The role of neutralizing antibodies in hepatitis C virus infection

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Hepatitis C virus (HCV) is a blood-borne virus estimated to infect around 170 million people worldwide and is, therefore, a major disease burden. In some individuals the virus is spontaneously cleared during the acute phase of infection, whilst in others a persistent infection ensues. Of those persistently infected, severe liver diseases such as cirrhosis and primary liver cancer may develop, although many individuals remain asymptomatic. A range of factors shape the course of HCV infection, not least host genetic polymorphisms and host immunity. A number of studies have shown that neutralizing antibodies (nAb) arise during HCV infection, but that these antibodies differ in their breadth and mechanism of neutralization. Recent studies, using both mAbs and polyclonal sera, have provided an insight into neutralizing determinants and the likely protective role of antibodies during infection. This understanding has helped to shape our knowledge of the overall structure of the HCV envelope glycoproteins – the natural target for nAb. Most nAb identified to date target receptor-binding sites within the envelope glycoprotein E2. However, there is some evidence that other viral epitopes may be targets for antibody neutralization, suggesting the need to broaden the search for neutralization epitopes beyond E2. This review provides a comprehensive overview of our current understanding of the role played by nAb in HCV infection and disease outcome and explores the limitations in the study systems currently used. In addition, we briefly discuss the potential therapeutic benefits of nAb and efforts to develop nAb-based therapies.

Introduction

Hepatitis C virus (HCV) infects approximately 170 million people worldwide (Ascione et al., 2007) and as many as 3 million individuals are newly infected each year (Anonymous, 1999). Currently, there is no available vaccine to prevent HCV infection. In 20–30 % of infections the virus is cleared spontaneously (Santantonio et al., 2008); however, in the majority of patients the virus persists. The mechanism by which some individuals spontaneously resolve infection, while others become chronically infected is not clearly understood. Chronic HCV infection can lead to cirrhosis of the liver and, in some cases, hepatocellular carcinoma (HCC), which ultimately requires a liver transplantation. HCV infection is the predominant indication for liver transplantation and no treatments are currently available to prevent reinfection of a grafted liver. The current standard therapy for chronic infection – pegylated alpha interferon (IFN-α) and ribavirin – is only effective in 40–60 % of cases and response to therapy varies between different viral genotypes. Two recently developed protease inhibitors, boceprevir and telaprevir, given in combination with IFN-α and ribavirin, improve sustained virological response (SVR) rates to 75 % in genotype 1-infected patients (Kwo et al., 2010; McHutchison et al., 2010). This development in the treatment of hepatitis C is encouraging; however, HCV still represents a major disease burden. Recent developments in the model systems used to study virus–receptor and virus–antibody interactions have led to advances in our understanding of the nature of the anti-HCV antibody response and demonstrate the complexity of the virus–host relationship. However, there are still large gaps in our knowledge and understanding of the interplay between HCV and neutralizing antibodies (nAb).

Virus targets of the antibody response

Antibodies generated during acute infection may be targeted against epitopes within structural and non-structural viral proteins; however, the majority of nAb have been mapped to the envelope glycoproteins E1 and E2 (Johansson et al., 2007; Kato et al., 1993; Keck et al., 2008b; Meunier et al., 2008; Owsianka et al., 2005; Perotti et al., 2008; Shimizu et al., 1996). E1 and E2 are produced by cellular signal peptidase cleavage from the viral polyprotein
thought to anchor the glycoproteins within lipid membranes endoplasmic reticulum (ER)-retention signals that are thought to anchor the glycoproteins within lipid membranes (Op De Beeck et al., 2001). The N-terminal ectodomain of E2 possesses the entry determinants for infection of the host cell (Burlone & Budkowska, 2009). A 3D model of the structure of E2 has recently been proposed (Krey et al., 2010). This model assigns a typical class II fusion protein structure to E2, akin to the fusion proteins of members of the alpha- and flaviviruses, consisting of three distinct domains. The structure of E1 is less well defined, although it has been proposed to function as the fusion determinant, triggering fusion of the viral and cellular membranes during entry (Lavillette et al., 2007). However, in other viruses belonging to the family Flaviviridae, the proteins analogous to E1 chaperone the fusion protein to ensure correct folding; this is reflected in the model by Krey et al. (2010) where the putative fusion peptide is located in domain II of the E2 protein. E1 and E2 form non-covalent heterodimers (Dubuisson et al., 1994) and these were considered to be the mature functional forms on the surface of the virus. However, more recent studies of E1 and E2 present in cell culture infectious HCV particles indicated the presence of larger covalent complexes stabilized by disulphide bridges (Vieyres et al., 2010); it remains to be fully determined which represents the functional form. Despite conserved function between clinical isolates, the E2 glycoprotein tolerates great genetic diversity (Fig. 1, Supplementary Fig. S1, available in JGV Online). The ectodomain contains three highly variable regions. Hypervariable region (HVR) 1 is a 26–28 aa region located at the N terminus of E2 that plays an important role in entry, antibody binding and disease outcome (Bartosch et al., 2003c; Farci et al., 2000; Vieyres et al., 2011). Two additional hypervariable regions, HVR2 and the intergenotypic variable region (igVR) are thought to be involved in E1E2 heterodimerization and virus infectivity (Albecka et al., 2011; McCaffrey et al., 2011). Two additional hypervariable regions have been identified within the subtype 3a E2 glycoprotein. These regions, designated HVR495 and HR575, are located downstream of HVR1 at aa 495–501 and 575–578, respectively. Both regions are under positive selection during acute (Humphreys et al., 2009) and chronic infection (Brown et al., 2007). Whether or not substitution in these additional variable regions is being driven by antibody responses, and the clinical relevance of this variability, is unknown.

Early evidence of nAb in HCV infection

The earliest studies of the antibody response to HCV were carried out in chimpanzees. Such studies showed that serum from HCV-infected individuals can neutralize virus infectivity in vitro and subsequently protect chimpanzees against challenge with HCV (Farci et al., 1994). Similarly, hyper-immune sera raised against peptides homologous to HVR1 also neutralized HCV infectivity in vitro (Shimizu et al., 1996) and in vivo (Farci et al., 1996). In a separate study, chimpanzees immunized with the envelope glycoproteins E1E2 generated a strong antibody response that partially protected against experimental challenge with autologous HCV (Choo et al., 1994). Evidence that antibodies could protect from natural infection in humans arose from a retrospective study of a cohort of patients receiving polyclonal immunoglobulins against hepatitis B virus surface antigen (HBIG). Patients who received HBIG prior to the introduction of routine screening for HCV infection were less likely to develop HCV than those who received HBIG screened for HCV. Anti-HCV antibodies were detected in HCV-negative patients who had undergone HBIG treatment, indicating a passive transfer of anti-HCV antibodies within HBIG to the recipient (Feray et al., 1998). Furthermore, individuals with hypogammaglobulinaemia (Bjørø et al., 1994) who became infected with HCV experienced a very rapid disease progression, highlighting the potential importance of antibodies in controlling chronic infection. It has however been reported that some hypogammaglobulinaemic patients are able to spontaneously resolve acute HCV infection (Razvi et al., 2001), indicating the importance of cell-mediated immunity in resolved infection.

Model systems to study nAb response to HCV

Chimpanzees are the only species other than humans permissive for HCV infection. Due to ethical and financial constraints, however, they are not an ideal system in which to study the virus and these in vivo studies have largely been replaced by in vitro systems.

Early in vitro methods to study virus–antibody and virus–cell interactions relied upon soluble, recombinant, truncated or full-length versions of E2 expressed in mammalian cells. This system enabled the identification of the first cellular receptors for HCV, namely CD81 (Pileri et al., 1998). Based on this discovery, many early studies to isolate nAb utilized the neutralization of binding (NOB) assay that relies upon E2 binding to Molt-4 cells expressing CD81 (Flint et al., 1999; Rosa et al., 1996). However, as will be discussed later, this system possessed several shortcomings and was superseded by the development of virus-like particles (VLPs), generated in insect cells (Baumert et al., 1998). These VLPs showed structural similarities to the virions isolated from HCV-infected humans and chimpanzees (Baumert et al., 1998) and therefore more accurately represented the native conformation of the envelope glycoproteins. Indeed, comparison of the neutralizing activity of a panel of mAbs to VLPs and soluble E2 (E2661) showed a marked difference in their ability to bind the different forms of E2 (Clayton et al., 2002) and to block E2–CD81 interactions (Owsianka et al., 2001). These studies highlight the importance of using E2 representative of the native glycoprotein found on the surface of the viral particle. However, these systems have since been replaced by the HCV pseudoparticle (HCVpp) and HCV cell culture (HCVcc) assays.
HCVpp are generated by displaying HCV envelope glycoproteins on the surface of retroviral or lentiviral core particles (Bartosch et al., 2003b). This has facilitated studies of viral attachment and entry into target cells and led to the identification of neutralizing sera and antibodies (Bartosch et al., 2003a; Flint et al., 2004; Yu et al., 2004). One advantage of this system is the ability to generate HCVpp displaying E1E2 isolated from a variety of sources (Fafi-Kremer et al., 2010; Tarr et al., 2011). Although useful for studying entry and neutralization, this model system does have its drawbacks. Not all patient-derived E1E2 form functional pseudoparticles (Dowd et al., 2009; Flint et al., 2004; Lavillette et al., 2005), and the reasons for this are unknown. Also, the cell-type used to generate infectious pseudoparticles (human embryonic kidney cells) lacks lipid metabolism machinery (Burlone & Budkowska, 2009); therefore, HCVpp are not associated with any host lipoproteins, unlike serum-derived viral particles (see below). This limitation may greatly affect the interaction of HCVpp with nAb.

HCVcc is based on replication of the genotype 2a stain, JFH-1, in human hepatocarcinoma cells. This is the only strain of HCV so far found to replicate in cell culture without accumulating adaptive mutations. The virus particles produced are infectious allowing studies of the complete virus life cycle (Kato et al., 2001; Wakita et al., 2005). This assay system has been modified to study the properties of genetically diverse viruses by the development of chimeric infectious clones encoding the structural proteins (core, E1, E2 and p7) and non-structural protein 2 (NS2) of all major genotypes (1–7). However, these chimeras often show poor replication kinetics and also acquire cell-culture adaptive mutations (Gottwein et al., 2006; Pietschmann et al., 2006). There is emerging evidence that at least some culture-adaptive mutations render isolates more sensitive to neutralization (Dhillon et al., 2010; Grove et al., 2008). This phenomenon has been widely documented for other viruses, including human immunodeficiency virus (HIV) (Pugach et al., 2004) and respiratory syncytial virus (Marsh et al., 2007). Therefore, an HCV-based single-cycle infection system, particularly one that could be easily supplemented with E1E2 cloned directly ex vivo, would provide a more robust method for studying antibody neutralization. Trans-complementation of HCV replicons with constructs expressing the HCV structural genes has been shown to result in the production of infectious particles containing a packaged replicon genome, which can then be used to infect permissive cells (Adair et al., 2009). However, the relatively low virus titres produced limit the general applicability of this system.

A small animal model capable of supporting the complete replicative cycle is potentially a better system in which to study systemic virus–host interactions. However, current animal models are far from perfect. The uPA-SCID mouse model uses immunosuppressed chimeric mice transplanted with human hepatocytes, which renders them susceptible to HCV infection (Lindenbach et al., 2006; Mercer et al., 2001). Generation of the chimeric livers is technically difficult and the mice are immunodeficient, preventing any study of the host-adaptive immune response. They have, however, been used to study virus neutralization by passively transferred antiviral antibodies (Law et al., 2008; Vanwollegem et al., 2008) and anti-receptor antibodies (Meuleman et al., 2008). More recently, a transgenic immunocompetent mouse, expressing the essential HCV entry factors, has been developed. Whilst incapable of supporting significant HCV replication, it supports HCV entry and has been used to demonstrate entry inhibition by virus- and receptor-specific antibodies (Dorner et al., 2011).

The nature of epitopes recognized by nAb

To inform vaccine design and to understand the process of neutralization, it is important to define the nature and location of neutralizing epitopes. Epitopes can be categorized as being either linear or conformational. A number of linear HCV epitopes have been mapped using peptide scanning, a technique where reactivity to a panel of overlapping peptides corresponding to the protein of interest is determined (Clayton et al., 2002; Owianska et al., 2001). In addition, iterative enrichment of random peptide display libraries can be used to identify peptides capable of binding to mAbs and serum (Tarr et al., 2006; Zhang et al., 2007, 2009). The technique utilizes a suitable host, phage or bacterium, which is genetically modified to display 6–40mer peptides of a random nature, which can be either linear or constrained in a disulphide loop. Multiple rounds of biopanning then enrich peptides able to bind specifically to the target molecule. Alignments of the resulting peptide sequences identify likely antibody contact residues within the linear peptide sequence. Identifying residues constituting conformational epitopes is more challenging. A common approach is to probe panels of glycoproteins possessing single amino acid substitutions (most often using alanine replacement) with the antibody of interest. Marked loss of reactivity indicates that the substituted residue is involved in binding (Roben et al., 1994). However, introduction of substitutions, particularly in structurally important regions of the protein, can have an adverse effect on the overall conformation of the protein. It is important to ensure that the global structure of a mutated protein is maintained, by ensuring that the binding of control, non-competing, conformation-dependent antibodies is unaltered. Using this mutant panel approach, we have successfully mapped the epitopes recognized by a large number of conformation-sensitive and -insensitive antibodies (Johansson et al., 2007; Law et al., 2008; Owianska et al., 2008; Perotti et al., 2008; Tarr et al., 2006). However, such a directional mutagenesis approach is labour-intensive and requires prior knowledge of the possible contact residues. More recently, a number of novel mAbs were mapped using yeast surface-expressed random- and deletion-mutant E2 libraries (Sabó et al., 2011). Such an approach enables a large number of different residues to be interrogated simultaneously. However, the overall fold of the yeast-expressed proteins was not verified, and this will need to be performed before this approach can be adopted widely. Finally, studies
of antibody escape, particularly in vitro, can provide insight into the location of key epitopes. Passing virus in the presence of nAb and characterizing the emergence of escape mutations has been carried out for HIV (Mo et al., 1997), foot-and-mouth disease virus (Crowther et al., 1993), hepatitis A virus (Ping & Lemon, 1992), influenza A virus (Kaverin et al., 2002) and, more recently, HCV (Gal-Tanany et al., 2008; Keck et al., 2008b, 2011).

**Potential nAb epitopes: HVR1**

Early chimpanzee studies demonstrated that nAb have the potential to protect against HCV infection or to reduce the severity of the disease. They identified the HVR1 region of E2 as a major target for nAb. HVR1 possesses multiple linear epitopes between aa 384 and 410. The HVR1 region plays an important role in antibody recognition and disease outcome, and is necessary for binding to scavenger receptor class B type I (SR-BI), a lipoprotein receptor molecule involved in HCV entry (Fig. 2) (Bartosch et al., 2003c; Scarselli et al., 2002). Antibodies targeting HVR1 have been identified in vivo (Kato et al., 1993, 1994; Weiner et al., 1992); however, they tend to be highly strain specific (Bartosch et al., 2003a; Shimizu et al., 1996; Vieyres et al., 2011). It has also been suggested that high concentrations of HVR1-specific nAbs are required for effective neutralization (Bartosch et al., 2003a). A number of HVR1-specific mAbs have been generated and characterized (Table 1, Figs 1 and 3, and Supplementary Fig. S1). The rat mAb 9/27 inhibits SR-BI binding to E2, neutralizing infectivity of retroviral pseudotypes bearing HCV E1E2 glycoproteins derived from a genotype 1a virus (Bartosch et al., 2003a; Hsu et al., 2003). It also possesses the ability to neutralize cell-to-cell transfer of HCV (Brimacombe et al., 2011). The epitope recognized by this mAb has been localized to the C-terminal portion of HVR1 (aa 396–407) (Hsu et al., 2003). Other HVR1-specific antibodies (the mouse mAb AP213 and the polyclonal sera R1020 and R140) that show neutralizing activity against HCVpp and HCVcc also map to epitopes within the C-terminal portion of HVR1 (Vieyres et al., 2011). In contrast, the non-neutralizing mAbs 7/59, 6/16 and 6/82a bind to the N-terminal portion of HVR1 (aa 384–395) (Hsu et al., 2003). Thus, it appears that there are two immunogenic regions within HVR1, with the C-terminal portion containing the neutralization determinants.

In addition to mediating the direct binding of E2 and SR-BI, HVR1 is also necessary for interaction between SR-BI and HDL, which has been shown to augment entry (Bartosch et al., 2005; Voisset et al., 2005). This same interaction inhibits neutralization of HCVpp (Bartosch et al., 2005) and HCVcc (Dreux et al., 2006) by anti-HCV antibodies. However, dependency and function of HVR1 in infectivity might vary between different genotypes (Prentoe et al., 2011). Recent studies have also shown that HVR1 is able to mask nAb epitopes within E2, as HVR1 deletion mutants are much more susceptible to neutralization by a panel of human mAbs and patient sera targeting the CD81-binding site within the E2 protein (Bankwitz et al., 2010; Prentoe et al., 2011). This is probably due to masking of the CD81-binding site. Therefore, HVR1 may function to protect viral entry determinants within E2 from neutralization during the early stages of entry (Bankwitz et al., 2010). These myriad roles played by HVR1, and their somewhat contradictory nature, has led some to suggest that HVR1 functions as an immunological decoy, stimulating a strong antibody response towards HVR1 that does not result in viral clearance, but instead drives the selection of antibody-escape mutants (Ray et al., 1999).

**Potential nAb epitopes: interactions with CD81**

The lack of broadly nAb targeting HVR1 led to the search for other, well-conserved antibody targets. CD81 was the first host factor identified as critical for virus entry and quickly became the focus of the search for nAb. Its role in virus entry is reviewed by Burlone & Budkowska (2009) and is outlined in Fig. 2. CD81 is a member of the tetraspanin family of transmembrane proteins found on most cell types. It was first identified as a receptor due to its ability to bind a recombinant soluble ectodomain of the E2 protein (sE2), a reaction that occurs via the CD81 large extracellular loop (LEL) (Pileri et al., 1998). Both anti-CD81 mAb and soluble CD81 LEL are able to inhibit the entry of HCVpp and HCVcc into hepatoma cells (Bartosch et al., 2003b; Wakita et al., 2005).
Early studies showed that anti-HCV serum from chimpanzees and humans was able to block E2 binding to target cells due to the presence of NOB antibodies (Ishii et al., 1998; Rosa et al., 1996). The identification of CD81 as a cellular receptor by Pileri et al. (1998) confirmed that NOB antibodies are able to inhibit HCV binding to CD81.

Antibody competition studies provided the first insight into regions of E2 involved in CD81 binding. These studies suggested that CD81-binding sites exist within E2 regions aa 412–423 (Owsianka et al., 2001), aa 432–447 (Clayton et al., 2002), aa 480–493 (Flint et al., 1999), aa 528–535 (Owsianka et al., 2001) and aa 544–551 (Flint et al., 1999). Subsequent mutagenesis studies confirmed the importance of most of these regions by showing that the specific residues critical for E2 binding to CD81 include W420, Y527, W529, G530 and D535 (Owsianka et al., 2006) and the 436GWLAGLFY443 motif (Drummer et al., 2006). However,
there was no evidence for the involvement of conserved residues within region aa 480–493 (Owsianka et al., 2006). These somewhat conflicting data demonstrate the importance of the choice of E2 used in mapping studies. The initial suggestion that aa 480–493 function as a CD81-binding site was based on studies with a soluble, truncated form of E2 (Flint et al., 1999), whereas subsequent studies used E2 expressed in the context of VLPs (Owsianka et al., 2001) or E1E2 (Owsianka et al., 2006). Therefore, the antigenic exposure of epitopes in different forms of E2 can greatly affect our understanding of E2–receptor and E2–antibody interactions.

There is a high degree of conservation of the residues W420, Y527, W529, G530 and D535 and the 436GWLAGLFY443 across different genotypes. Analysis of human and murine mAbs recognizing both linear and conformational epitopes, demonstrated that the most broadly nAb are targeted to the CD81-binding site (Johansson et al., 2007; Law et al., 2008; Owsianka et al., 2005, 2008; Perotti et al., 2008) (Table 1 and Fig. 3). The region immediately downstream of HVR1 is recognized by the broadly neutralizing mouse mAb AP33 (Owsianka et al., 2005). Key residues within E2 that are essential for AP33 binding, identified by analysis of alanine replacement glycoproteins and enrichment of random peptide display libraries, are L413, N415, G418 and W420 (Tarr et al., 2006). Whilst its epitope is predominantly linear in nature, maximal binding is dependent on the overall local conformation of the E2 protein. The neutralizing rat mAb 3/11 recognizes an epitope overlapping that of the mAb AP33 (Tarr et al., 2006) (Fig. 1 and Supplementary Fig. S1). However, <5% of individuals with resolved or chronic infection harbour antibodies targeting this region of the E2 protein (Tarr et al., 2007), suggesting that this region is less immunogenic in humans than in rodents, or that the presentation of this region is different in experimental immunogens compared with the native viral particle.

The majority of human mAbs targeting the CD81-binding site recognize conformational epitopes. These mAbs exhibit either a cross-reactive broadly neutralizing or an intermediate neutralizing phenotype (Table 1 and Fig. 3). Our mapping studies showed that all the broadly neutralizing conformation-sensitive antibodies targeted the highly conserved E2 residues W529, G530 and D535 (Johansson et al., 2007; Keck et al., 2008b; Law et al., 2008; Owsianka et al., 2006; Tarr et al., 2006). In addition to these residues, the human Fab fragment e137 also required T416 and W420 (Perotti et al., 2008). Thus, based on their epitopes, the broadly neutralizing CD81-binding site antibodies fall into three groups: those that recognize predominantly linear epitopes located between E2 residues 412 and 423 (e.g. AP33 and 3/11); those that recognize conformational epitopes where key contact residues are located between residues 529 and 535 (e.g. 1:7, A8, AR3A and CBH2); and those whose epitopes span these two important CD81-binding regions (e.g. e137).

A number of antibodies recognizing the CD81-binding site that exhibit intermediate or restricted neutralization breadth have also been described. These include antibodies targeting the region 436GWLAGLFY443 (7/16b and 11/20), although there is conflicting data about the ability of 7/16b to compete for CD81 binding (Flint et al., 1999) and the region 524APTYSWGA531 of E2 (2/64a). However, mAb 9/75, whose minimal epitope is also located between residues 528 and 535, was unable to neutralize HCVpp at a concentration of 50 μg ml⁻¹ (Hsu et al., 2003). Thus, subtle differences in the specificity of antibodies targeting CD81-binding sites might influence their neutralizing potency. In addition, accessibility of the antibody-binding site will also influence neutralizing potency. Early studies comparing the accessibility of epitopes on various forms of E2 protein, including E1E2 heterodimers, soluble truncated E2 ectodomain and E2 in the context of insect expressed VLPs, demonstrated differential exposure of epitopes in the various presentations of E2 (Clayton et al., 2002). Finally, it is worth highlighting the apparent differences between the human and the murine antibody response. The most potent and broadly neutralizing murine antibodies target linear epitopes within residues 412–423, whereas similar human antibodies are rare. By contrast, the most broadly neutralizing human antibodies are conformation sensitive and target epitopes centred on the key CD81-binding residues W529, G530 and D535, whereas murine antibodies targeting this region (e.g. mAb H35 and H48) are highly restricted (Owsianka et al., 2006).

**Are there other targets for nAb?**

The majority of antibodies identified to date target receptor-binding epitopes within E2. This can, in part, be attributed to the use of assays, such as mammalian-expressed sE2 (Pileri et al., 1998) and NOB assays (Rosa et al., 1996), which are biased towards E2. It is important to note that the NOB assays that first identified nAb targeting CD81 were carried out on Molt-4 cells, a lymphoma cell line that expresses only CD81 but none of the other HCV entry receptors. Therefore, the presence of antibodies inhibiting interactions between the virus and other receptors may have been overlooked. Indeed, nAb which do not target the E2–CD81 interaction have been found (Hsu et al., 2003) (Table 1). Subsequent studies comparing Molt-4 cells and Huh7 human hepatoma cells showed that NOB antibodies inhibit the binding of E2 to Molt-4 cells more readily than to Huh7 cells (Heo et al., 2004). Additionally, in early studies the NOB assay served as an initial screen for ‘neutralizing’ potential of mAbs, promoting the selection of anti-E2 antibodies targeting this site for further study. This has introduced an inherent bias towards reports of nAb that disrupt the interaction between E2 and CD81. Multiple cell surface receptors are involved in HCV entry to target cells; however, they do not all interact directly with E2 (Fig. 2). In addition to CD81, SR-BI and the tight junction protein occludin are both thought to interact with E2 directly. Whilst HVR1 is thought to be important for SR-BI interaction, and mAbs targeting linear
Table 1. mAbs targeting the envelope glycoproteins of HCV

All amino acid numbering is based on the H77 reference strain.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Epitope (aa)</th>
<th>Epitope class</th>
<th>Interaction targeted</th>
<th>Source of antibody</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-111</td>
<td>192–211</td>
<td>Linear</td>
<td>Unknown</td>
<td>Human</td>
<td>Keck et al. (2004b)</td>
</tr>
<tr>
<td>IGHS05</td>
<td>312–327 (E1)</td>
<td>Linear</td>
<td>Unknown</td>
<td>Human</td>
<td>Meunier et al. (2008)</td>
</tr>
<tr>
<td>IGHS26</td>
<td>312–327 (E1)</td>
<td>Linear</td>
<td>Unknown</td>
<td>Human</td>
<td>Meunier et al. (2008)</td>
</tr>
<tr>
<td>H77.39</td>
<td>415 and 417</td>
<td>Linear</td>
<td>CD81/SR-B1</td>
<td>Mouse</td>
<td>Sabo et al. (2011)</td>
</tr>
<tr>
<td>3/11</td>
<td>412–423</td>
<td>Linear</td>
<td>CD81</td>
<td>Rat</td>
<td>Flint et al. (1999)</td>
</tr>
<tr>
<td>HCV1</td>
<td>412–423</td>
<td>Linear</td>
<td>CD81</td>
<td>Human*</td>
<td>Broering et al. (2009)</td>
</tr>
<tr>
<td>95-2</td>
<td>412–423</td>
<td>Linear</td>
<td>CD81</td>
<td>Human*</td>
<td>Broering et al. (2009)</td>
</tr>
<tr>
<td>AP33</td>
<td>412–423</td>
<td>Linear</td>
<td>CD81</td>
<td>Mouse</td>
<td>Owsianka et al. (2005); Tarr et al. (2006)</td>
</tr>
<tr>
<td>2/69a</td>
<td>436–443</td>
<td>Linear</td>
<td>Unknown</td>
<td>Rat</td>
<td>Flint et al. (1999); Hsu et al. (2003)</td>
</tr>
<tr>
<td>1/39</td>
<td>442–443</td>
<td>Linear</td>
<td>CD81?</td>
<td>Rat</td>
<td>Flint et al. (1999)</td>
</tr>
<tr>
<td>AP320</td>
<td>464–471</td>
<td>Linear</td>
<td>Unknown</td>
<td>Mouse</td>
<td>Clayton et al. (2002)</td>
</tr>
<tr>
<td>6/41a</td>
<td>480–492</td>
<td>Linear</td>
<td>Unknown</td>
<td>Rat</td>
<td>Flint et al. (1999)</td>
</tr>
<tr>
<td>11/20</td>
<td>436–447</td>
<td>Linear</td>
<td>CD81</td>
<td>Rat</td>
<td>Hsu et al. (2003)</td>
</tr>
<tr>
<td>2/64a</td>
<td>524–531</td>
<td>Linear</td>
<td>CD81</td>
<td>Rat</td>
<td>Hsu et al. (2003)</td>
</tr>
<tr>
<td>H53</td>
<td>540–550</td>
<td>Conformation dependent</td>
<td>Unknown</td>
<td>Mouse</td>
<td>Cocquerel et al. (1998); Owsianka et al. (2006)</td>
</tr>
<tr>
<td>H35</td>
<td>523 and 530</td>
<td>Conformation dependent</td>
<td>CD81</td>
<td>Mouse</td>
<td>Cocquerel et al. (1998); Owsianka et al. (2006)</td>
</tr>
<tr>
<td>H48</td>
<td>530</td>
<td>Conformation dependent</td>
<td>Cd81</td>
<td>Mouse</td>
<td>Cocquerel et al. (1998); Owsianka et al. (2006)</td>
</tr>
<tr>
<td>Fab e137</td>
<td>416, 420, 529, 530 and 535</td>
<td>Conformation dependent</td>
<td>CD81</td>
<td>Human</td>
<td>Perotti et al. (2008)</td>
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<tr>
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<td>529, 530 and 535</td>
<td>Conformation dependent</td>
<td>CD81</td>
<td>Human</td>
<td>Mancini et al. (2009)</td>
</tr>
<tr>
<td>AR3C</td>
<td>424, 530, 535, 538 and 540</td>
<td>Conformation dependent</td>
<td>CD81</td>
<td>Human</td>
<td>Law et al. (2008)</td>
</tr>
<tr>
<td>AR3D</td>
<td>424, 436–447, 530 and 535</td>
<td>Conformation dependent</td>
<td>CD81</td>
<td>Human</td>
<td>Law et al. (2008)</td>
</tr>
<tr>
<td>1:7</td>
<td>523, 529, 530 and 535</td>
<td>Conformation dependent</td>
<td>CD81</td>
<td>Human</td>
<td>Allander et al. (2000); Johansson et al. (2007)</td>
</tr>
<tr>
<td>A8</td>
<td>523, 529, 530 and 535</td>
<td>Conformation dependent</td>
<td>CD81</td>
<td>Human</td>
<td>Allander et al. (2000); Johansson et al. (2007)</td>
</tr>
<tr>
<td>L1</td>
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<td>Conformation dependent</td>
<td>Unknown</td>
<td>Human</td>
<td>Allander et al. (2000); Johansson et al. (2007)</td>
</tr>
<tr>
<td>CBH4B</td>
<td>Unknown</td>
<td>Conformation dependent</td>
<td>Unknown</td>
<td>Human</td>
<td>Hadlock et al. (2000); Keck et al. (2004a)</td>
</tr>
<tr>
<td>CBH4D</td>
<td>Unknown</td>
<td>Conformation dependent</td>
<td>Unknown</td>
<td>Human</td>
<td>Hadlock et al. (2000); Keck et al. (2004a)</td>
</tr>
<tr>
<td>CBH4G</td>
<td>Unknown</td>
<td>Conformation dependent</td>
<td>Unknown</td>
<td>Human</td>
<td>Hadlock et al. (2000); Keck et al. (2004a)</td>
</tr>
</tbody>
</table>
epitopes within HVR1 block E2 interaction, efficient binding is E2 conformation-dependent (Scarselli et al., 2002). Recently, a number of mAbs have been described which appear to interfere with both CD81 and SR-BI binding. Whilst most of these have limited breadth, mAb H77.39, whose epitope overlaps that recognized by mAb AP33, was shown to inhibit E2 binding to both CD81 and SR-BI (Scarselli et al., 2009). Therefore, this presents another promising target for virus neutralization that requires further investigation.

Antibodies targeting epitopes within the envelope glycoprotein E1 have been identified in some patient sera, but these are generally rare (Pestka et al., 2007). The reasons for this are unclear but might be due to technical difficulties in detecting E1 responses, as the protein misfolds unless it is co-translated with E2 (Dubuisson et al., 1994). Alternatively, the presence of E2 might mask E1 epitopes or be immunologically dominant (Garrone et al., 2011). Despite these challenges, there are reports of anti-E1 responses in the literature. For example, mAb H-111 binds the 192YEVRNVSGVYH211 region of E1 and is cross-reactive to genotypes 1a, 1b, 2b and 3a. In studies, this mAb partially reduced VLP entry into Molt-4 cells (Keck et al., 2004b). The mAbs IGH505 and IGH526 recognize a linear epitope encompassing aa 313–327 and show broad neutralization of HCVpp bearing E1E2 derived from different genotypes (Meunier et al., 2008). Previous trials of E1 protein vaccine candidates induced E1-specific antibody responses (Garrone et al., 2011; Leroux-Roels et al., 2004; Nevens et al., 2003), but these did not reduce viral load in chronically infected patients (Nevens et al., 2003). A recent study in chimpanzees showed that anti-E1 antibodies induced by immunization with a recombinant form of E1 conferred protection against experimental infection with heterologous HCV (Verstrepen et al., 2011). The authors reported that the protective effect of the E1-based vaccine was better than that induced by an E2-based vaccine (Verstrepen et al., 2011). More recently, VLPs expressing E1E2 and E1 constructs were used in prime–boost experiments in an attempt to elicit nAb. Despite being able to elicit E1-specific responses, these were not neutralizing, and

Table 1. cont.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Epitope (aa)</th>
<th>Epitope class</th>
<th>Interaction targeted</th>
<th>Source of antibody</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBH5</td>
<td>523, 525, 530, 535 and 540</td>
<td>Conformation dependent</td>
<td>CD81</td>
<td>Human</td>
<td>Hadlock et al. (2000); Owsianka et al. (2008)</td>
</tr>
<tr>
<td>CBH7</td>
<td>540 and 549</td>
<td>Conformation dependent</td>
<td>CD81</td>
<td>Human</td>
<td>Hadlock et al. (2000); Owsianka et al. (2008)</td>
</tr>
<tr>
<td>ALP98</td>
<td>644–651</td>
<td>Linear</td>
<td>Unknown</td>
<td>Mouse</td>
<td>Clayton et al. (2002); Owsianka et al. (2001)</td>
</tr>
<tr>
<td>ALP1</td>
<td>647–658</td>
<td>Linear</td>
<td>Unknown</td>
<td>Mouse</td>
<td>Clayton et al. (2002); Owsianka et al. (2001)</td>
</tr>
</tbody>
</table>

*Denotes antibodies generated in transgenic mice containing human antibody genes.

Occludin co-precipitates with E2 (Benedicto et al., 2008; Liu et al., 2009); however, no virus-specific antibodies that can neutralize this interaction have been identified. To date, direct interaction between E2 and claudin-1 has not been demonstrated (Evans et al., 2007; Krieger et al., 2010). Experimentally derived anti-claudin-1 antibodies have been shown to neutralize HCV infectivity by inhibiting the interaction between CD81 and claudin-1, which is necessary for the formation of the E2-CD81-claudin-1 co-receptor complex (Fofana et al., 2010; Krieger et al., 2010). Although these antibodies do not target the virus particle itself, targeting host factors may represent an alternative approach to preventing HCV entry. Indeed a number of anti-receptor antibodies targeting CD81 (Meuleman et al., 2008; Molina et al., 2008) and SR-BI (Catanese et al., 2010) have been shown to block viral entry.

HCV entry into target cells occurs via clathrin-mediated endocytosis of the viral particle (Blanchard et al., 2006). Subsequent release of the viral genome into the cytosol requires the pH-dependent fusion of viral and cellular membranes. There is evidence to suggest that patient-derived antibodies may be able to inhibit this fusion process and thus block virus infectivity (Haberstroh et al., 2008; Kobayashi et al., 2006). The fusion determinant or determinants within the envelope glycoproteins are still incompletely defined; Therefore, it is not possible to identify the epitopes to which these antibodies may bind. A precedent for virus neutralization by targeting the fusion determinant has been set by studies into other viruses. For example, the highly conserved HIV-1 fusion determinant lies within the gp41 envelope glycoprotein and is targeted by broadly nAb (reviewed by Karlsson Hedestam et al., 2008). Antibodies have also been identified that target the fusion determinant within the influenza virus haemagglutinin (HA) and these show broad neutralizing activity against all HA subtypes within a specific group (Sui et al., 2009).
Neutralization was only observed when animals were boosted with E1E2 (Garrone et al., 2011). Due to the limited understanding of the role and structure of E1 and how it interacts with E2, the mechanism by which these antibodies act remains unknown. It is also important to remember that the HCV fusion determinant has yet to be identified but has been proposed to reside within E1 (Lavillette et al., 2007). If this were the case, E1 may become an important target for antibodies inhibiting the fusion step.

Do nAb influence infection outcome?

Due to the asymptomatic nature of acute HCV infection it is difficult to identify, and therefore study, patients in the 

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**Fig. 3.** Organization of the antigenic regions (AR) of the E2 glycoprotein. E2 has previously been classified into three antigenic 'domains' (Keck et al., 2004a) or three AR (Law et al., 2008). This Venn diagram summarizes all previous data on the specificity of mAbs directed to E2, as determined by competition assays and point mutagenesis studies. Antibody names are placed relative to their location on this map, with non-nAb highlighted in green text and nAb in red text. This organization highlights at least six partially overlapping AR (denoted by coloured circles and rectangles), as well as other discrete epitopes recognized by mAbs. Anti-E2 mAbs can be clearly classified as non-neutralizing, broadly neutralizing, or narrowly neutralizing, which generally correlate with the three AR. AR1, 2 and 3 (Law et al., 2008) are highlighted as yellow, red and blue circles, respectively. These AR partially overlap, with the epitope recognized by mAb CBH7 overlapping all three epitope clusters. Many of the reported antibodies map to AR3. Interestingly, all broadly nAb exclusively recognize the discontinuous AR3. AR1 contains epitopes that are exclusively recognized by non-nAb, with part of this region possessing epitopes that overlap with AR3. AR2 is defined by a single antibody, AR2A, which neutralizes a restricted number of HCV isolates. In addition to these three AR, a discrete region is defined by the conformation-sensitive antibodies CBH4B, CBH4D and CBH4G, as well as two conformation-independent regions defined by ALP98 and ALP1, and 7/16b, 11/20, 1/39 and 2/69a. The previously described antigenic ‘domains’ (Keck et al., 2004a) broadly agree with these AR. Domain ‘A’ appears to be a separate AR that does not overlap with other epitope clusters. Domain ‘B’ significantly overlaps AR3, and domain C is a shared epitope that overlaps AR1, AR2 and AR3. Mapping the relative specificities of these mAbs reveals complex immunogenic surfaces of E2 that broadly equate to a non-neutralizing face, a broadly neutralizing face and a restricted neutralizing face to the E2 protein. It remains to be demonstrated if these antibody reactivity data agree with structural analyses of the E2 protein.
early phase of disease. Despite this, several studies of acute HCV infection have been performed and these have shown that development of a broad and potent T-cell response is important for clearance (reviewed by Bowen & Walker, 2005). More recent studies have also indicated that nAb also play a critical role in disease outcome. Rapid induction of nAb early during infection is associated with spontaneous recovery and these antibodies appear to be more cross-neutralizing (Dowd et al., 2009; Pestka et al., 2007). By contrast, clearance in chimpanzees is associated with cellular immunity (Barth et al., 2011; Grakoui et al., 2003; Shoukry et al., 2003) rather than nAb (Logvinoff et al., 2004; Meunier et al., 2005). There are some important differences in the outcome of HCV infection in chimpanzees compared with humans. Chimpanzees tend to suffer a milder disease, show a higher rate of viral clearance and do not appear to develop liver fibrosis or cirrhosis (reviewed by Jo et al., 2011). Therefore, chimpanzees may not be the most reliable indicator of the role played by the humoral immune response in clearance of the virus during acute infection. In contrast to spontaneous resolvers, patients who developed a chronic infection generated no antibodies or a very low titre antibody response in the early phase of the infection (Dowd et al., 2009; Pestka et al., 2007). Assessment of nAb levels several years later demonstrated high antibody titres in chronically infected patients, although these were unable to clear the virus (Pestka et al., 2007).

Intriguingly, limited studies of viral evolution during acute infection have shown that resolved infection is associated with stable HVR1 sequences, whereas persistence is accompanied by noticeable HVR1 sequence change (Farci et al., 2000; Ray et al., 1999). This observation led to the hypothesis that HVR1 is acting as an immunological decoy (Ray et al., 1999) – targeting the antibody response to this region of E2 at the expense of other neutralization epitopes that are functionally or structurally less amenable to change. This hypothesis gained support from a detailed analysis of the nAb response in patient H, over a period of 26 years. This study found that the early antibody response was targeted towards HVR1, which led to HVR1 sequence change. A more broadly nAb response was only observed much later during chronic infection (von Hahn et al., 2007). These findings can be interpreted in one of two ways. In those virus populations able to undergo rapid HVR1 evolution, mutations arise and lead to antibody escape, whilst in those populations where HVR1 evolution is limited, escape does not occur and the virus is cleared (Ray et al., 1999). It is, however, equally plausible that the antibody response in individuals who experienced viral clearance targeted more conserved epitopes outside of HVR1 and, therefore, HVR1 sequence evolution was not observed. The finding that the early antibody response in acute resolvers is more broadly neutralizing supports our alternative hypothesis.

Even in chronically infected individuals, there is evidence that antibodies partially control the virus. Firstly, hypogammaglobulinaemic individuals exhibit a marked rapidity and severity in disease progression (Bjørø et al., 1994). Secondly, B-cell depletion during rituximab therapy has been reported to cause an increase in viral load, which returns to normal after cessation of therapy (Ennishi et al., 2008), presumably as the B-cell population recovered. It is likely that during chronic infection there is an intricate interplay between host and virus such that mutations that lead to immune escape might also reduce viral fitness. This is particularly compelling given that most nAb responses are targeted to functionally important regions.

**Viral persistence in the presence of nAb**

Early studies using the HCVpp system suggested that broadly nAb were a common feature of long-term chronic HCV infection. Therefore, how does the virus persist in the face of this response? Very often antibodies or sera have only been tested against a limited number of genotypes or subtypes (Bartosch et al., 2003a; Broering et al., 2009) and therefore may not truly be broadly neutralizing. Also, data from our laboratory showed that E1E2 clones differ greatly in their sensitivity to neutralization, and the E1E2 derived from H77c, which has been most frequently used in the HCVpp studies, is easily neutralized by the majority of sera. By contrast, most E1E2 clones derived ex vivo are far more resistant to neutralization and truly broadly neutralizing sera are rare (Tarr et al., 2011).

Even so, HCV can still persist in those patients with more broadly neutralizing responses and the virus must utilize mechanisms to evade these. The most widely reported evasion mechanism is mutational escape. HCV contains a positive-sense ssRNA genome that is replicated by a virus-encoded RNA-dependent RNA polymerase. This polymerase lacks proof-reading capabilities which, when coupled with the high replication rate of the virus, results in the generation of a highly diverse population of viral variants (reviewed by Simmonds, 2004). This population is often referred to as a quasispecies, although it is questionable if this assignment is strictly accurate (Holmes, 2010). The diverse virus population will harbour neutralization escape variants that have a selective advantage over neutralization-sensitive variants. Positively selected amino acid sites are located within and around known receptor- and nAb-binding regions (Brown et al., 2005, 2007). Recent work has shown that E1E2 evolution is driven by the nAb response and escape variants have been reported to occur in the dominant circulating strain (Dowd et al., 2009; Farci et al., 2000; von Hahn et al., 2007). Both von Hahn et al. (2007) and Dowd et al. (2009) show that sequential serum samples are limited in their ability to neutralize the concurrently circulating viral strains, but are able efficiently to neutralize virus strains from earlier time points. Therefore even within one individual, a single viral envelope sequence may not be sufficient to ‘pull-out’ all nAb.

The most broadly nAb described to date target the CD81-binding site and HCV has evolved various methods of shielding this region of the E2 protein. E2 contains up to 11 potential N-linked glycosylation sites, nine of which are conserved across genotypes (>97%) (Helle et al., 2007).
Glycans are important for the structure and function of glycoproteins (Goffard et al., 2005) and are critical for HCVpp entry into target cells (Falkowska et al., 2007). Specific glycans are known to mask the CD81-binding site and, therefore, nAb epitopes. Removal of these glycans results in increased binding to CD81 and increased sensitivity to neutralization by patient sera and mAb (Falkowska et al., 2007; Helle et al., 2007, 2010). Changes occur in the frequency and position of glycans on both HIV-1 gp120 and influenza HA glycoproteins, and these ‘evolving glycans shields’ have been shown to decrease sensitivity to antibody neutralization (Abe et al., 2004; Wei et al., 2003). Whilst there is some variability in the location and number of glycosylation sites across different functional E1E2 clones (Helle et al., 2007), there is little evidence that the glycans undergo significant intra-host evolution (Brown et al., 2007; Helle et al., 2007) and some highly conserved glycans are critical for entry (Falkowska et al., 2007).

Lipid shielding may represent an additional strategy used by HCV to evade the antibody response. In patient sera, HCV exists as a heterogeneous population with a range of buoyant densities. Low density particles, termed lipoviral-particles (LVPs) are the most infectious, and current data suggest that key neutralizing epitopes are less accessible on LVPs. These are associated with very low density lipoproteins (VLDL), such as the apolipoproteins apoB and apoE (reviewed by Burlone & Budkowska, 2009). In addition, several in vitro studies have demonstrated that high density lipoprotein (HDL) components of human serum, such as apoCI, can enhance the infectivity of HCVpp and HCVcc via an HVR1-dependent mechanism (Bartosch et al., 2005; Dreux et al., 2007; Meunier et al., 2005). HDL also reduces the sensitivity of HCVpp to antibody neutralization, possibly by accelerating the entry of HCV via SR-BI-mediated lipid uptake (Dreux et al., 2006). ApoCI is able to further enhance infectivity by promoting fusion between viral and cellular membranes (Dreux et al., 2007). Lipids clearly play a crucial role in the infectivity and entry of viral particles as well as the neutralization sensitivity, and further studies are required to fully elucidate this interplay.

More recently, HCV has been found to be capable of direct cell-to-cell transmission, which is largely resistant to antibody neutralization (Timpe et al., 2008; Witteveldt et al., 2009), although the rat mAbs 9/27 (targeting aa 396–407 within HVR1) and 11/20c (targeting the CD81-binding residues aa 412–423 and 436–447) are able to partially inhibit cell-to-cell transmission (Brimacombe et al., 2011). Cell-to-cell transmission requires all four entry receptors: CD81, SR-BI, claudin-1 and occludin, although SR-BI appears particularly important (Brimacombe et al., 2011). Many other enveloped viruses, including herpes simplex virus 1, human T-cell lymphotropic virus and measles virus, utilize direct cell-to-cell transmission in a bid to evade the host immune response (reviewed by Mothes et al., 2010). HIV-1 also employs a number of mechanisms to spread directly from cell to cell. These include promoting the fusion of infected and uninfected cells; hijacking actin-containing structures which physically join infected and uninfected cells; and exploiting the immunological synapse formed between dendritic cells and T-cells (reviewed by Sattentau, 2008). The exact mechanism of HCV cell-to-cell transmission is still unknown, but regardless of the mechanism, direct cell-to-cell transmission represents an ideal method of immune evasion and may explain why nAb do not always clear the virus.

It has also been postulated that non-nAb present within patient sera bind distinct epitopes within E2 and block or inhibit the binding of nAb to neutralization epitopes (Zhang et al., 2007, 2009). This has been proposed as a reason for the failure of polyclonal immunoglobulin preparations to successfully treat HCV (Davis et al., 2005). This does, however, contradict observations made by Féra et al. (1998) in which polyclonal immunoglobulins were found to protect against HCV infection. Zhang and colleagues reported that antibodies targeting the region of E2 encompassing aa 434–446 strongly interfere and abrogate the neutralization exhibited by antibodies targeting epitopes located between residues 412 and 423. However, we have shown that murine mAbs and affinity enriched human immunoglobulin fractions targeting epitopes overlapping the aa 434–446 region of E2 exhibit restrictive neutralization of both HCVpp and HCVcc (A. W. Tarr, unpublished data). Previously, some of the mAbs targeting this region of E2 had been shown to neutralize E2–CD81 binding (Clayton et al., 2002; Owsianka et al., 2001) as well as HCVpp supplemented with autologous H77c E1E2 (Hsu et al., 2003).

Insights into the structure of HCV envelope glycoproteins

Studies of murine and human mAbs also provide insight into the structure of the E1E2 glycoproteins. HCV belongs to the family Flaviviridae and its genomic organization is characteristic of other members of this group (Lemon et al., 2001). Similarities between the primary amino acid sequence of HCV E2 and the envelope glycoproteins of flavivirus (E) and alphavirus (E1) suggest that E2 is a class II fusion protein (Krey et al., 2010). Several conserved cysteine residues within E2 form disulphide bonds to stabilize the structure (Dubuisson & Rice, 1996). The identification of conformation-dependent antibodies, such as H53 and CBH5 (Table 1) demonstrated that the structure of E2 is highly ordered (Hadlock et al., 2000; Owsianka et al., 2006). Delineation of the disulphide bridged cysteine partners, together with knowledge of the CD81-binding sites, antibody epitopes and location of potential N-linked glycosylation sites facilitated the generation of a 3D model of E2 based on the structure of other class II fusion proteins. In this model, E2 is composed of three domains (DI, DII and DIII). HVR1 precedes DI, HVR2 is found within DII, and IgV links DI and DIII (Krey et al., 2010). In this model, key CD81-binding regions are brought together into DI, with the glycosylation sites of E2 clustering around the CD81-binding surface. A putative highly conserved fusion peptide is located within DII, which may also form a
region of contact with E1 (Krey et al., 2010). The study by Krey et al. (2010) used a soluble E2 construct truncated at amino acid residue 715 and therefore does not precisely represent the native E2 glycoprotein found on the surface of viral particles. However, it does provide us with the best indication yet of the structure of E2. A model such as this could help to focus future studies at those regions of E2 that are surface exposed and therefore likely to be a target for antibodies. It could also help to identify highly conserved features or domains of E2 that might elicit broadly nAb and thus aid in the rational design of therapeutic immunogens. Indeed, if the fusion determinants within the envelope glycoproteins are identified, it would greatly facilitate the search for antibodies capable of neutralizing this important step in viral entry.

**Potential application of nAb in vaccines or therapeutics**

Although our knowledge of HCV has increased dramatically in the two decades since it was first identified, development of an effective vaccine to prevent or treat infection has yet to be achieved. Studies of the immune response suggest that any vaccine would need to target both cellular and humoral arms of the immune system in order to be effective. A strong CD8+ and CD4+ T-cell response targeting multiple epitopes is associated with spontaneous viral clearance (reviewed by Thimme et al., 2008) and both cellular and humoral arms of the immune response drive evolution of HCV sequences leading to the generation of escape mutations (von Hahn et al., 2007). Any vaccine or therapy targeting a single viral epitope would lead to the development of resistant strains. Therefore, the rational design of vaccine or therapeutic immunogens requires an approach similar to that adopted for the treatment of HIV; that is, the simultaneous targeting of multiple viral (and possibly host) targets to prevent the emergence of resistance mutations.

The protective effect of a high titre, broadly nAb response in the acute phase of the disease (Dowd et al., 2009; Pestka et al., 2007) and evidence that spontaneous resolution of primary infection affords some protection against persistent reinfection in IVDUs (Osburn et al., 2010) give strong support to the idea that immunity to HCV can be elicited by immunization. In addition, it is believed that passive immunotherapy may be able to reduce viral titres to undetectable levels. A number of products assessed in clinical trials have attempted to replicate this protective immunity but have met with mixed results. Trials of polyclonal anti-HCV immunoglobulins (Civacir) (Davis et al., 2005) and an anti-E2 mAb (HCV-Ab XTI68) (Schiano et al., 2006) in liver transplant patients resulted in high levels of serum antibodies, which correlated with a reduction in HCV RNA levels. However, sustained reduction of HCV RNA has not been reported and no long-term protection against liver pathology has been demonstrated (Davis et al., 2005; Schiano et al., 2006). Perhaps the most promising vaccine candidate to date is a recombinant form of the envelope glycoproteins E1E2 (Chiron Corp.). It induced a strong cellular and humoral response in chimpanzees, resulting in sterilizing immunity against homologous viral challenge. This vaccine also prevented development of chronic infection following heterologous viral challenge (Houghton & Abrignani, 2005). Preliminary safety and immunogenicity studies of an adjuvanted form of the vaccine (HCV E1E2/MF59.C1) suggest that it is capable of inducing both cellular and humoral immunity against the envelope glycoproteins in healthy humans with few adverse events (Frey et al., 2010). Phase I clinical trials of this vaccine candidate have been completed and are awaiting publication of the results.

This review has focused on the role played by nAb in preventing or limiting HCV infection of target cells and much effort has been invested in this area of research. However, non-nAb may also play a role in disease outcome through the action of Fc-mediated effector functions including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). ADCC and CDC are both involved in the immune response to HIV (Chung et al., 2008), but Fc-mediated effector functions in the context of HCV infection are less well understood. Sera from both the acute and chronic phase of infection can mediate ADCC via binding to E2 expressed at the cell surface (Nattermann et al., 2005), while several E2-specific mAbs are able to induce CDC of E2-expressing cells (Machida et al., 2008). Viral mechanisms to evade the effects of ADCC and CDC have also been reported (Machida et al., 2008; Maillard et al., 2004). Optimizing these non-nAb effector functions may prove critical in the design of effective therapeutic antibodies (Jiang et al., 2011).

**Conclusion**

Our current understanding of the nAb response raised against HCV suggests that E2 is the major target and that multiple epitopes within E2 may be targeted by both linear- and conformation-dependent antibodies. Predominantly these neutralization epitopes overlap with CD81-binding sites and clearly demonstrate a role in inhibition of entry. However, this interaction is not the only target for nAb. Antibodies are able to inhibit the fusion of viral and cellular membranes, target as-yet-unidentified epitopes within E2, bind E1 and may be able to inhibit the interaction between the virus and other cellular receptors. Claudin-1 and occludin have only recently been identified as entry receptors and as such are less well studied than CD81. Switching the focus to these receptors may lead to the discovery of new nAb epitopes. Defining the fusion determinants within the envelope glycoproteins would facilitate the discovery of antibodies targeting these epitopes. This may be a step closer thanks to the recently described 3D structural model of E2 (Krey et al., 2010). It will be interesting to see whether the proposed fusion domains are targeted by patient-derived antibodies capable of inhibiting fusion. It will also be interesting to see if E1 has a role to play in fusion, as has been suggested. Very few
antibodies to E1 have been identified so far (Keck et al., 2004b; Meunier et al., 2008); however, the exposed nature of this glycoprotein on the surface of the viral particle makes it a natural target for the immune response. If fusion determinants within E1 are identified, it would be an ideal candidate for the design of rational immunogens.

The protective effect of nAb in vivo in some individuals gives hope that such antibodies can be used therapeutically. The challenge for researchers will be to find antibodies which target highly conserved epitopes with broad neutralizing capabilities that will be effective against all genotypes of HCV. Identifying antibodies which inhibit many different virus–cell interactions would greatly enhance our repertoire of agents to use in the fight against this virus and we should continue searching for these antibodies.

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