Determination of the human antibody response to the epitope defined by the hepatitis C virus-neutralizing monoclonal antibody AP33

Alexander W. Tarr,1 Ania M. Owsianka,2 Dhanya Jayaraj,1 Richard J. P. Brown,1 Timothy P. Hickling,1 William L. Irving,1 Arvind H. Patel2 and Jonathan K. Ball1

Correspondence
Jonathan K. Ball
Jonathan.Ball@nottingham.ac.uk
Arvind H. Patel
a.patel@vir.gla.ac.uk

1The Institute of Infection, Immunity and Inflammation and Division of Microbiology, The University of Nottingham, Queen’s Medical Centre, Nottingham NG7 2UH, UK
2MRC Virology Unit, Institute of Virology, University of Glasgow, Church Street, Glasgow G11 5JR, UK

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Hepatitis C virus (HCV) is a major cause of liver disease worldwide and there is a pressing need for the development of a preventative vaccine as well as new treatments. It was recently demonstrated that the mouse monoclonal antibody (mAb) AP33 potently neutralizes infectivity of HCV pseudoparticles (HCVpp) carrying E1E2 envelopes representative of all of the major genotypes of HCV. This study determined the prevalence of human serum antibodies reactive to the region of HCV E2 recognized by AP33. Antibodies recognizing this region were present in less than 2.5% of sera obtained from individuals with chronic HCV infection. A similar prevalence was found in a smaller cohort of individuals who had experienced an acute infection, suggesting that AP33-like antibodies do not play a major role in natural clearance of HCV infection. Sera exhibited different patterns of reactivity to a panel of peptides representing circulating variants, highlighting the presence of distinct epitopes in this region. Only two sera contained antibodies that could recognize a specific AP33-reactive peptide mimotope. AP33-like antibodies made a measurable contribution to the ability of these sera to inhibit E2–CD81 interaction, but not to the overall neutralization of cell entry. Together, these data show that antibodies to the AP33 epitope are not commonly generated during natural infection and that generation of such antibodies via vaccination may require modified immunogens to focus the generation of specific antibodies. Importantly, individuals harbouring AP33-like antibodies are an important potential source of human mAbs for future therapeutic development.

INTRODUCTION

Worldwide, approximately 180 million people are infected with hepatitis C virus (HCV). Of those infected, 80% experience a chronic infection that frequently leads to serious liver disease such as cirrhosis and primary liver cancer (Alter, 2000; Makris et al., 1996). With up to 15 million people newly infected each year (Anon., 1999), there is a pressing need for the development of an effective vaccine and new therapeutic interventions.

HCV is classified into six distinct genotypes and more than 90 subtypes (Simmonds, 2001). Extensive heterogeneity is also observed within an infected individual, such that the virus population exists as a quasispecies. This enables the virus population to respond to selective pressures including host immunity (Cooreman & Schoondermark-Van de Ven, 1996; Eckels et al., 1999). Identification of protective determinants is crucial to our understanding of the role of neutralizing responses in disease pathogenesis, as well as in the development of appropriate vaccine candidates and antibody-based therapies.

There is increasing evidence for a role of neutralizing antibodies in controlling HCV during all stages of infection (Pestka et al., 2007; Rosa et al., 1996; Zhu & Eckels, 2002). Neutralizing antibodies targeting the hypervariable region 1 (HVR1) at the N terminus of the E2 protein were the first to be identified, but it was demonstrated that a virus population can evolve to escape neutralization by these antibodies (Farci et al., 1996; van Doorn et al., 1995). Neutralizing antibodies recognizing more constrained conformational epitopes have also been isolated (Allander et al., 2000; Bugli et al., 2001; Habersetzer et al., 1998; Hadlock et al., 2000; Ishii et al., 1998; Owsianka et al., 2005). However, it is unclear what role conformation-sensitive antibodies have in controlling infection. Previously, we and others have shown that the region
immediately downstream of HVR1 can elicit antibodies capable of inhibiting E2 binding to the HCV receptor, CD81 (Clayton et al., 2002; Flint et al., 1999; Owsianka et al., 2001; Patel et al., 2000; Triyatni et al., 2002). The mouse monoclonal antibody (mAb) AP33, whose epitope includes amino acid residues between 413 and 420 of the H77c isolate polyprotein (Tarr et al., 2006), inhibits the interaction between CD81 and a range of presentations of E2, including soluble E2, E1E2 heterodimers and virus-like particles (Owsianka et al., 2001). This antibody also potently neutralizes infection of retroviral pseudoparticles (HCVpp) bearing glycoproteins representative of all six genotypes of HCV, and inhibits infection in cell-culture models of HCV (Owsianka et al., 2005; Tarr et al., 2006). The rat mAb 3/11, which recognizes an overlapping epitope in the E2 protein (Tarr et al., 2006), has also been shown to neutralize CD81 binding (Flint et al., 1999) and infectivity of HCVpp reconstituted with autologous strain H77 E1E2 glycoproteins (Logvinoff et al., 2004).

In the current study, we have shown that the prevalence of human serum antibodies directed to epitopes within the region recognized by mAb AP33 is low. Where present, these antibodies are able to inhibit E2 binding to CD81. These findings suggest that induction of AP33-like antibodies by vaccination will require innovative approaches to increase the immunogenicity of this region. However, identification of sera harbouring AP33-like antibodies will prove an invaluable resource for lead therapeutic and prophylactic human mAb discovery.

**METHODS**

**Patient samples.** Serum samples were obtained from 277 HCV antibody-positive individuals, as tested by third-generation ELISA (Ortho Diagnostics) and Roche Amplicor version II (Roche). Of these, 245 were from individuals chronically infected with diverse HCV genotypes. The remaining 32 samples were isolated from patients who had resolved HCV infection (anti-core- and anti-E2-positive, RNA-negative). Samples were taken with ethics committee approval as part of the Trent HCV Cohort Study Group. Sera were inactivated by heating to 56 °C for 30 min before use.

**Peptides and mAbs.** Peptides were synthesized using SynPhase PA lanterns (Mimotopes). Each linear peptide was synthesized on an 8 μm scale, using a C-terminal biocytin molecule separated from the epitope sequence by a Gly-Ser-Gly spacer sequence. Peptides were resuspended in DMSO and diluted to a concentration of 1 mg ml⁻¹ in PBS. Peptides were assessed for purity by matrix-assisted laser desorption/ionization time-of-flight spectrometry and quantified using a NanoDrop spectrophotometer. Peptides representative of the major variants of the region encompassing aa 412–423 of the HCV H77c polyprotein (QLINTNGSWHIN; Table 2) were synthesized. In addition, a panel of peptides were also synthesized where the alanine residue of the AP33 epitope was replaced with an alanine residue (Table 2). A peptide containing a Gly-Ser-Gly spacer sequence. Peptides were inactivated by heating to 56 °C for 30 minutes before use.

**HCV neutralization assays.** Human hepatoma Huh-7 cells (Nakabayashi et al., 1982) and human epithelial kidney 293T cells (ATCC CRL-1573) were propagated as described previously (Clayton et al., 2002). HCVpp were produced essentially as reported previously (Bartosch et al., 2003b; Owsianka et al., 2005) and used for infection of Huh-7 cells in the presence of dilutions of human sera or mAb AP33. Peptide inhibition of neutralization was performed by pre-incubating sera with monomeric AP33 peptide or control HSV-1 UL9 peptide. The cells infected with HCVpp were lysed with 100 μl Gly lysis buffer (Promega); a 50 μl aliquot of the lysate was then mixed with an equal volume Bright Glo luciferase substrate (Promega) and the luminescence was measured in a Hidex Chameleon micro-plate reader.

**Inhibition of CD81 binding.** CD81 large extracellular loop (LEL)–glutathione S-transferase (CD81-LEL–GST) fusion protein (Flint et al., 1999) immobilized onto the wells of a microtitre plate was used to capture 293FT cell lysates containing HCV H77c E1E2 in the absence or presence of either test or control human sera, using dilutions of sera at or near to their IC₅₀. Bound E1E2 was detected by incubation with a rabbit anti-E2 antisera (R646; Owsianka et al., 2005), followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (Dako) with 3,3',5,5'-tetramethylbenzidine substrate. Reactions were stopped with 2 M H₂SO₄ and the absorbance measured at 450 nm. The ability of the AP33 or control peptide G7 to eliminate inhibition of binding was determined by performing the inhibition assay in the presence of 20 μg each peptide ml⁻¹.

**Sequence analysis of the AP33 epitope region of the E2 protein.** Full-length E1E2 clones were recovered from patient sera as described previously (Lavillette et al., 2005; Tarr et al., 2007). The sequence of a fragment of the E2 gene encompassing the AP33 epitope and inhibition of CD81-binding assays corresponded to aa 141–154 of the herpes simplex virus type 1 (HSV-1) UL9 protein (IMNDRPFHRLIVQV; McGeoch et al., 1988) and control biotinylated peptide G7 (GRYPFDSAENH; previously shown not to bind to mAbs AP33 and 3/11), respectively. The anti-E2 mAbs AP33, ALP98 and 3/11 have been described previously (Flint et al., 1999; Owsianka et al., 2005).

**Peptide enzyme immunoassay and competition assays.** Biotinylated peptides were captured on the wells of a Maxisorp microtitre plate (Nunc) each coated with 500 ng neutravidin (Pierce). Fifty microlitre aliquots of human serum, diluted 1:40 in 0.05 % Tween 20 in PBS, were allowed to bind to peptide, and bound serum antibodies were detected using an alkaline phosphatase (AP)-conjugated anti-human IgG antibody (Sigma), followed by p-nitrophenol phosphate substrate (pNPP; Sigma). Absorbance at 405 nm was determined and the assay cut-off calculated as twice the mean of six HCV antibody-negative human serum control wells.

Competition assays were carried out using the enzyme immunoassay described above, except that immobilized peptide was used to capture human serum, diluted between 1:40 and 1:320, containing either mAb AP33, mAb 3/11 or the negative-control mAb ALP98, at final concentrations of 50 μg ml⁻¹.

**Antibody competition assay for binding to recombinant E1E2.** Envelope glycoproteins from the H77.20 clone of HCV (Owsianka et al., 2006) were captured on Galanthus nivalis agglutinin (GNA)-coated ELISA plates. Binding of mAb AP33 was assessed in the presence of the peptide-reactive serum, and HCV-positive, peptide-unreactive serum and normal human serum. Bound mAb AP33 was detected with an AP-conjugated anti-mouse IgG antibody (Sigma) and pNPP substrate.
was determined using Prism Big Dye dideoxy terminator sequencing chemistry. Sequencing products were resolved using a 3130 genetic analyser (Perkin Elmer).

**Statistical testing and hierarchical grouping.** Statistical analyses of data were performed using tests available in the GraphPad Prism 4 software. Hierarchical clustering was performed with between-groups linkage, using Euclidean distance intervals. This was performed using spss version 11.0 for Windows (SPSS Inc.).

**RESULTS**

**Low prevalence of antibodies to the E2 region recognized by AP33 in natural human infection**

The prevalence of human antibodies reactive to a peptide encompassing the AP33 epitope (sequence QLINTNGSWHIN; aa 412–423 of H77c polyprotein; referred to in the following text as AP33 peptide) in sera obtained following acute and chronic infection was determined by immunoassay (Table 1). The peptide represented the most frequent sequence identified amongst viral isolates representative of diverse genotypes (unpublished data). Only six (UKN1A37, UKN1A54, UKN1B15, UKN1B25, UKN2A28 and UKN3A41) of the 245 sera obtained during chronic HCV infection recognized the AP33 peptide; this equated to an overall prevalence of just less than 2.5%. Repeat assays using dilutions of either the capture peptide or the AP33 peptide-reactive serum showed that the response was dose-dependent (not shown). To confirm the accuracy of seroprevalence estimates for genotypes other than genotype 1, 66 genotype

2 and 80 genotype 3a sera were retested using the most frequent variant peptide sequence present on circulating genotype 2 and 3a variants (see peptides described in Table 3). No additional reactive sera were identified in this screening (not shown). Finally, a subset of sera was also tested for reactivity to an NS4 peptide previously shown to be immunogenic. Approximately 36% were positive. The frequency of both AP33 peptide- and NS4 peptide-reactive sera in individuals infected with genotype 1 HCV was greater than for individuals infected with other genotypes ($P<0.0001$, $\chi^2$ test).

To assess whether AP33-like antibodies are associated with control of HCV infection, the prevalence of AP33 peptide-reactive antibodies in serum samples obtained from individuals with resolved infection was also investigated. Of the 21 samples initially tested (of undetermined HCV genotype), only one sample (UKNX18) contained antibodies reactive to the AP33 peptide. Whilst these patients had resolved infection and still possessed detectable anti-E2 antibodies (data not shown), the duration of time post-resolution was unknown. To address this, sera that had been obtained between 2 and 18 weeks following resolution of infection were screened. Despite detectable anti-E2 responses in nine out of 11 patients (not shown), antibodies recognizing the AP33 peptide were not identified in any of these samples.

**Serum antibodies possess epitopes that overlap those recognized by mAbs AP33 and 3/11**

Having identified serum samples that contained antibodies reactive to the AP33 peptide, we next assessed the specificity of this response. Specificity was initially tested in competition assays using mAbs AP33 and 3/11 and the negative control mAb ALP98 (Fig. 1a). AP33 and 3/11 showed similar levels of competition against sera UKN2A28 and UKN1B25. Neither antibody was able to reduce serum UKN1A54 binding by more than 50%. For the remaining sera, each mAb was able to compete to differing degrees. For example, mAb 3/11 shows lower levels of competition than mAb AP33 against sera UKN1A37, UKN1A54 and UKN1B15.

To verify further the specificity of the antibody response to the AP33 epitope, reactive sera and control sera were used to compete with AP33 binding to GNA-captured H77c E1E2 (Fig. 1b). HCV antibody-negative serum and those HCV-positive sera that were unreactive to the AP33 peptide failed to inhibit mAb AP33 binding to E1E2. In contrast, all of the sera that harboured AP33-peptide reactive antibodies were able to compete with mAb AP33 for binding to E1E2 by between 65 and 85%.

**Identification of amino acid residues critical for serum binding**

Having demonstrated that the serum antibodies recognized epitopes within the aa 412–423 region of E2, we went on to...
Table 2. Reactivity of serum to peptides containing point substitutions across the region representative of aa 412–423 of HCV E2

Reactivity is expressed as the percentage of binding compared with the consensus peptide sequence and was assessed by ELISA; the mean result is representative of at least two independent experiments. Reactivity: ■, <10%; □, 10–79.9%; △, 80–120%; ◻, >120%.

<table>
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<tr>
<th>Sequence Amino acid replacement</th>
<th>AP33 3/11</th>
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<th>UKN1A54</th>
<th>UKN1B15</th>
<th>UKN1B25</th>
<th>UKN2A28</th>
<th>UKN3A41</th>
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Table 3. Reactivity of mAbs and AP33 peptide-reactive sera with peptides representative of natural variants of the aa 412–423 sequence of E2

Reactivity is expressed as the percentage of binding compared with the reference consensus peptide sequence and was assessed by ELISA. Results represent the mean of at least two independent experiments. Reactivity: ■, <10%; □, 10–79.9%; △, 80–120%; ◻, >120%.

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<th>Peptide no.</th>
<th>Sequence Amino acid replacement</th>
<th>Genotype distribution (%)*</th>
<th>Antibody reactivity</th>
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*The frequency of a particular epitope sequence within a given genotype of HCV, sampled from 5800 sequences retrieved from GenBank.
map specific residues critical for serum and mAb binding. This was performed by testing serum and mAb reactivity to a panel of alanine-substitution AP33 peptides (Table 2). mAb AP33 binding was reduced by the substitutions L413A, I414A, T416A, G418A, W420A and H421A, whereas mAb 3/11 binding was susceptible to the mutations I414A, W420A and H421A. These data demonstrated that these mAbs recognize distinct, yet overlapping, epitopes. Alanine replacement at positions N415, T416, G418 and W420 resulted in reduced binding by all sera. In contrast, the Q412A mutation had an enhancing effect for six of the seven sera tested. Binding of the serum from the resolved infection (UKNX18) was unusual in that reactivity was eliminated by the S419A substitution, whereas for a number of the other sera, for example UKN1A37, this substitution enhanced binding. To assess whether the effect of mutation on binding segregated according to the genotype of the infecting virus, hierarchical cluster analysis was performed to group similar patterns of seroreactivity to the mutant peptides. The Euclidean distances for each group were calculated, demonstrating that sera obtained from genotype 1-infected individuals grouped together, although the reactivity of serum UKN1A37 was highly divergent from the other genotype 1 sera (Fig. 2).

**Reactivity of patient antibodies with an AP33 mimotope**

We previously described a random peptide display library-enriched peptide mimotope of the AP33 epitope (VELRNLLGTTWRP), which is highly specific to mAb AP33 (Tarr et al., 2006). To dissect further the antibody specificities detected in human sera, binding to this peptide was assessed. Only two of the sera (UKN1B15 and UKN3A41) recognized this mimotope (Fig. 3), and the reactivity was similar to that observed for the aa 412–423 screening peptide. By contrast, reactivity of mAb AP33 to this mimetic was more than twice that observed for the peptide corresponding to the native epitope. Together, these data indicated that antibodies present in sera UKN1B15 and UKN3A41 share similar contact residues to those of AP33, whilst the other sera

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**Fig. 1.** Competition between mAbs and human sera for binding either to the AP33 peptide or to immobilized H77c E1E2. (a) Human sera were mixed with either the mAb AP33 (▲), mAb 3/11 (◇) or control antibody ALP98 (●) at a final concentration of 50 μg ml⁻¹. Binding to target AP33 peptide was detected by enzyme immunoassay. (b) Serum blocking of mAb binding to H77c E1E2 protein. Saturating amounts of GNA-captured H77c E1E2 were detected by 1 μg mAb AP33 ml⁻¹ in the presence of different human sera at a final dilution of 1:40. Control sera obtained from HCV E1E2-antibody positive sera that were non-reactive to the AP33 peptide were included as controls together with a normal human serum (NHS). mAb AP33 binding was expressed as a percentage of that observed in the presence of the NHS. All AP33 peptide-reactive sera blocked binding of mAb AP33, whilst HCV-antibody positive, AP33 peptide-non-reactive sera (controls 1, 2 and 3) all failed to inhibit binding.

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harbour antibodies that recognize distinct but overlapping epitopes.

**Reactivity of sera to peptides representative of natural HCV variant sequences of the AP33 epitope**

We next assessed whether naturally occurring variability within region aa 412–423 of E2 could affect serum recognition. A panel of peptides was synthesized representing the most common variant sequences observed across genotypes 1 to 6. These peptides were tested for their ability to bind to reactive sera (Table 3). One of the most striking findings was that binding by mAb AP33 was unaffected by all of the naturally occurring substitutions, and this property was shared by serum UKN3A41. Binding observed for the remaining sera was affected by at least one of the amino acid substitutions. The substitution T416S generally resulted in reduced binding, as did mutation of I414V in conjunction with T416S. Interestingly, the mutation I422V, which was also present in the Glasgow strain immunogen used to raise mAb AP33 antibody (Owsianka et al., 2001), reduced binding in four of the seven sera tested. Finally, mAb 3/11 showed similar reactivity to AP33 except that binding was reduced by the double substitution I414V and T416S, although individually these substitutions had minimal effect.

**Inhibition of CD81 binding**

We have previously demonstrated that mAb AP33 inhibits binding of E2 to the receptor CD81, a component of the entry pathway for HCV (Owsianka et al., 2001). We therefore assessed the potency of AP33 peptide-reactive sera to inhibit CD81 binding in a plate-based assay for neutralization of CD81-LEL binding, and the ability of a saturating amount of the AP33 peptide to eliminate any inhibitory effect (Fig. 4a). Having determined that all of the HCV antibody-positive sera tested were capable of inhibiting H77c E1E2 binding to CD81-LEL–GST, these sera were diluted to achieve inhibition within the dynamic range of the assay, as close to 50% inhibition as possible. Addition of the AP33 peptide reduced the inhibitory effects by varying degrees, with serum UKN1A54 and UKN2A28 being most and least susceptible to its effects, respectively. These data indicated that the AP33 peptide-reactive antibodies make varying contributions to the serum’s ability to inhibit the E2–CD81 interaction.

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**Fig. 2.** AP33-reactive sera derived from genotype 1-infected individuals cluster based on their reactivity to a panel of alanine-replacement point-mutated peptides. The unrooted tree was derived from Euclidean distances calculated from each serum’s relative reactivity to the mutated peptides shown in Table 2.

**Fig. 3.** Reactivity of mAbs and sera to a peptide representing a mAb AP33-reactive phage library-selected peptide mimotope is restricted to a subset of AP33 peptide-reactive sera. Immobilized peptides representing the consensus aa 412–423 (WT), mimotope (P1) or negative-control peptide (NEG) were probed with AP33 peptide-reactive sera or control antibodies mAb AP33, mAb 3/11 or mAb ALP98. In addition to the positive-control mAb AP33, only UKN1B15 and UKN3A41 reacted with the AP33-selected peptide.
Neutralization of HCV pseudotype particle infectivity by patient sera

Having shown that patients’ sera contain antibodies that recognize epitopes that overlap the AP33 epitope, and that some of these are likely to contribute to the sera’s ability to inhibit E2 binding to CD81, we next assessed the contribution of these antibodies to the neutralizing potential of these sera. HCVpp incorporating E1E2 derived from the infectious clone H77c were mixed with sera at a dilution that was approximately the IC50 value, in the presence of the AP33 peptide or an irrelevant negative-control peptide, and the resulting HCVpp infectivity was determined (Fig. 4b). A peptide concentration of 10 μg ml−1 was used for inhibition, as this concentration of peptide had no direct effect on the infectivity of the HCVpp (not shown). A noticeable reduction in neutralization in the presence of the AP33 peptide was only evident for mAb AP33 and not for the sera. Therefore, whilst AP33-like antibodies are present in natural infection, these do not make a measurable contribution to the overall neutralizing potency of these sera. To verify this finding further, peptide inhibition of neutralization assays were repeated with peptide concentrations ranging from 0 to 100 μg ml−1. Increasing peptide concentration did not affect the inhibition of HCVpp neutralization (not shown).

The presence of AP33-like antibodies is not associated with AP33 epitope variation in the autologous viral quasispecies

To determine whether the presence of antibodies recognizing the AP33 epitope could drive sequence change within the aa 412–423 region of the HCV polyprotein, this region in the HCV quasispecies present in some of the patients’ autologous serum samples was assessed (Fig. 5). Despite the presence of AP33-like antibodies in these patients, the viral quasispecies possessed a peptide sequence known to be recognized by mAb AP33. This demonstrated that either escape was mediated by sequence change outside of this region or, more likely, the neutralizing (selective) effect of these antibodies was not potent enough to drive escape.

DISCUSSION

mAb AP33 recognizes a highly conserved epitope located in the E2 protein and is capable of potent neutralization of cell culture-grown HCV strain JFH-1, as well as HCVpp bearing E1E2 glycoproteins representative of the major HCV genotypes (Owsianka et al., 2005; Tarr et al., 2006). Here, we assessed the immunogenicity of the epitope defined by the AP33 antibody in natural infection. Reactivity to the AP33 epitope was confirmed by competition assays. The prevalence of antibodies reactive to the AP33 epitope, in sera from both chronic and acute resolved infections, was less than 2.5 %. To ensure that this low prevalence was not due to limitations in the peptide capture assay used, a subset of samples was tested for reactivity to an immunogenic epitope in the NS4 protein. Reactivity was observed in 36 % of sera tested. This is slightly lower than the previously reported detection frequency, although this is most likely due to differences
in the genotype distribution between the samples analysed in the two studies and the genotype-restricted specificity of this epitope (Simmonds et al., 1993). This observation was supported by our finding of greater reactivity of the NS4 peptide with genotype 1 sera, as the epitope sequence was isolated from a genotype 1a sequence.

We have previously mapped the epitopes recognized by mAb AP33 by measuring the effects of alanine replacement mutagenesis of E2 on mAb–E2 binding, as well as by enrichment of random peptide display libraries; alanine replacement at positions L413, N415, G418 and W420 resulted in greater than 75% reduction in binding, whilst alanine substitution at other sites had either a negligible effect or enhanced binding (Tarr et al., 2006). In the present study, using the corresponding peptide panel, a different profile was observed. In particular, mutation N415A had no effect on binding, whilst I414A and T416A both reduced binding by greater than 60%. The reason(s) for these discrepancies are not clear, but one possibility is that mutagenesis of the peptide and of the full-length E2 protein has different effects on the conformation of the epitope in each context and therefore different effects on mAb recognition. Indeed, we have shown previously that the AP33 epitope is partially sensitive to conformation (Tarr et al., 2006). The sera exhibited quite distinct patterns of reactivity to the alanine replacement peptides, although hierarchical clustering, based on the effects of the substitutions on binding, led to grouping according to genotype. This suggests that the presentation of the AP33 epitope is genotype-specific and that this skews the host antibody response. Only two sera (UKN1B15 and UKN3A41) recognized a peptide sequence corresponding to a previously isolated AP33 mimotope. Together, these findings indicated that only a minority of the sera reactive to the aa 412–423 region of E2 contained antibodies with the same specificity as mAb AP33, and that antibodies with differing and possibly overlapping specificities occur.

To investigate further the apparent restriction of AP33-reactive antibodies in those patients infected with genotype 1 HCV, as well as the apparent genotype-based clustering, seroreactivity to peptides representative of the HCV genotypes was assessed. However, this analysis showed that recognition of the genotype variant peptides did not correlate with the infecting HCV genotype, suggesting that, when present, antibodies binding to this region in natural infection are able to cross-react with different strains of HCV. Binding of serum UKN3A41 was unaffected by all of the naturally occurring mutations, as was binding of mAb AP33. This, together with its clustering with AP33 in the hierarchical cluster analysis and recognition of the AP33 mimotope, suggested that a proportion of UKN3A41 serum antibodies were of similar specificity to mAb AP33.

We then went on to determine whether these antibodies contributed to the sera’s ability to neutralize HCVpp infectivity and inhibit CD81 binding. All of the sera were capable of neutralizing HCVpp entry, in line with other reports (Bartosch et al., 2003a; Hsu et al., 2003; Lavillette et al., 2005). However, addition of AP33 peptide had minimal effect on neutralization, suggesting that most of the neutralizing antibodies were directed to epitopes outside the aa 412–423 region of E2. By contrast, AP33 peptide was able to reduce the inhibition of CD81 binding by these sera. Attenuation of binding inhibition by the peptide was not absolute, highlighting the fact that antibodies targeting other regions of the CD81-binding
domain (Allander et al., 2000; Flint et al., 1999; Yagnik et al., 2000) are also involved. Together, our data support the hypothesis that antibodies directed towards the AP33 epitope make a significant contribution to inhibition of CD81 binding, in the context of a polyclonal neutralizing antibody response that includes epitopes outside the AP33 epitope region. It is possible that the human antibodies shown to bind to the AP33 epitope region have lower affinity for the peptide than for the recombinant protein, resulting in reduced elimination of peptide blocking. The interpretation of HCVpp neutralization data are also complicated by the enhancement of infectivity by human serum components, particularly high-density lipoprotein (HDL), as described previously (Bartosch et al., 2005; Voisset et al., 2005). Recent studies have shown that HDL can attenuate the HCVpp-neutralizing potency of some mAbs, including mAb AP33, that target the CD81-binding domain (Dreux et al., 2006). This observation might explain the low contribution of the AP33-like antibodies in HCVpp neutralization. Our finding that the region of E2 encompassing the AP33 epitope was highly conserved within the viral quasispecies present in the sera containing AP33-like responses suggests that HDL-mediated attenuation of infection may still have relevance in vivo and these issues need to be resolved using suitable model systems.

However, to minimize the effect of serum components in our neutralization assays, we used a serum dilution at which little enhancement by the control serum was observed. An alternative approach would be to use purified immunoglobulin (Ig) fractions, but this was not possible due to the small amounts of sera available. Recently, Zhang et al. (2007) were able to purify Igs reactive to a peptide that included the AP33 epitope, and found that these Igs were neutralizing in the HCVpp assay, confirming the presence of naturally occurring antibodies directed to this region. Although they were unable to dissect fully the epitopes recognized, our data would suggest that a range of epitopes within the aa 412–423 region of the E2 protein would be targeted by their purified Igs.

Identifying potently neutralizing antibodies with epitopes conserved across all isolates of HCV is an essential step in the development of a successful vaccine. The high degree of positive selection observed in regions involved in CD81 binding during natural infection is probably driven, at least in part, by antibody escape (Brown et al., 2005, 2007). Therefore, any interplay between neutralizing antibodies and serum components is complex, and consequently antibodies that target CD81 binding should not be dismissed as potential vaccine targets until these issues have been better resolved. The constrained linear nature of the AP33 epitope, together with the broad neutralizing phenotype of AP33, renders this region a highly promising target for vaccine-induced neutralizing antibodies. However, our current data show that this region is poorly immunogenic in natural infection. Whilst this region is predicted to be solvent exposed (Jackson et al., 1997; Yagnik et al., 2000) and is clearly targeted by at least a proportion of infected individuals, it is still possible that mechanisms such as glycan (Wei et al., 2003) or lipid (Andre et al., 2002) shielding may reduce its immunogenicity. Indeed, the AP33 epitope contains a putative N-linked glycosylation site at position 417 (Slater-Handshy et al., 2004), which has recently been described as important in evasion of antibody neutralization of HCVpp (Helle et al., 2007). Therefore, it will be interesting to determine whether this glycan affects the neutralizing potency of these serum antibodies targeting the AP33 epitope.

Despite their rarity, AP33-like antibodies and their epitopes can still have a major role in the development of future vaccines and antibody therapies. The data presented here provide evidence that AP33-like antibodies might be generated in humans by immunization. Focusing the human response to generate antibodies specific for the AP33 epitope is likely to require alternative approaches to developing an appropriate immunogen. This scenario is not unprecedented: much of the current vaccine effort towards human immunodeficiency virus type 1 is concentrated on engineering immunogens to elicit immune responses akin to a handful of potent broadly neutralizing mAbs (reviewed by Burton et al., 2004). Such an approach, in the context of HCV, will require in-depth knowledge and understanding of the antibody–antigen interaction. Only with correct presentation of an immunogen will a vaccine generate the desired immune responses. Prophylactic administration of neutralizing antibodies may have an important role in post-liver-transplant management, as is the case for hepatitis B virus infection (Shouval & Samuel, 2000). Therefore, individuals harbouring antibodies with similar specificities to the broadly and potently neutralizing mAb AP33 will be an important source for human mAb isolation and future therapy.

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REFERENCES


Anon. (1999). Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the
Viral Hepatitis Prevention Board, Antwerp, Belgium. J Viral Hepat 6, 35–47.


