely explanation of the neutralizing activity of VF-Fab1b-5 is that it targeted a highly neutralizing epitope and captured
viral variants in all the antibody challenge experiments, when compared to VF-Fab1b-10 (Table 2). However, nAbs against HVR1 are mostly strain-specific and of limited cross-reactivity; hence, VF-Fab1b-10 is the lesser neutralizing between the two VF-Fabs (reviewed previously [36, 37]). A well-known caveat that needs to be appreciated when studying neutralization potential is that the pseudoparticle system provides a homogenous population of E1E2 glycoprotein in HCV virions. The column was then incubated for 5 min mixing. The first flow-through (W0) was retained as the AFV fraction. Eight washes (W1-W8) of 300 µl binding buffer were applied to the column, while the last wash (W8) was tested by PCR ampiclon analysis to confirm the absence of HCV virions. The column was then incubated for 5 min with 200 µl elution buffer with end-over-end mixing. The elute is now identified as total IgG. Total IgG contains AAV fraction of 1b-10. This may indicate a possible conformational or discontinuous epitope outside the HVR1, which is not included in our study. Our total IgG and VF-Fab binding studies strongly suggest that nAb responses are clonotypic in nature. However, this statement must be qualified by the fact that we have only examined AAV in the context of eight samples.

In conclusion, we show differential binding behaviour of patient-derived anti-HCV antibodies, VF-Fab and mAb AP33 targeting clonotypic populations in unrelated viraemic sera. We have identified two epitopes that are subjected to humoral immune attack in vivo. Importantly, our data add new information on the in vivo humoral immune response to chronic HCV infection.

METHODS

Serum

This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, and written consent from patients was obtained. A panel of viraemic sera positive for HCV genotypes 1a (n=3), 1b (n=12) and 3a (n=3) was randomly selected (Table 1). Ten out of 12 1b serum samples belonged to a cohort of Irish women infected with a single source of HCV genotype 1b via contaminated anti-D immunoglobulin [35]. Patient 1b-1 was the only patient whose serum samples were obtained at three different time intervals (Table 1). The VERSANT HCV Genotype Assay was used to confirm the HCV genotype.

Nucleic acid extraction and E1E2 gene junction amplification

HCV RNA was extracted with QIAamp Viral RNA mini kit where applicable (Qiagen). The E1E2 gene junction (318 bp) of HCV was amplified as previously described [29]. All PCRs were carried out using Pfu DNA polymerase (Thermo Scientific) to guard against unequal template selection due to the quasispecies nature of the virus [46].

Fractionation of serum samples into AFV and AAV

The Ab Spin Trap columns were used to separate the samples into (i) AFV and (ii) AAV populations following the manufacturer’s protocol with a few modifications (GE Healthcare Life Sciences). The protein G columns were used to purify total IgG from serum or plasma. Briefly, 200 µl of patient sera was applied to the column followed by incubation for 15 min at room temperature with end-over-end mixing. The first flow-through (W0) was retained as the AFV fraction. Eight washes (W1–W8) of 300 µl binding buffer were applied to the column, while the last wash (W8) was tested by PCR ampiclon analysis to confirm the absence of HCV virions. The column was then incubated for 5 min with 200 µl elution buffer with end-over-end mixing. The elute is now identified as total IgG. Total IgG contains AAV (HCV targeted by antibodies), along with free antibodies bound by the column.

Dissociation of antibody–virus complex and collection of VF-Fab, λ-VF-Fab and κ-VF-Fab

Proteinase K was used to dissociate the antibodies from the antibody–virus complex. This was achieved by adding 1 : 1 (v/v) of proteinase K (5 mg ml−1) to AAV-positive sera. The Ab Spin Trap protocol was followed post-protease K treatment. We analysed the functional component in post-protease-K-treated samples using HiTrap LambdaFabSelect.
and KappaSelect pre-packed columns (GE Healthcare Life Sciences). These columns have a ligand that binds to the constant region of lambda or the kappa light chain of human IgG, respectively. Briefly, 1 ml proteinase-K-treated serum samples were passed through both the columns as per the manufacturer’s protocol. Columns were washed to remove unbound material with 5 vols binding buffer (PBS, pH 7.4). Fab fragments were eluted with 0.5 ml elution

Fig. 3. Heatmap overview of binding motifs targeted by VF-Fab1b-5 and VF-Fab1b-10. 1^AAV fractions of 1b-5 and 1b-10 and 2^AAV fractions of 1b-4, 1b-7 and 1b-8 were assessed for epitope mapping with VF-Fab1b-5 and VF-Fab1b-10 from amino acid 364–430 including the HVR1 in E2. Individual peptides are listed on the right-hand side, and VF-Fab is indicated at the base of the heatmap. Black horizontal lines show the start position of a new target sequence in that particular set of peptides. Target sequences are flanked by black brackets on the left. The magnitude of colour (dark magenta) with higher z score represents the binding affinity of Fab to the peptide. The sequence in a red box (on the right) represents a motif targeted by VF-Fab1b-5. The sequence in a blue box (on the right) represents a motif targeted by VF-Fab1b-10. In order to make the heatmap legible, only every second peptide in the study has been included in the figure.
buffer (0.1 M glycine buffer, pH 2.5, for KappaSelect, 0.1 M acetate buffer, pH 3.5, for LambdaFabSelect). The eluted fractions were concentrated by an Amicon Ultra-0.5 centrifugal unit with Ultracel 50 (Millipore). Confirmation of the virus-free status of this proteinase-K-treated preparation was determined by the absence of an E1E2-specific amplification following reverse transcription PCR (RT-PCR). Furthermore, elutes obtained post-proteinase K treatment from Ab Spin Trap (VF-Fab), LambdaFabSelect (\(\lambda\)-VF-Fab) and KappaSelect (\(\kappa\)-VF-Fab) were analysed by Western blotting. Elutes, natural human IgG Fab fragment protein (ab90352, Abcam) and control IgG obtained from human plasma CTM (/C0\(\alpha\)C/Roche Molecular Systems), were blotted on a nitrocellulose membrane. The samples were then incubated with HRP-labelled mouse anti-human IgG Fab antibody (Biorbyt) at 1 : 10 000 concentration in 0.05 % (v/v) PBS Tween-20.

**Antibody-sera (non-detectable AAV) challenge**

Total IgG that contains AAV (hereafter referred to as 1’AAV) along with free antibodies, VF-Fab, \(\lambda\)-VF-Fab and \(\kappa\)-VF-Fab, were used to challenge the AAV-negative sera in 1 : 5 ratios (Table 2). This mixture was then incubated at 37 °C for 2 h. AP33 is a mouse mAb that targets the partially confirmation-dependent epitope within amino acid residues 412–423 (a kind gift from Dr Arvind Patel, University of Glasgow, UK). Simultaneously, we challenged the sera (Table 2) with both intact AP33 and Ab-Spin-Trap-eluted post-proteinase-K-treated AP33 (25 µg ml\(^{-1}\)). The Ab Spin Trap protocol was followed, and the challenged samples were tested for the presence of a newly formed 2 AAV by PCR.

**Molecular cloning and sequencing analysis**

The AFV and 1’AAV fractions obtained on initial analysis of sera were analysed by direct sequencing only. cDNA obtained from 1’AAV and 2’AAV was diluted 1 : 100 to ensure that there was no template resampling. AFV and 2’AAV amplicon obtained from total IgG, VF-Fab and AP33 antibody (Table 2) were cloned into pJET1.2/blunt cloning vector (Thermo Scientific). Ten colonies each for AFV and three colonies each for 2’AAV fractions were analysed for sequencing (MWG Operon).

**Epitope mapping**

Linear peptides were synthesized for 1’AAV 1b-5 [KU888834] and 1b-10 [KT873219] and 2’AAV 1b-4 [KU888835], 1b-7 [KT873204] and 1b-8 [KU888836] covering amino acid region 364–430 in the E1E2 glycoprotein [ref:
AF011751] to study the epitopes targeted by the host immune system. (For 1b-7 [KT873204] and 1b-10 [KT873219], since we did not have full-length sequences at the time, peptides were designed for region 364–423.)

Two sets (hence, set 1 and set 2) of overlapping peptides of 15 amino acid lengths with an overlap of 14 were synthesized for these sequences. Set 2 comprised linear peptides of 15 amino acid length; however, amino acids at positions 10 and 11 were replaced by Ala. When a native Ala would occur on either position, it was replaced by Gly. Control peptides unrelated to our test sequences that are propriety of Pepsan were designed based on epitopes of monoclonal antibodies 57.9 and 3C9 [47]. The binding of VF-Fab1b-5 and VF-Fab1b-10 to peptides was assessed in a Pepsan-based ELISA as described below (Pepsan Presto) [48]. Each well in the card contained covalently linked peptides that were incubated overnight at 4°C with VF-Fab1b-5 and VF-Fab1b-10, between 0.1 and 0.0005 Pepsan buffer and preconditioning blocking buffer (SQ) (a mixture of horse serum, Tween 80 and ovalbumin in PBS). After washing, the plates were incubated with goat anti-human HRP conjugate (1:1000, Southern Biotech 2010). Incubation for >15 min. The lysate was then transferred to a white low-luminescence 96 well plate, and luciferase activity was quantified after 60 min using a charge-coupled device camera and an image-processing system.

**Production of infectious HCVpp**

Full-length E1E2 glycoprotein sequences 1b-5 [KU888834] and 1b-10 [KU888837] positive for 1 AAV on initial serum analysis and 1b-4 [KU888835] and 1b-8 [KU888836] targeted by VF-Fab1b-5 and VF-Fab1b-10 forming 2 AAV (Table 2), respectively, were cloned in pcDNA3.1V5hS TOPO expression vector (Life Technologies) as previously described [28]. HCVpp were generated according to the protocol of Bartosch et al. [25]. Briefly, HEK-293T cells were co-transfected with plasmids expressing the HCV E1E2 glycoproteins, the murine leukemia virus Gag-Pol packaging vector (Inserm Transfert) and a transfer construct with luciferase reporter gene (a gift from Dr Arvind Patel, University of Glasgow, UK). Pseudoparticles generated without E1E2 glycoprotein (no envelope) were used as a negative control. pHCMV-ΔC/E1/E2 H77 clone was used as a positive control (Inserm Transfert). Infectivity of the HCVpp was tested as previously described [25, 28]. To characterize the capacity of our VF-Fab to recognize different isolates, E1E2 glycoproteins from the clarified lysates of transfected HEK 293T cells were captured onto GNA (Galanthus nivalis) lectin (Sigma Aldrich)-coated microtitre plates and then detected by the anti-E2 mouse mAb AP33 and VF-Fab1b-5, VF-Fab1b-10 and control Fab [human plasma CTM (–)C, Roche Molecular Systems].

**Neutralization of HCVpp infection in Huh7 cells**

Neutralization was tested only for those HCVpp with infectivity at least 10 times greater than mock pseudoparticle (no envelope) values. Huh7 cells were cultured in 24 well plates at 2.5×10⁴ cells density for 2 AAV HCVpp1b-4 and 4×10⁴ cells in 96 well plates for HCVpp-H77. HCVpp-H77 were mixed with VF-Fab1b-5, VF-Fab1b-10 and control Fab [human plasma CTM (–)C, Roche Molecular Systems], at concentrations from 0.015 to 0.400 mg ml⁻¹ to estimate the highest neutralization working concentration. HCVpp1b-4 were mixed with VF-Fab1b-5 (0.167 mg ml⁻¹), VF-Fab1b-10 (0.400 mg ml⁻¹) and control Fab (0.400 mg ml⁻¹) and incubated for 1 h at 37°C. The mixture was added to the Huh7 cells and was incubated for 4 h at 37°C. Inoculum was removed and replaced with fresh media and incubated at 37°C for 72 h. After 72 h, media was removed from the cells, and 50 µl Cell Culture Lysis Reagent (Promega) for 96 well plate and 100 µl for 24 well plate were added and left to incubate for >15 min. The lysate was then transferred to a white low-luminescence 96 well plate, and luciferase activity was measured in RU’s in a GloMax. The percentage of neutralization was calculated as 100%×[1–(HCVppRLUtest/HCVppRLUcontrol)]. Each sample was tested in duplicate following three independent experiments.

**Acknowledgements**

The authors declare that there are no conflicts of interest.

**Funding information**

A.N.’s work was funded by Molecular Medicine Ireland as part of the Clinical and Translational Research Programme.

**References**


36. Tarr AW, Khera T, Hueging K, Sheldon J, Steimann E et al. Genetic diversity underlying the envelope glycoproteins of

