

Therapeutic Control of Hepatitis C Virus: The Role of Neutralizing Monoclonal Antibodies

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Abstract Liver failure associated with hepatitis C virus (HCV) accounts for a substantial portion of liver transplantation. Although current therapy helps some patients with chronic HCV infection, adverse side effects and a high relapse rate are major problems. These problems are compounded in liver transplant recipients as reinfection occurs shortly after transplantation. One approach to control reinfection is the combined use of specific antivirals together with HCV-specific antibodies. Indeed, a number of human and mouse monoclonal antibodies to conformational and linear epitopes on HCV envelope proteins are potential candidates, since they have high virus neutralization potency and are directed to epitopes conserved across

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diverse HCV genotypes. However, a greater understanding of the factors contributing to virus escape and the role of lipoproteins in masking virion surface domains involved in virus entry will be required to help define those protective determinants most likely to give broad protection. An approach to immune escape is potentially caused by viral infection of immune cells leading to the induction hypermutation of the immunoglobulin gene in B cells. These effects may contribute to HCV persistence and B cell lymphoproliferative diseases.

1 Introduction

Over 170 million people worldwide are infected with hepatitis C virus (HCV). A large proportion of these individuals will harbor a chronic infection that eventually leads to the development of severe liver disease, liver failure, and hepatocellular carcinoma (Major et al. 2001). In the United States, 40% of patients needing liver transplantation have HCV-associated diseases. While current therapy with pegylated interferon and ribavirin is effective in some patients with chronic HCV hepatitis, adverse side effects and a high relapse rate, particularly in individuals infected with genotype 1 virus, are major problems. These problems are compounded in liver transplant recipients, where HCV reinfection occurs shortly after transplantation and antiviral treatment can only start at a later time point (Biggins and Terrault 2005). Allograft failure due to HCV reinfection is the most common cause of retransplantation and death (Charlton et al. 1998). HCV reinfection occurs immediately after transplantation and viral replication starts within hours of surgery (Garcia-Retortillo et al. 2002). Risk factors associated with recurrent and severe liver disease include donor and recipient age, acute rejection, human cytomegalovirus infection, and higher levels of HCV viral load. Serum viral load increases rapidly and peaks by 4 months after transplantation with titers up to 20 times higher than pretransplant levels at 1 year after transplantation (Everhart et al. 1999). Treatment of acute rejection with steroids leads to further increases in viral load. Combined treatment with pegylated interferon and ribavirin is more effective than interferon alone (Biggins and Terrault 2005), but combined therapy is associated with more than 50% of patients needing to stop treatment because of ribavirin-associated anemia. The occurrence of HCV reinfection in liver transplant recipients indicates that the prior immunity in HCV patients does not provide protection. Clearly, more effective strategies are needed for treating and preventing HCV reinfection among liver transplant recipients, and more understanding on the mechanisms of HCV immune escape is needed.

One possibility to control posttransplantation HCV reinfection is the combined use of specific antivirals together with HCV-specific antibodies. This type of approach is widely used for the control of hepatitis B virus (HBV) after liver transplantation, where the standard of care is pretransplant treatment with lamivudine for at least 4 weeks followed by posttransplant treatment with combined lamivudine and HBV immunoglobulin (Schreibman and Schiff 2006). When lamivudine or other

nucleoside analogs are used alone, emergence of HBV escape mutants is a major concern (Perrillo et al. 2001; Fung et al. 2006). Similarly for HCV, a number of small molecule inhibitors of HCV NS3-4A protease and NS5B polymerase are in advanced stage clinical studies, potentially providing new therapeutic options (reviewed in De Francesco and Migliaccio 2005). However, a growing concern is that a high and error-prone viral replication will eventually lead to resistance to these antivirals. Our proposal is that virus neutralizing (Vn) antibodies when used in combination with antivirals could achieve the needed therapeutic outcome and minimize escape virus mutants. Selected antibodies ideally should be broadly reactive to different HCV genotypes, each inhibiting at different steps of virus entry, and be synergistic in their ability to control virus infection. At least two antibodies to different epitopes are proposed to minimize the concern of escape mutants. We and others have shown that the majority of HCV antibodies with the broadest and most potent Vn activities recognize conformational epitopes (Habersetzer et al. 1998; Allander et al. 2000; Ishii et al. 1998). Nonetheless, Vn monoclonal antibodies (MAbs) to conserved linear epitopes have been identified and should be considered as therapeutic candidates (Owsianka et al. 2001, 2005; Tarr et al. 2006). While antibodies offer an as yet under-explored avenue for HCV treatment, there are a number of factors that will influence the efficacy of this approach and therefore dictate the most appropriate strategy. These factors are considered below.

2 Evolutionary Dynamics of HCV Envelope Genes

HCV can be classified into six genetically distinct genotypes and further subdivided into a large number of subtypes, of which the six major genotypes differ by approximately 30% and the subtypes differ by 20%–25% at the nucleotide level (Simmonds et al. 2005). A significant challenge for vaccine and immunotherapeutic development is the identification of protective epitopes conserved in the majority of viral genotypes and subtypes. This problem is compounded by the fact that the envelope E1E2 proteins, the natural targets for the Vn response, are two of the most variable proteins (Bukh et al. 1995). The error-prone nature of the RNA-dependent RNA polymerase together with the high HCV replicative rate in vivo (Neumann et al. 1998) results in the production of viral quasispecies (Martell et al. 1994; Bukh et al. 1995). Quasispecies can respond to and overcome a variety of selective pressures including host immunity (Cooreman and Schoondermark-Van de Ven 1996; Cooreman and Schoondermark-Van de Ven 1996); chronic infection arises, at least in part, through the outgrowth of immune escape mutants (Frasca et al. 1999; Farci et al. 2000; Majid et al. 1999; Ray et al. 1999; Sullivan et al. 1999). The envelope glycoprotein genes display some of the highest levels of genetic heterogeneity, with E2 exhibiting greater variability at the quasispecies level than E1. Mean pair-wise genetic distances, calculated for translated HCV protein sequences spanning the entire E1E2 coding region, highlight regions of high and low variability and are distributed throughout all of the E1E2 genes (Fig. 1).

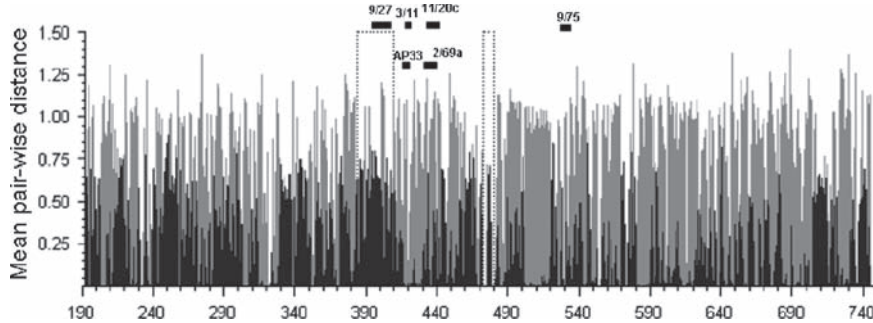


Fig. 1 Mean pair-wise distance plot for E1E2 amino acids and synonymous sites generated for HCV sequences representative of genotypes 1 through 6 (98 isolates, 543 codons). Mean p-distance at specific amino acid positions are represented by *black bars* in the foreground. Mean p-distance at synonymous sites in homologous codons are represented by *gray bars* in the background. Positions are relative to homologous codons in the H77 reference strain polyprotein. *Dotted columns* represent HVR1 and HVR2. The labeled *black bars* located above the diversity plot represent the relative positions of characterized Vn and non-Vn MAbs recognizing linear epitopes

While high levels of amino acid diversity are evident, pair-wise distances calculated at synonymous (silent) sites revealed that a considerable proportion of genetic variation across HCV genotypes 1–6 is due to synonymous substitution. These high levels of synonymous site diversity observed throughout E1 and E2 indicate these genes are under strong purifying selection. Much of the current knowledge of adaptive evolution within the envelope genes during HCV chronic infection is based on averaged estimates of synonymous (d_s) and nonsynonymous (d_N) nucleotide substitution rates across very small regions of the envelope genes such as the first hypervariable region (HVR1; Curran et al. 2002; Gretch et al. 1996; Honda et al. 1994; McAllister et al. 1998; Smith 1999). However, these averaging types of analysis are too blunt to dissect out exact evolutionary mechanisms leading to antibody escape. Averaged d_N/d_s ratios across regions are highly conservative, as only a few codons within the protein may be under diversifying selection. The signal is therefore diluted in an overbearing purifying selective background that arises through strong functional constraint. To overcome analytical problems associated with differential selection across a region, the distribution of the d_N/d_s ratio (ω) can now be estimated for individual amino acids by assessing competing models of codon substitution within a maximum likelihood (ML) framework (Yang and Bielawski 2000). These ML methods have recently been applied to the identification of site-specific adaptive mutations in human immunodeficiency virus (HIV) *env* genes (Choisy et al. 2004) and partial E1E2 sequence data sets from individuals with chronic (Brown et al. 2005) and acute phases of HCV infection (Sheridan et al. 2004). The latter study extended earlier findings (Puig et al. 2004; Farci et al. 2000) revealing a statistically significant association between outcome of acute phase infection and the number of positively selected sites (Sheridan et al. 2004). Importantly, these methods can help pinpoint those residues and regions that the virus can easily change to

help evade both cellular and humoral responses as well as those that are under strong functional constraint. This information is key to devising appropriate immunotherapeutic approaches, since utilizing antibodies that target highly conserved functionally constrained regions will minimize the likelihood of antibody escape and maximize breadth of action and therapeutic potential.

Analysis of viral evolution has shown that the amino terminus of the E2 envelope protein contains residues that have a very high propensity for adaptive change. Indeed, this is the only locus within E1E2 that harbors positively selected sites that are common across all genotypes (Fig. 2). This region, known as the first hyper-variable region (HVR1), is the major determinant for strain-specific Vn antibody responses (Farci et al. 1994, 1996; Bartosch et al. 2003a; Rosa et al. 1996; Shimizu et al. 1994). There is increasing evidence that acute infection outcome is correlated to the rate and nature of nucleotide substitutions within the envelope genes (Farci et al. 2000; Ray et al. 1999; Sheridan et al. 2004). Patients harboring stable HVR1 quasispecies frequently resolve infection, while those with evidence of a rapidly evolving quasispecies develop chronic infection (Farci et al. 2000; Ray et al. 1999). Evolution of the viral quasispecies continues during the chronic phase (Brown et al. 2005; Gretch et al. 1996), and differences in evolutionary rates and disease severity in individuals with differing levels of immunocompetency highlight the importance of antibody responses in controlling the infection (Booth et al. 1998; Kumar et al. 1994). While there is strong evidence that antibodies directed to this region correlate with a beneficial outcome, the limited cross-reactivity of most HVR1-specific responses presents a serious problem to their wider therapeutic potential. A recent

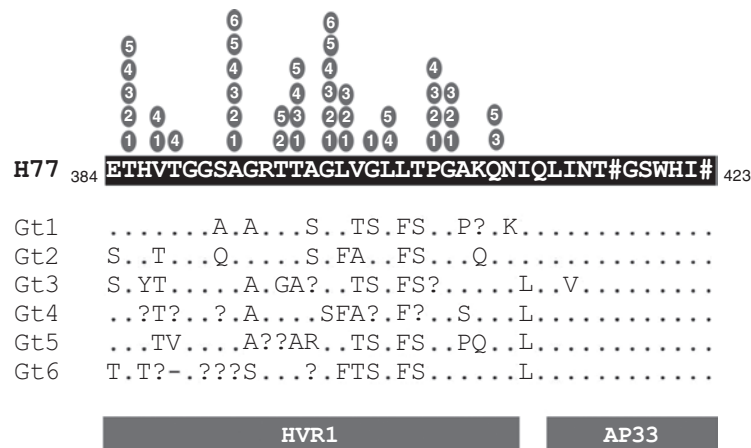


Fig. 2 Alignment of genotype consensus amino acid sequences relative to the H77 reference strain, showing distribution of amino acid sites identified as under diversifying selection in E1E2 sequences relative to their homologous positions in the H77 reference strain polyprotein (accession no. NC_004102). Symbols *above* the sequence indicate positively selected sites for sequences representative of each genotype 1 through 6. Also shown is the region targeted by the MAbs AP33. #, putative N-linked glycosylation site (NXT or NXS). ?, no overall consensus amino acid at this position. Modified from Brown et al. (2007)

study examining a series of E1E2 sequences in the form of retroviral pseudoparticles from samples collected over a 26-year period of one patient found that sequential serum antibodies obtained at the same time points of the E1E2 sequences either fail or showed reduced Vn activity demonstrating a continuous generation of escape mutants predominately in HVR1 (von Hahn et al. 2007). Despite this caveat, studies have shown that positive selection is not evenly distributed across HVR1 and instead is focused on a few discontinuous residues (Brown et al. 2007; McAllister et al. 1998; Penin et al. 2001). By using consensus HVR1 sequence mimotopes, Puntoriero et al. (1998) were able to induce cross-reactive antibody responses in immunized mice. Therefore, isolation of antibodies focused on the conserved HVR1 residues may still have therapeutic promise.

We recently performed in-depth analysis of the E1E2 evolution, both across diverse genotypes as well as within individual-patient quasispecies (Brown et al. 2005, 2007), and this work has shown that much of the E1E2 coding region is under very strong purifying selective pressure. This is particularly evident in regions of known or suspected functional importance. We have shown that several discontinuous regions of E2 contain highly conserved residues that are involved in binding to CD81, a receptor for HCV (Owsianka et al. 2006) and, importantly, some of these residues are targeted by Vn antibodies. For example, MAb AP33 (see Sect. 3.5, “Antibodies to Linear Epitopes” below) has a broadly Vn phenotype that can be attributed to the extreme conservation of its epitope and the importance of this region of E2 in CD81 binding (Owsianka et al. 2005, 2006; Tarr et al. 2006). Utilizing Vn antibodies that target such highly conserved, functionally constrained epitopes will limit the likelihood of viral escape, and therefore maximize Vn antibodies’ therapeutic potential. Even if escape occurs within these conserved epitopes, it is probable that this will come at a replicative cost to the virus, such as decreased receptor binding. Such a relationship is not unprecedented; there is mounting evidence that mutations in and around the CD4 binding domain of gp120 are associated with reduced sensitivity to Vn antibodies but at the cost of efficient CD4 receptor binding (Beaumont et al. 2004; Pinter et al. 2004; Pugach et al. 2004).

3 Humoral Immunity

What is the evidence that Vn antibodies can be protective? Until recently, the role of antibodies in preventing and controlling HCV infection has been less defined because of difficulties in having efficient and reliable *in vitro* systems to grow HCV. Early clinical trials with IgG therapy achieved significant prophylactic effects on transfusion-associated HCV hepatitis (Sugg et al. 1985; Sanchez-Quijano et al. 1988) and a reduction of infection among sex partners of HCV-infected patients receiving gamma-globulin (Piazza et al. 1997). Experimental animal studies have shown that IgG therapy delayed the onset of acute infection in HCV-challenged chimpanzees (Krawczynski et al. 1996). If the challenge HCV inoculum is first pre-incubated with HCV-specific Ig plasma, complete protection can be achieved (Farci et al. 1994). This led to studies

showing that serum antibodies from patients with chronic HCV are directed at HVR1 (Shimizu et al. 1994, 1996; Zibert et al. 1995; Rosa et al. 1996). Additional findings were later obtained in chimpanzees by pre-incubating the infectious plasma with a rabbit antiserum specific for HVR1 (Cooreman and Schoondermark-Van de Ven 1996; Farci et al. 1996). Vaccination with recombinant HCV E1 and E2 proteins also provided some protection (Choo et al. 1994). Supporting the view that the humoral immune response is at least partly protective is the finding that circulating HCV virions in a chronically infected chimpanzee exist in two forms, as free virions and as immune complexes, with the latter displaying lower infectivity (Hijikata et al. 1993).

Acute infection with HCV is usually silent, with most infected individuals going on to develop persistent infections (Alter and Seeff 2000). Nonetheless, 20%–25% of acutely infected individuals clear viremia without disease progression. Also, a study of injecting drug users (IDU) comparing previously HCV-infected individuals (HCV antibody-positive and HCV RNA-negative) and those without evidence of prior infection (HCV antibody-negative and RNA-negative) found previously infected IDU were 12 times less likely than those with first time exposure to develop persistent infection (Mehta et al. 2002). Peak viral load on average was $10^{-1.8}$ lower in previously infected IDU. These findings indicate that adaptive immunity can prevent disease progression. Understanding the mechanisms contributing to acute infection resolution and control of disease persistence will be essential for vaccine and immunotherapeutic development. Conversely, identifying factors contributing to virus escape will help define those protective determinants most likely to give broad protection. In a recent study of a single-source outbreak of hepatitis C in a cohort of women, viral clearance was found to be associated with a rapid induction of Vn antibodies in the early phase of infection, the amounts of which were reduced or lost altogether following recovery from virus infection. In contrast, chronic HCV infection in this cohort was characterized by absent or low-titer Vn antibodies in the early phase and during persistence of infection despite the induction of cross-neutralizing antibodies in the late phase of infection. These data suggest that rapid induction of Vn antibodies during the early phase of infection may contribute to control of HCV infection (Pestka et al. 2006).

Vn epitopes can be broadly classified as either isolate-specific or broadly cross-reactive. The N-terminus of E2, encompassing HVR1 and the region immediately downstream contains potent linear epitopes (Flint et al. 2000; Flint et al. 1999; Owsianka et al. 2001; Triyatni et al. 2002; Zibert et al. 1997). Conservation in the region downstream of HVR1 leads to some cross-reactivity, although high levels of diversity seen between different genotypes can abrogate Vn (Bartosch et al. 2003a). More broadly, Vn antibodies have been demonstrated (Logvinoff et al. 2004) and these are usually directed against conformational epitopes within E2 (Allander et al. 2000; Bugli et al. 2001; Habersetzer et al. 1998; Hadlock et al. 2000; Ishii et al. 1998). E1-specific Vn antibodies are rare (reviewed in Depraetere and Leroux-Roels 1999), probably because this protein has low immunogenicity. However, there is evidence that E1-specific responses can be invoked following experimental vaccination and these responses may be protective (Rollier et al. 2004). Further, E1-specific Vn antibodies are present in some HCV-infected patients. We have previously described the use of an HCV-producing lymphoma cell line (Sung et al. 2003)

to confirm Vn activity of a human monoclonal antibody (HMAb), H-111, to an epitope located on the N-terminal end of E1 that also blocks baculovirus-expressed HCV-like particles binding to target cells (Baumert et al. 1998; Keck et al. 2004b). The presence of E1 Vn antibodies is supported by recent studies showing that E1-specific sera are capable of neutralization of both retroviral pseudoparticles carrying HCV E1E2 proteins as well as cell cultured HCV (Dreux et al. 2006). Different groups have suggested that both E1 and E2 contain putative fusion domains and the development of HMABs to E1 should facilitate functional and structural analyses of this HCV envelope protein.

3.1 Human Antibodies to Conformational Epitopes

There are two fundamental approaches to characterize the antibody response through the generation of HMABs: the immortalization of antigen-specific B cells and the combinatorial approach of cloning Ig gene repertoires; each with its distinct attributes. The first approach includes Epstein-Barr virus (EBV) transformation, which itself is of limited utility because of the inherent instability of antibody secretion in EBV-transformed B cells. This instability can be addressed by fusing EBV-activated B cells to a human heteromyeloma fusion partner (Foung et al. 1984). The refinements of cell fusion methods have led to successes in generating relevant HMABs with as few as 10^5 human B cells from peripheral blood (Foung et al. 1984, 1990; Zimmermann et al. 1990; Perkins et al. 1991). The advantages of this approach are the relative ease in obtaining antigen-specific B cells from peripheral blood and the ability to more directly analyze the B cell response under disease constraints. EBV transformation or activation of peripheral B cells followed by fusion to a human heteromyeloma has been used successfully by a number of groups to isolate HCV-specific HMABs (Siemoneit et al. 1995; Habersetzer et al. 1998; Hadlock et al. 2000; Keck et al. 2004b). The second approach involves the construction of combinatorial libraries expressing human Fab fragments on the surface of M13 phage (Burton and Barbas 1994). This approach has the theoretical ability to examine millions of Fabs and consequently the full display of possible antibody diversity in the human B cell repertoire. Enrichment of combinatorial libraries constructed from the bone marrow of HCV-infected individuals has led to the recovery of a number of recombinant HMABs to HCV E1E2 glycoproteins (Allander et al. 2000; Burioni et al. 1998; Burioni et al. 2001; Schofield et al. 2005; Zhong et al. 2005).

The majority of antibodies capable of interacting with diverse strains of HCV are directed to conformational epitopes within E2 (Allander et al. 2000; Bugli et al. 2001; Habersetzer et al. 1998; Bartosch et al. 2003b; Hadlock et al. 2000). In a panel of HCV HMABs, cross-competition studies showed that the E2 glycoprotein contains three immunogenic conformational domains, designated A, B, and C, and these are accessible on the surface of retrovirus particles incorporating HCV glycoproteins, HCVpp (Keck et al. 2004a). Without knowing the exact location of Vn and non-Vn HMABs to conformational epitopes on E2, their spatial relationship

was assessed based on competition studies (Keck et al. 2005). Each HMAb was biotinylated and binding was tested with competing HMAb. Relatedness of the HMABs to each other was determined by a modified unweighted pair-group method using arithmetic averages (UPGMA) (Fitch and Margoliash 1967; Keck et al. 2004a; Sneath and Sokal 1973). This method assumes the extent of bidirectional inhibition as the extent of epitope overlap by the competing antibodies. Unidirectional inhibition or enhancement is interpreted as proximal but not overlapping epitopes. This approach spatially places paired antibodies with the highest bidirectional inhibition as most related and an example of such an analysis is shown in Fig. 3. The cluster analysis yielded three distinct immunogenic domains. The epitopes recognized by HMABs CBH-4G, -4B, and -4D constitute domain A.

A second domain, B, is characterized by CBH-2, -5, -8C, -8E, and -11, and a third domain C by CBH-7 and -23. HMABs within a domain have significant bidirectional inhibition and minimal competition with HMABs in other domains. For recently isolated HMABs (Fig. 3 in italics), their placements were based on cross-competition with representative antibodies from each domain. CBH-20, -21, and -22 belong to domain A, and CBH-23 to domain C. HMABs to domain B and C inhibit E2-CD81 interaction and HMABs to domain A do not (Hadlock et al. 2000).

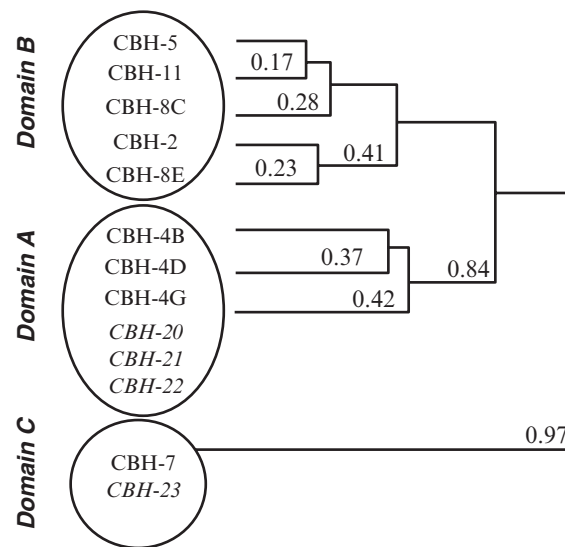


Fig. 3 Competition analysis of HCV HMABs and proposed model for three immunogenic domains. Phylogenetic grouping of HCV HMABs is based on cross-competition. *Solid lines with numbers* indicate the relatedness of the two adjoining antibodies, the smaller the number, the greater the relatedness. *Circles* are clusters of antibodies in a specific domain. Competition results used for calculation are the mean values obtained from 2–5 separate experiments (Keck et al. 2004a)

3.2 Virus Neutralization with HCV Retroviral Pseudoparticles

A robust means to analyze Vn potency and breadth is the infectious HCV retroviral pseudoparticle (HCVpp) system, whereby infection by retroviral particles containing an appropriate reporter gene is mediated by HCV E1E2 envelope proteins (Bartosch et al. 2003b; Bartosch et al. 2003c; Hsu et al. 2003). These HCVpp preferentially infect human hepatocytes and hepatocellular cell lines, and the E1E2 present in these particles has been shown to represent the functional and antigenic noncovalent E1E2 heterodimer formed by their recognition by a panel of conformation-dependent E2-specific HMABs (Op De Beeck et al. 2004). All E2 HMABs to conformational epitopes were able to immunoprecipitate HCVpp, suggesting their expression on the HCVpp surface (Keck et al. 2004a). Studies with HCVpp have provided important insights into the early stages of virus entry (Bartosch et al. 2005, 2003c; Goffard et al. 2005; Lavillette et al. 2005; Voisset et al. 2006). With genotype 1b HCVpp infection on Huh-7 cells, HCV E2 HMABs in domains B (CBH-2, -5, -8C, -11) and C (CBH-7, -23) have Vn activities as measured by reduction of luciferase activity compared with the infection medium contains phosphate-buffered solution (PBS) (no antibody) or RO4 (Keck et al. 2004a, 2005). All domain A HMABs were non-Vn (Fig. 4).

To assess the breadth of Vn, three HCV HMABs with broad binding profiles to different genotypes, CBH-2, -5, and -7, were tested with six HCV genotype HCVpp (Fig. 5). CBH-2 showed Vn with one of two isolates of genotype 1a, genotypes 1b, 2a, 2b, 5, 6, and weak Vn with 4. CBH-5 showed Vn with 1a, 1b, 2a, 2b, 4, 6, and weak Vn with 5. CBH-7 showed Vn with only 1a, weak Vn with other genotypes, and no activity with one of two isolates of genotype 4. The breadth of Vn is roughly correlated with the breadth of binding with each Vn HMAb (A.M. Owsianka, A.W. Tarr, Z.Y. Keck, T.K. Li, J. Witteveldt, R. Adair, S.K.H. Fong, J.K. Ball, and A.H. Patel, manuscript in preparation).

The relationship between inhibiting E2-CD81 interaction and HCVpp neutralization also holds true with recombinant HMABs to E2 (Schofield et al. 2005). Cross-competition studies with HMABs from phage display libraries mapped a cluster of

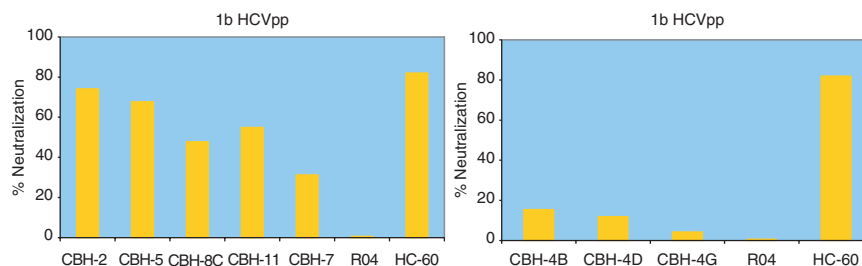


Fig. 4 Genotype 1b HCVpp Vn with E2 HMABs. The HCVpp neutralization assay was performed as described by Keck et al. (2005) with each HMAb tested at 20 µg/ml. HC60, an HCV-positive human serum, was tested at 1:100 dilution. RO4 is an isotype-matched HMAb to CMV

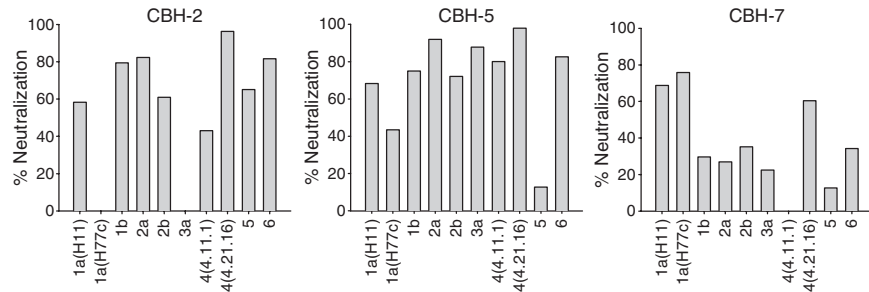


Fig. 5 Neutralization of six genotype HCVpp (ten isolates). Huh-7 cells were seeded at 8×10^3 cells per well in a 96-well plate 24 h before infection. HCVpp bearing envelopes from HCV genotype 1a (H11, H77c), 1b (1B5.23), 2a (2A1.2), 2b (2B2.8), 3a (3A13.6), 4 (4.11.1, 4.21.16), 5 (5.15.7), and 6 (6.5.8) were incubated with HMABs (CBH), an irrelevant isotype control HMAB RO4, or PBS (no MAb control) for 1 h at 37°C. After 15 h the HCVpp medium was replaced with fresh complete medium and incubated for an additional 72 h. Vn activity of an antibody was determined by the percentage reduction of luciferase activity compared with the infection medium containing PBS (not shown)

related epitopes inhibiting E2-CD81 interaction or neutralization of binding (NOB)-positive and three unrelated epitopes that are NOB-negative (Bugli et al. 2001).

3.3 Virus Neutralization with Cell Culture Infectious HCV Virions

Three groups recently developed full-length HCV RNA genomes that are able to replicate efficiently and produce infectious viral particles when transfected into human hepatoma cells (Huh-7) (Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005). The availability of these and other cell-cultured infectious HCV virions (HCVcc) should greatly accelerate studies of HCV biology (Cai et al. 2005; Lindenbach et al. 2005; Pietschmann et al. 2006; Wakita et al. 2005; Yi et al. 2006; Zhong et al. 2005). Another group reported that stable human hepatoma cell lines containing a chromosomally integrated cDNA of HCV genotype 2a (JFH1) RNA constitutively secrete HCVcc into the medium (Cai et al. 2005). Employing this source of HCV virions, HMABs to three distinct immunogenic domains on HCV E2 to neutralize HCVcc were tested (Keck et al. 2007). Vn was determined by measuring the levels of HCV NS3 protein expression by Western blot analysis. Figure 6 shows the inability of each domain A HMAB and the abilities of representative domain B (CBH-5) and C (CBH-7) HMABs to neutralize HCV infectivity to Huh-7.5 cells. All domain A HMABs had no effect on NS3 protein levels (Fig. 6A). HCVcc was completely neutralized at low antibody concentrations by all domain B HMABs (as represented by CBH-5 in Fig. 2B and more fully discussed below). However, domain C HMAB, CBH-7, showed Vn activity only at 5 µg/ml IgG (Fig. 6B). The GAPDH

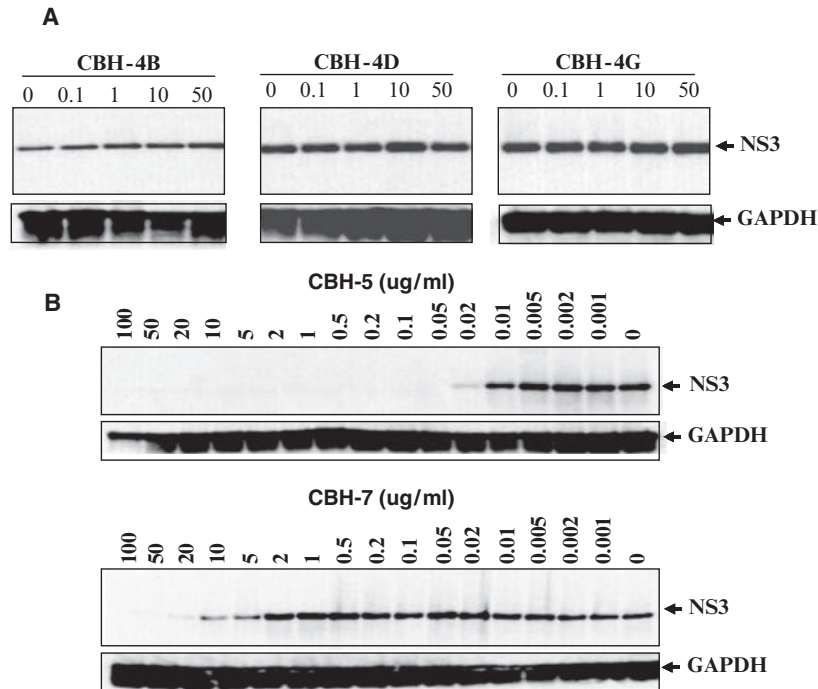


Fig. 6 Effect of HMABs on genotype 2a HCVcc infectivity as determined by NS3 expression. HCVcc was incubated with each HMAB at increasing concentrations prior to infection to Huh-7.5 cells pre-seeded in a 24-well plate. At 3 h post infection (p.i.) the HCVcc/antibody-containing medium was removed, the cells washed twice with PBS followed by incubation at 37°C in fresh medium. Cells were harvested for Western blotting analysis at 72 h p.i. **A** Domain A HMABs showed no reduction of NS3 expression. **B** Quantitative HCVcc Vn with CBH-5 and -7 shown as representative domain B and C antibodies. HCV NS3 protein expression was determined by a MAb specific to NS3 (Cai, Zhang et al. 2005). The GAPDH protein used as an internal control was detected using an anti-GAPDH MAb (Abcam)

protein used as an internal control was detected by using an anti-GAPDH MAb (in Fig. 6A and 6B; Abcam, Cambridge, MA). Similar findings of Vn neutralization with domain B and C HMABs were obtained with infectious genotype 2b HCV virions from a B cell lymphoma-derived cell line that continuously produces infectious HCV virions in culture (Sung et al. 2003; M. Lai, personal communication). Collectively, these findings support an immunogenic model of HCVcc E2 containing three distinct functional domains as previously shown with HCVpp studies.

3.4 Virus Neutralization Potency with HCV Virions

A more quantitative measure of neutralization potency was determined by the inhibitory antibody concentration that reduces HCV infectivity 90%, IC_{90} (Keck et al. 2007).

Table 1 Neutralization titer

Antibodies	IC ₅₀ ^a		IC ₉₀ ^a	
	µg/ml	nmol/l	µg/ml	nmol/l
CBH-2	0.057	0.378	0.350	2.330
CBH-5	0.056	0.375	0.096	0.640
CBH-8C	0.205	1.367	0.980	6.530
CBH-11	0.283	1.886	4.061	27.070
CBH-7	25.580	170.530	49.800	332.000

^aAntibody concentration to reach 50% and 90% viral neutralization

HCVcc neutralization conferred by each HMAb is summarized in Table 1. The IC₉₀ for HMAb CBH-5 was 0.01 µg/ml, while CBH-7 required approximately 50 µg/ml. The neutralization potency is in the order of CBH-5>CBH-2>CBH-8C>CBH-11>CBH-7. Another observation was that the HCVcc neutralization profiles for CBH-5 and CBH-7 displayed a linear relationship with antibody concentration from IC₅₀ to IC₉₀ (Fig. 6B and Table 1). However, the profiles for CBH-2, -8C, and -11 displayed a nonlinear relationship between IC₅₀ and IC₉₀, in which 5 to 14 times more antibodies were required to achieve IC₉₀ than IC₅₀. The possible contributions to differences with Vn profiles include the affinity of antibody binding to different viral epitopes, kinetics of antibody association and dissociation with targeted antigen, IgG subclass, and mechanisms of Vn whereby these antibodies inhibit different facets of HCVcc interactions with its putative receptor(s) (Harris et al. 1997). However, IgG subclass and antibody affinity are unlikely factors in this case as these antibodies are all IgG₁ and their relative K_d values are similar except for CBH-5 (Keck et al. 2007). More studies will be required to find out whether these antibodies block at distinct steps in virus–receptor interactions. In summary, the immunogenic organization of HCVcc E2 glycoprotein consists of three distinct domains, with Vn epitopes restricted to two domains as described for HCVpp. The fact that the ability to block E2 binding to CD81 is predictive of Vn provides additional support that CD81 is a required molecule for HCVpp and HCVcc entry.

3.5 Antibodies to Linear Epitopes

While Vn E1 antibodies have been described (Keck et al. 2004a; Dreux et al. 2006; Pietschmann et al. 2006), the majority of antibodies in natural infection are targeted to the E2 protein. Antibodies that demonstrate broad Vn capacity described to date generally tend to be directed against conformational epitopes within E2 (Habersetzer et al. 1998; Ishii et al. 1998; Allander et al. 2000; Hadlock et al. 2000; Bugli et al. 2001). However, Vn antibodies that recognize linear epitopes within HCV E2 have also been reported. Farci et al. (1996) first described a rabbit hyperimmune serum directed against HVR1 of E2 that was able to neutralize virus in vitro and thus protect chimpanzees in experimental infections. Subsequently, Zibert et al. (1995, 1997) showed that patient antibodies to HVR1 as well as a rabbit immune serum to

that region were able to specifically block virus binding to cells. More recently, antibodies targeting this region have been shown to inhibit binding of E2 to cells (Scarselli et al. 2002), HCV virus-like particles (VLPs), and serum-derived HCV (Steinmann et al. 2004; Zhou et al. 2000; Barth et al. 2005), as well as HCVpp entry into target cells (Bartosch et al. 2003a; Hsu et al. 2003; Owsianka et al. 2005). The HVR1, which comprises the N-terminal 27 residues of E2 (aa 384–410), is highly variable among HCV genotypes as well as subtypes belonging to the same genotype. Deletion of HVR1 was shown to attenuate virus infectivity both in the chimpanzee model and also in HCVpp assay (Bartosch et al. 2003c; Fornis et al. 2000). Together, these indicate an important role of this region in virus entry. However, due to the high variability of this epitope, these antibodies exhibit poor cross-neutralization potency across different HCV isolates. The use of conserved HVR1 mimotopes has been proposed to overcome problems of restricted specificity (Cerino et al. 2001; Roccasecca et al. 2001; Zucchelli et al. 2001).

Several antibodies targeting different linear regions (other than HVR1) within E2 that inhibit E2–CD81 interaction in *in vitro* assays have been identified. The first of these regions lies immediately downstream of the second hypervariable region between positions 480 and 493 (Flint et al. 1999), the second spans residues 528 to 535 (Owsianka et al. 2001; Clayton et al. 2002), and a third region encompasses residues 544 to 551 (Flint et al. 1999; Owsianka et al. 2001). In addition, antibodies targeting amino acids 412–423 and 432–447 are also capable of blocking CD81 binding (Owsianka et al. 2001; Hsu et al. 2003). Barth et al. (2003, 2006) recently showed that antibodies specific to regions 516–530 block the interaction between E2 and heparin sulfate, a candidate receptor for HCV, and they also inhibit HCVpp entry. Interestingly, however, those antibodies recognizing regions 480–493, 528–535, and 544–551 failed to neutralize HCVpp infection of target cells (Hsu et al. 2003). Furthermore, the region 432–447 displays a high degree of variation among different HCV isolates, and therefore the antibodies targeting this region have restricted cross-neutralizing potency (Owsianka et al. 2005).

The region 412–423, which is located immediately downstream of HVR1, has been shown to contain a potent neutralizing epitope (Clayton et al. 2002; Flint et al. 2000; Triyatni et al. 2002). This epitope, defined by the mouse MAb AP33 and a rat MAb 3/11, inhibited the interaction between CD81 and a range of presentations of E2, including soluble E2, E1E2, and virus-like particles (VLPs) (Owsianka et al. 2001). Moreover, MAb AP33 inhibited the interaction between E2 and heparin sulfate (Barth et al. 2006). Indeed, the MAb AP33 was reported to potently neutralize HCVpp-bearing envelope glycoproteins derived from all six HCV genotypes and major subtypes (Owsianka et al. 2005). As shown in Fig. 7 and Table 2, the IC_{50} of AP33 when neutralizing HCVpp of diverse genotypes ranged from approximately 0.6 up to 32 μ g/ml. The rat MAb 3/11, which recognizes the same E2 region, also neutralized diverse HCVpp, albeit with a lower potency than MAb AP33 (Tarr et al. 2006). Similarly, both MAbs AP33 and 3/11 were capable of neutralizing the genotype 2a HCVcc in cell-culture infection assays, with the former more potent than the latter (Fig. 8). Fine mapping identified four highly conserved residues within the E2 region 412–423 crucial for MAb AP33 binding,

Fig. 7 MAb AP33-mediated neutralization of HCVpp-bearing envelope glycoproteins from HCV genotypes 1a (1A H77 and 1A14.36), 1b (1B12.6), 2a (2A2.4), 2b (2B1.1), 3a (3A13.6), 4 (4.21.16), 5 (5.15.11), and 6 (6.5.340). Taken from Owsianka et al. (2005)

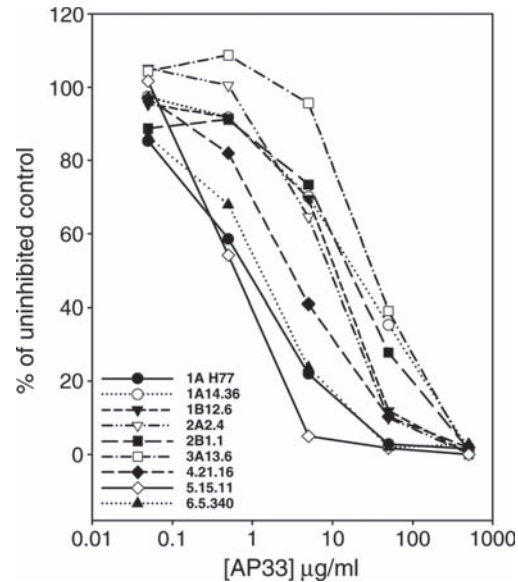


Table 2 MAb AP33 concentration required to achieve 50% (IC_{50}) or 90% (IC_{90}) inhibition of infection by diverse HCVpp shown in Fig. 7

Genotype	HCVpp isolate	IC_{50}	IC_{90}
1A	H77	5.9 nM (0.9 μ g/ml)	145 nM (22.0 μ g/ml)
1A	UKN1A.14.36	132.0 nM (20.0 μ g/ml)	198 mM (300.0 μ g/ml)
1B	UKN1B.12.6	72.0 nM (12.0 μ g/ml)	528 nM (80.0 μ g/ml)
2A	UKN2A2.4	59.4 nM (9.0 μ g/ml)	363 nM (55.0 μ g/ml)
2B	UKN2B1.1	119.0 nM (18.0 μ g/ml)	165 mM (250.0 μ g/ml)
3A	UKN3A13.6	211.0 nM (32.0 μ g/ml)	198 mM (300.0 μ g/ml)
4	UKN4.21.16	19.8 nM (3.0 μ g/ml)	396 nM (60.0 μ g/ml)
5	UKN5.15.11	4.0 nM (0.6 μ g/ml)	26.4 nM (4.0 μ g/ml)
6	UKN6.5.340	8.6 nM (1.3 μ g/ml)	145.2 nM (22.0 μ g/ml)

whereas three residues were found to be critical for MAb 3/11, only two of which were shared with MAb AP33 (Tarr et al. 2006). Thus, this MAb targets an overlapping yet distinct epitope, indicating that this region harbors multiple Vn epitopes (Tarr et al. 2006).

Analysis of over 5,500 sequences obtained from the Genbank database showed that the AP33 epitope is highly conserved. Identifying potentially Vn antibodies with epitopes conserved across all isolates of HCV is an essential step in the development of a successful vaccine. They could have a future role in the treatment of HCV infection and they might also serve to define future vaccine candidates. It will be important to define whether or not passive transfer of AP33 and 3/11 can protect in animal model-challenge experiments. Similarly, immunization and challenge studies

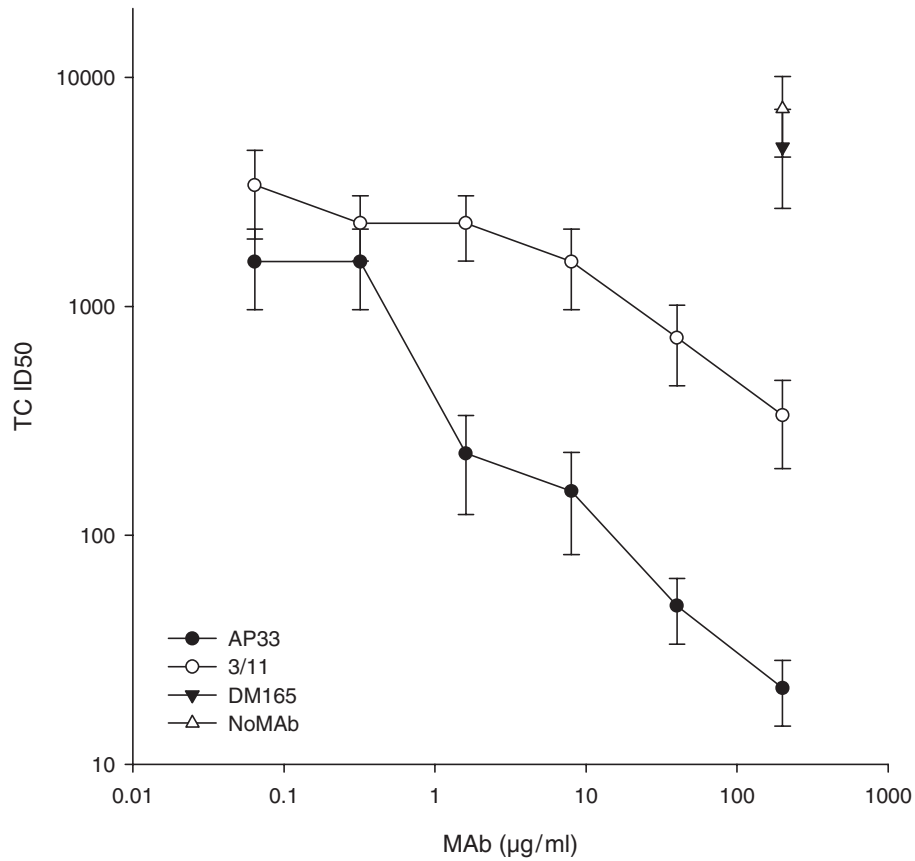


Fig. 8 Neutralization by MAbs AP33 and 3/11 of HCVcc infection of target cells in culture. HCVcc was pre-incubated with various concentrations of MAbs for 1 h at 37 °C. Serial dilution of each mixture was used to infect Huh-7 cells. Following incubation at 37 °C for 4 days the cells were fixed with methanol and analyzed by indirect immunofluorescence for the presence of viral protein NS5a. The wells of infected cells were scored for the presence or absence of fluorescing cells, and the virus infectivity was determined as TCID₅₀ (tissue culture infectious dose) essentially as described by Lindenbach et al. (2005). *DM165* denotes a irrelevant MAb control of the same isotype as AP33

using various immunogens containing the AP33 or 3/11 epitope will define its usefulness as a vaccine candidate. However, studies with HIV-1 have shown that focusing the immune response on epitopes recognized by broadly Vn antibodies is a significant challenge.

In this context, the finding that MAbs AP33 and 3/11 potently neutralize the entry of diverse HCVpp, and also HCVcc, is significant, particularly as their epitope is linear and highly conserved across different genotypes of HCV, and as such it offers significant hope for the development of a successful HCV vaccine. The exact mechanism of neutralization by these antibodies is unknown, although

inhibition of CD81-binding or heparin sulfate-binding (or both) is the most likely (Owsianka et al. 2001; Barth et al. 2006). Thus, the design of future vaccine candidates based on these epitopes and AP33 (or 3/11)-like therapeutic antibodies will require a better understanding, at the molecular level of the antigen–antibody interaction. Only with correct presentation of an immunogen will a vaccine generate the desired immune responses.

4 Mechanisms of Virus Neutralization

HCV is a member of the family Flaviviridae and is composed of three structural proteins, capsid, two envelope proteins, E1 and E2, and six nonstructural proteins (Robertson et al. 1998; Lindenbach and Rice 2001; McLauchlan et al. 2002). Similar to other flaviviruses, virus entry is thought to be mediated by envelope proteins, which are responsible for virus attachment and receptor-mediated endocytosis where a low pH environment in the endosomes triggers conformational structural changes leading to virus envelope fusion with the endosomal membrane (Bartosch et al. 2003c; Hsu et al. 2003; Op De Beeck et al. 2004). Antibody binding to E1 or E2 epitopes on virion surface should then lead to Vn by a number of mechanisms (Smith 2001; Fig. 9).

First, HCV antibodies may mediate aggregation of virus particles, leading to a reduced number of infectious virions, the pentameric status of IgM therefore being associated with increased neutralization potency. Second, the prevailing view for some

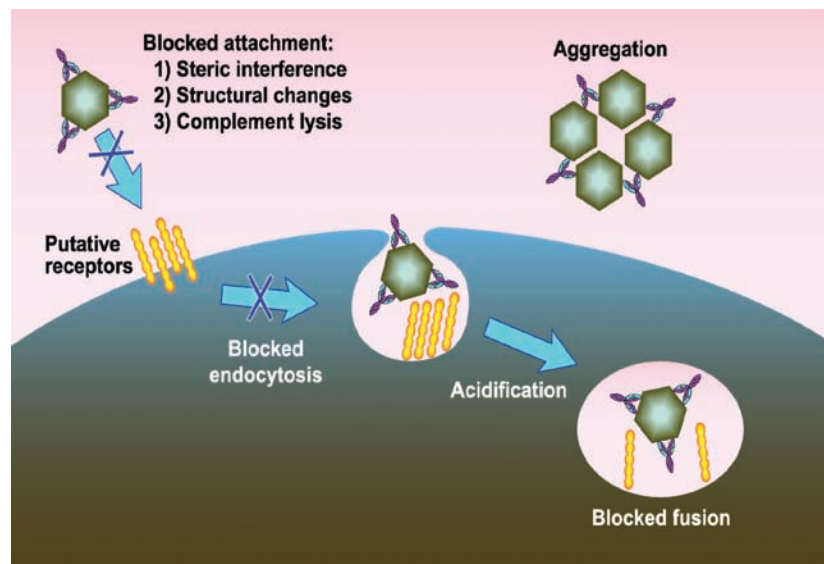


Fig. 9 Graphical representation of the mechanisms of virus neutralization

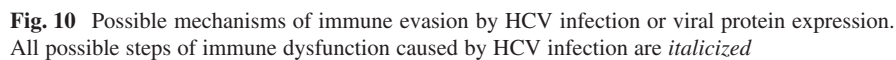
viruses is that Vn correlates with increased antibody binding to any virion surface site independent of the epitope recognized by the antibody. Vn is then the result of a critical number of binding sites being occupied, thus preventing virus entry through steric hindrance (Burton et al. 2001). Higher affinity antibodies will have a higher Vn while non-Vn antibodies either do not bind to virion surface or bind with low affinity. Alternatively, for some viruses, only specific surface epitopes that are involved in functional steps of virus entry have been proposed to be associated with neutralization (Houghton 1996). Steps targeted in this way include interaction with receptor and co-receptor, and initiation of viral envelope fusion with the cellular membrane. Our studies on HCVpp showed that E2 immunogenic domains A, B, and C are on the surface of these particles (Keck et al. 2004a). However, only domain B and C HMABs mediate Vn, and domain A HMABs are non-Vn, supporting the perspective that HCV virion attachment and entry are restricted to specific virion surface domains.

5 Mechanisms of Immune Escape

The mechanisms of viral escape include mutational escape from humoral (see Sect. 2, “Evolutionary Dynamics of HCV Envelope Genes”) and cellular immunity, and viral infection of immune cells that leads to the induction of Ig hypermutation. The latter phenomenon is associated with a reduction in binding affinity and Vn activity of antibodies that are specific for HCV envelope proteins. Furthermore, antibody-dependent enhancement of infection and potential roles of lipoproteins and glycans to modify antibody binding to epitopes mediating Vn have been described (Voisset et al. 2006; Nielsen et al. 2006; Germi et al. 2002).

5.1 *Mutational Escape from Cellular Immunity*

The relationship between cell-mediated immunity and the outcome of HCV infection has been established by numerous studies. Memory CD8⁺ cytotoxic T cells (CTL) are required for protection against persistent HCV infection; at the same time, durable intrahepatic memory is likely established during acute HCV infection, since T cells recognizing HCV antigens have been recovered from the livers of chimpanzees several years after spontaneous clearance of infection (Shoukry et al. 2004). Consequently, the outcome of HCV infection may be dictated by escape mutations in the epitopes targeted by CTL (Erickson et al. 2001). On this issue, one report demonstrated that CTL escape mutations occurred in persistent HCV infection (Cox et al. 2005). A second report described that divergent and convergent virus evolution after a common-source outbreak of HCV affected disease outcome (Ray et al. 2005). Immune evasion leading to persistent infection, in contrast to recovery from viral infection, after acute HCV infection from a shared source has been reported (Tester et al. 2005), reinforcing the general relevance of this immune evasion mechanism to persistence of RNA viruses in humans (Bowen and Walker 2005a). Amino acid changes also can alter



Successful immune responses in HCV infection generally target multiple major MHC class I-restricted epitopes in structural and nonstructural HCV proteins (Cooper et al. 1999; Shoukry et al. 2004). At the earliest time point studied in persons infected with HCV, highly activated CTL populations were observed that temporarily failed to secrete interferon (IFN)- γ , a “stunned” phenotype, from which they recovered as viremia declined (Lechner et al. 2000). In long-term HCV-seropositive persons, CTL responses were more common in those who had cleared viremia than those with persistent viremia, although the frequencies of HCV-specific CTL were lower than what was found in persons during and after resolution of acute HCV infection (Lechner et al. 2000).

CTL escape mutants are found during HCV infection in humans (Chang et al. 1997). Escape mutations in MHC class I-restricted epitopes are a feature of HCV infection that can diminish CTL responses via several mechanisms. For mutations in the CTL epitopes, marked fitness cost is not exacted by viral escape, and reversion to a more immunogenic ancestral state is not automatic upon passage to a host in which immune selection pressure is absent. It is tempting to speculate that this phenomenon might be due to low fitness cost associated with this particular mutation, thus allowing persistence of the variant sequence in the absence of immune selection pressure. A loss of epitope phenotype can also occur when amino acid anchor residues required for MHC binding are changed (Chang et al. 1997;

Erickson et al. 2001). Evidence for the emergence of escape mutations and their role in HCV infection are well-documented. The evolution of escape mutations in HCV is likely constrained by both intrinsic viral factors and certain characteristics of the adaptive immune response (see Sect. 2 above). There are several supporting reports on the lack of protective immunity against reinfection with HCV (Lai et al. 1994), the failure of naturally acquired antibodies to prevent reinfection of immune chimpanzees or humans (Farci et al. 1992), and emergence of CTL escape variants (Weiner et al. 1995).

Immune selection of HCV variants in humans includes the following steps (Bowen and Walker 2005a). First, mutations occur in immune epitopes of both structural and nonstructural viral proteins during acute infection. Second, evolution of quasispecies occurs in early infection. Third, a divergent or convergent CTL mutational evolutionary pattern from the prototypical epitope has been observed following a common-source HCV outbreak (Bowen and Walker 2005b; Ray et al. 2005; Tester et al. 2005). Some of these mutations are due to viral reversion to a more fit ancestral state (Cox et al. 2005; Ray et al. 2005). Nonetheless, these adaptive mutations can temper the effectiveness of CD8⁺ CTL function (Franzin et al. 1995). There is a complex interplay between the breadth and specificity of the antigen-specific immune response and the degree to which mutations selected by this immune pressure govern viral reproduction. Fourth, limited epitope variation occurs during the late or chronic infection phase. Last, epitope variation can be present (restricting MHC allele vs nonrestricting MHC) without seroconversion during chronic infection (Post et al. 2004). Similarly in chimpanzees, immune selection of HCV variants includes the following observations (Cooper et al. 1999; Bowen and Walker 2005b). First, there is a minimal viral variation prior to onset of adaptive immune response. Second, escape mutations abrogating CTL function do occur in acute phase infection. Third, escape mutations evading the antiviral CTL response occurs in MHC class I-restricted epitopes (Weiner et al. 1995; Sasso 2000; Erickson et al. 2001).

Immune escape by mutations in CTL epitopes occurs by at least two mechanisms (Drummer et al. 2002; Bowen and Walker 2005a, b), the loss of T cell receptor (TCR) recognition (Ivanovski et al. 1998; De Re et al. 2000; Sasso 2000; inhibition of CTL response, antigenic sin, and preferential stimulation of response) and the loss of epitope by altered proteasome processing and reduced MHC class I binding. The amino acid substitutions within or adjacent to CTL epitopes can alter proteasomal processing, causing epitope destruction before transport to the endoplasmic reticulum for MHC binding (Seifert et al. 2004; Timm et al. 2004; Kimura et al. 2005). In HLA-A*01- and B*08-negative individuals, absence of these alleles was associated with evolution toward consensus within epitopes restricted by these MHC molecules (Ray et al. 2005).

The viral epitopes on the virus-specific CD4⁺ and CD8⁺ T cell frequently evolve during HCV infection, resulting in impaired effector function of HCV-specific CTL (Gruener et al. 2001; Wedemeyer et al. 2002) and a lack of protective immunity against reinfection with HCV (Lai et al. 1994). In the presence of the restricting allele, mutational escape of MHC class I-restricted epitopes may occur in four ways. First,

sustained cellular immune responses are associated with resolution of infection during the acute phase (Zuckerman et al. 1997). The diverse clonal CTL TCR repertoire due to sustained CD4⁺ T cell response may constrain the development of escape mutations in the restricting MHC molecule. Second, in the case of absent or weak CD4⁺ T cell responses, CTL responses are weak and CTL-escape mutations may not develop (Hanley et al. 1996; De Vita et al. 2000). Third, where the CD4⁺ T cell response fails, escape mutations might emerge, particularly if their fitness cost is low. Lastly, if the TCR repertoire of the clonal CTL is narrow in the absent or weak CD4⁺ T cell response, escape mutations may emerge (Grakoui et al. 2003; Shoukry et al. 2004). In the absence of the restricting allele, when there is low associated fitness cost or well-developed compensatory mutations and if there is no CTL-mediated immune pressure, the mutated sequence may persist or may mutate to an equally “fit” alternative. Where there is high fitness cost associated with the presence of an escape mutation and if there is no CTL-mediated immune pressure (minimal immune selection pressure), reversion to the wild-type (“fitter”) sequence is likely to occur.

Epitope mutations in individual MHC alleles may alter the interaction of epitope with the immune system by at least four different mechanisms. First, mutational escape from CD8⁺ T cell immunity can occur (Bowen and Walker 2005a, b). Second, mutations in cognate epitopes in anchoring residues may lead to dissociation of the MHC–peptide complex. Third, mutations in the epitope or in flanking regions can alter proteasomal processing, leading to destruction of the epitope. Fourth, reduced TCR recognition of the neo-epitope–MHC complex is involved (Chang et al. 1997). TCR recognition may be reduced or possibly altered, leading to antagonism against responses to the wild-type peptide. Such mutated peptide–MHC complexes may alternatively antagonize responses to the wild-type epitope (Chang et al. 1997; Kaneko et al. 1997; Tsai et al. 1998). Nonsynonymous mutations may occur within CTL epitopes or within regions flanking these sequences.

Other immunological mechanisms (Bowen and Walker 2005b, c) are MHC class II-restricted escape mutations (Misiani et al. 1994), CD4⁺ T cell response from Th1 toward Th2 response (Casato et al. 2002), marked CD4⁺ CD25⁺ regulatory T cells (Rushbrook et al. 2005), regulatory CD8⁺ T cell, MHC class I-restricted antigen-specific regulatory activity with the potential to suppress antiviral T cells (Koziel et al. 1995), narrow CD8⁺ T cell receptor repertoire and impaired dendritic cell maturation in chronic hepatitis C patients (Auffermann-Gretzinger et al. 2001). Memory CD8⁺ T cells can vary in differentiation phenotype in different persistent virus infections (Appay et al. 2002). HCV persistence and immune evasion do occur in the absence of memory T cell help (Grakoui et al. 2003). A role of primary intra-hepatic T cell activation in the “liver tolerance effect” has been reported (Bertolino et al. 2002). Finally, the upregulation of inhibitory receptor programmed death-1 (PD-1) expression has been shown to lead to HCV-specific CD8 exhaustion (Tseng and Klimpel 2002). Thus, due likely to interplay between the opposing forces of immune selection pressure and viral fitness cost, a variety of outcomes are possible upon initial infection with HCV, or after subsequent transmission of the virus to a recipient in whom the initial MHC class I alleles are not expressed.

5.2 HCV Infection of B Cells Induces Ig Hypermutation, Altering B Cell Immunity

Besides chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, HCV infection is also frequently associated with B lymphocyte proliferative disorders, including mixed cryoglobulinemia, a disorder characterized by oligoclonal proliferation of B cells, and non-Hodgkin's B cell lymphoma (Silvestri et al. 1997). These B cell diseases may be the result of infection of B cells by HCV or the activation of B cells by HCV envelope proteins. The HCV envelope proteins E1 and E2 are type I transmembrane proteins, with N-terminal ectodomains and C-terminal hydrophobic anchors. It has been suggested that HCV infects human cells through the interaction of E2 with a tetraspanin molecule CD81. CD81 is thought to be a cellular receptor for HCV, based on its ability to bind E2 (Pileri et al. 1998; Hsu et al. 2003; McKeating et al. 2004; Zhang et al. 2004). CD81 is a member of the tetraspanin family and is a component of the multimeric B cell antigen receptor complex (Levy et al. 1998). It is associated with other membrane proteins, which vary in different B cell lineages and include the signaling molecule CD19, complement receptor 2 (CD21), and interferon-inducible Leu-13 (CD225) protein (Takahashi et al. 1990; Levy et al. 1998). Binding of CD81 with E2 or certain MAbs against CD81 induces B cell aggregation, inhibits Daudi cell proliferation (Flint et al. 1999), stimulates T cells (Soldaini et al. 2003), and inhibits natural killer cell functions (Crotta et al. 2002; Tseng and Klimpel 2002). In addition, triggering of the CD81 signaling pathway in B cells enhances the production of tumor necrosis factor- α (TNF- α) (Altomonte et al. 1996). Correspondingly, HCV infection of primary macrophages has been reported to induce TNF- α production (Radkowski et al. 2004). Coengagement of the CD19-CD21-CD81 complex and the B cell antigen receptor lowers the B cell activation threshold by antigen-presenting cells or lipopolysaccharide (Carter and Fearon 1992). Lymphocytes in mice lacking CD81 develop normally but have altered proliferative responses and are deficient in antibody production, suggesting that CD81 is one of the essential receptors for the production of antibodies (Miyazaki et al. 1997). These observations suggest that HCV may modify the B cell receptor-associated signaling pathway by binding to CD81 or by infecting B cells.

Evidence has been presented that at least certain strains of HCV can infect and replicate in B cells (Sung et al. 2003; Machida et al. 2004b, 2005). HCV infection of B cells triggers double-strand DNA breaks in many cellular genes (Machida et al. 2004a, b). Interestingly, the mere engagement of B cells by purified E2 alone, without viral replication, induces double-strand DNA breaks specifically in the variable region of the Ig gene locus, leading to hypermutation in Ig of B cells (Machida et al. 2005). Other gene loci are not affected by the E2-CD81 interaction. Pre-incubation with the anti-CD81 MAb blocks this effect. E2-CD81 interaction on B cells triggers the enhanced expression of activation-induced cytidine deaminase (AID) and also stimulates the production of TNF- α (Machida et al. 2005).

Knockdown of AID by the specific small interfering RNA (siRNA) blocked the E2-induced double-strand DNA breaks (DSBs) and the hypermutation of the Ig gene (Machida et al. 2005). Therefore, HCV infection, through the E2–CD81 interaction, may modulate host's innate or adaptive immune response by activation of AID and ensuing hypermutation of immunoglobulin gene in B cells. These effects may contribute to HCV persistence and B cell lymphoproliferative diseases.

In addition to the increased mutation frequency in the Ig gene, HCV infection also induces hypermutation of many other cellular genes, including p53 genes (Machida et al. 2004b). Subsequent studies showed that the HCV-induced mutations of somatic genes, such as *p53*, are mediated by nitric oxide (NO) and reactive oxygen species (ROS) (Machida et al. 2004a). In contrast, as stated above, the Ig mutations are mediated by AID activation through the binding of HCV E2 protein to CD81. AID is involved in both the somatic hypermutation and class-switching recombination of the Ig gene in normal B cell development; it causes deamination of deoxycytidine to deoxyuracil (dU) in the template DNA strand, with preference for certain hot-spot motifs (Petersen-Mahrt et al. 2002). The resulting dU/dG pairs can be resolved by the mismatch repair system (Papavasiliou and Schatz 2002), uracil glycosylase endonuclease (Di Noia and Neuberger 2002), and error-prone DNA polymerases (Radkowski et al. 2004). Among the latter, Pol ι , Pol η , and Pol ζ are involved in these pathways (Zan et al. 2001; Zeng et al. 2001; Faili et al. 2002). Interestingly, AID, Pol ζ , and Pol ι are induced in HCV-infected B cells (Machida et al. 2004b), at least partially caused by the binding of HCV E2 protein to CD81, which, in turn, triggers DSBs and subsequent Ig hypermutations (Fig. 11).

Furthermore, this interaction induces TNF- α production by B cells. These effects have been confirmed in the natural HCV infection of B cell in vitro and in vivo. These findings implicate that, even in the absence of virus replication, the very act of virus binding to B cells can contribute to the pathogenesis of HCV by Ig hypermutation and TNF- α production.

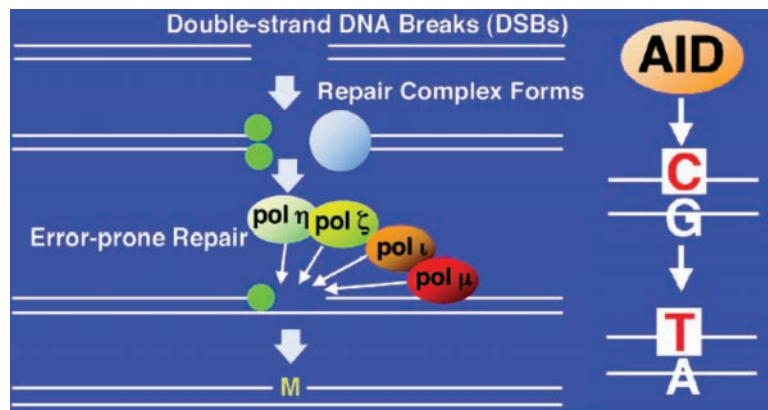


Fig. 11 Postulated mechanism of Ig hypermutation

The E2–CD81 interaction enhances mutation frequencies specifically in the Ig gene, but not in other genes, such as *p53*. The basis for such a specific effect is still not completely clear. E2–CD81 interaction may trigger a signaling response similar to that triggered by anti-CD40, interleukin (IL)-4, and other cytokines in B cells (Muramatsu et al. 1999; Chaudhuri et al. 2003). The normal somatic hypermutation mechanism of the Ig gene in B cells typically affects the genomic sequences within approx. 2 kb downstream from the transcription initiation site of the Ig gene (Rada and Milstein 2001), under the influence of the Ig gene enhancer (Goyenechea et al. 1997). The HCV E2-induced Ig hypermutation mirrors precisely this pattern, indicating that it is the result of hyper-activity of the normal Ig mutation mechanism. This specificity may explain the differential effects of E2–CD81 interactions on the Ig and *p53* genes. E2 likely will bind most cell types, since CD81 is expressed ubiquitously. However, although E2 can bind to hepatocyte cell lines expressing CD81, it does not induce enhancement of expression of AID or DSBs in this non-B cell line (Machida et al. 2005). These findings suggest that the other components of the CD81 complex, including CD21 and CD19, are important for the signal transduction involved in the induction of AID (Fig. 12). Several protein kinases have been shown to associate with CD19 and CD21 but not with CD81 (Fearon and Carter 1995).

TNF- α is one of the earliest host responses to viral infections (Guidotti and Chisari 2001); it is an inflammatory cytokine, which can contribute directly or indirectly to viral pathogenesis. On the other hand, it may purge viruses from infected cells noncytolytically and mediate intracellular signaling by adjusting the redox potential of the cell (Wong and Goeddel 1988; Kizaki et al. 1993). Thus, the E2–CD81 interaction may contribute to HCV pathogenesis through TNF- α production. The activation of AID and subsequent Ig mutations may also contribute to the development of B cell lymphoma.

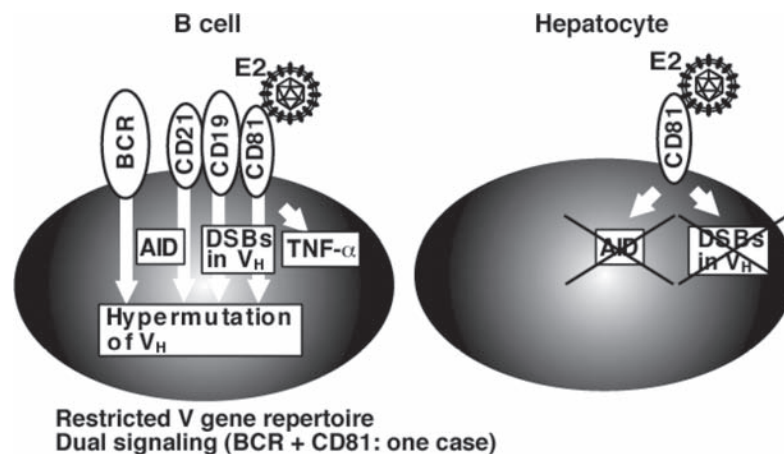


Fig. 12 A postulated signaling pathway for induction of hypermutation of the Ig gene in B cells or lack of induction in hepatocytes by E2 binding. *BCR*, B-cell receptor complex. A dual signaling model for B cell activation by HCV antigens has been reported (Weng and Levy 2003)

Another consequence of Ig hypermutation is that the specificity and avidity of antibodies produced from B cells will likely diverge following HCV infection. If the B cells producing HCV-specific antibodies are infected, it is predicted that the HCV-specific antibodies produced from these cells may lose their avidity and specificity within a short period of time. As a consequence, the neutralization activity or the antibody-mediated cell lysing activity may decline following HCV infection. This effect will enable the virus to escape from humoral immunity. This prediction has recently been proved correct by showing that HCV can infect human hybridoma cells producing E2-specific HMABs, causing the decline of the avidity and specificity of the HCV-specific antibodies (K. Machida, Y. Kondo, J. Hwang, Y.C. Chen, K.T. Cheng, V.M. Sung, Z. Keck, S. Fount, J. Dubuisson, and M.C. Lai, unpublished observation). This mechanism will contribute to immune escape of HCV. Similarly, at least some strains of HCV can infect certain T cells, affecting T cell functions, including IFN- γ signaling (Kondo et al. 2007). The suppression of T cell functions may contribute to T cell anergy and immune escape of HCV.

5.3 Antibody-Dependent Enhancement of Infection

Complement-mediated enhancement of antibody function for neutralization of pseudotype virus containing HCV E2 chimeric glycoprotein has been reported (Meyer et al. 2002). Significant increases in the neutralization titers of E2 HMABs and rabbit antiserum to HVR1 mimotopes have been observed upon addition of guinea pig complement. Complement activation occurred primarily by the classical pathway, since a deficiency in the C4 component led to a significant decrease in the level of Vn. During infection, HCV E2 glycoprotein induces a weak Vn antibody response; these antibodies can be measured in vitro by the surrogate pseudotype virus plaque reduction assay, and the neutralization function can be augmented by complement (Meyer et al. 2002). Therefore, it is possible that complement activation enhances infection, and lipoproteins and glycans can modify antibody binding to epitopes mediating Vn.

5.4 Potential Roles of Lipoproteins and Glycans to Modify Antibody Binding to Epitopes Mediating Virus Neutralization

Lipoproteins, high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL) could potentially reduce the neutralizing effect of the HCV-specific antibodies by promoting HCV entry (Voisset et al. 2006). In studies on HCVpp, HDL inhibits HCV Vn antibodies by stimulating cell entry via activation of the scavenger receptor BI (SR-B1). SR-B1 mediates lipid transfer and is proposed as a cell entry cofactor of HCV (Dreux et al. 2006). HDL interaction with

the SR-BI has been shown to strongly reduce Vn of HCVpp by Vn MAbs and HMABs (Dreux et al. 2006). For serum and liver HCV virions, it is probable that VLDL/LDL are involved in virus entry (Nielsen et al. 2006). The majority of serum and liver HCV RNA can be precipitated by antibodies to apolipoprotein B and apolipoprotein E. As to the site of lipoprotein linkage to virions, a study on intracellular infectious HCVcc and extracellular infectious HCVcc suggests that acquisition of lipoproteins might occur during viral secretion from infected cells (Gastaminza et al. 2006). While it is possible that some Vn E2 epitopes are masked by lipoproteins as suggested by HCVpp studies, some HCVcc E2 epitopes remain exposed as shown by Vn HMABs to domain B (Keck et al. 2007). In addition, some E2 HMABs will immunoprecipitate serum and liver virions (Kumar et al. 1994). Antibodies targeted to E1 protein of HCV efficiently neutralize HCVpp and HCVcc in the presence of human serum. Functional features of HCV glycoproteins for pseudotype virus entry into mammalian cells have been reported (Meyer et al. 2000; Beyene et al. 2002). LDL receptor-related molecules partially inhibit E1 pseudotype virus infectivity, while CD81-related molecules interfere with E2 pseudotype virus infectivity. A further understanding of HCV entry and strategies appropriate for mimicking cell surface molecules may help in the development of new therapeutic modalities against HCV infection.

6 Final Remarks

Vn monoclonal antibodies will likely succeed in the prevention of HCV reinfection after liver transplantation. However, it should be noted that a small trial was recently performed where liver transplant recipients with HCV-associated liver failure received multiple infusions of high-dose immunoglobulin of 200 mg/kg per dose over 14 weeks, but no viral load decrease was detected (Davis et al. 2005). However, this study should be considered with caution. In addition to the concern that the dosage used was based on the protective amount previously observed in chimpanzees (Krawczynski et al. 1996), there are a number of other issues. First, specific HCV antibodies are only a small fraction of the total Ig and of these only a smaller fraction will be directed to the important targets involved in entry, i.e., the E1E2 envelope glycoproteins. Second, not all antibodies to E1 or E2 mediate Vn (Keck et al. 2004a). Both Vn and non-Vn HMABs to HCV E2 have been described. The existence of non-Vn HMABs to HCV E2 has similarly been observed in sera of patients with chronic HCV infection (Burioni et al. 2004). As chronically infected patients were probably the primary source of IgG used in this trial, it is probable that large portions of these antibodies were non-Vn. Finally, the viral load in the patients enlisted for this clinical trial was many logs higher than in the challenged animals, and the supposedly “high” dose thus may still be inadequate. Supporting this possibility is the observation that liver tissue viral load in biopsies tended to be lower in high-dose Ig recipients compared to low-dose recipients (Davis et al. 2005).

The studies summarized in this review support the feasibility of isolating Vn antibodies to broadly conserved epitopes among different HCV genotypes. While candidate Vn antibodies to highly conserved conformational and linear epitopes have been identified, a greater understanding of the factors contributing to virus escape will be required to help define those protective determinants most likely to give broad protection. The emergence of the escape viral mutants may be slowed by the combination of Vn antibodies and antivirals. In addition, the antibodies used should be broadly reactive to different HCV genotypes, each inhibiting at different steps of virus entry, and be synergistic in their ability to control virus infection. At least two antibodies to different epitopes are needed to minimize the concern of escape mutants. Additional studies will also be required on the possible role of lipoproteins in masking virion surface domains involved in virus entry, although current data suggest that a specific cluster of epitopes (designated domain B) on HCV E2 remains exposed on low-density cell culture infectious HCV virions. In summary, the Vn antibodies clearly merit consideration as a therapeutic and preventative strategy against HCV reinfection in the liver transplantation setting.

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