

Variability or conservation of hepatitis C virus hypervariable region 1? Implications for immune responses

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The hypervariable region 1 (HVR1) of the E2 protein of hepatitis C virus (HCV) is highly heterogeneous in its primary sequence and is responsible for significant inter- and intra-individual variation of the infecting virus, which may represent an important pathogenetic mechanism leading to immune escape and persistent infection. A binding site for neutralizing antibodies (Ab) has also been allegedly identified in this region. Prospective studies of serological responses to synthetic oligopeptides derived from naturally-occurring HVR1 sequences showed promiscuous recognition of HVR1 variants in most patients via binding to C-terminal amino acid residues with conserved physicochemical properties. Monoclonal antibodies generated by immunization of mice with peptides derived from natural HVR1 sequences were shown to recognize several HVR1 variants in line with evidence gathered from studies using human sera. In addition, selected mAbs were able to bind HVR1 in the context of a complete soluble form of the E2 glycoprotein, indicating recognition of correctly folded sequences, and were shown to specifically capture circulating and recombinant HCV particles, suggesting that HVR1 is expressed on intact virus particles and therefore potentially able to interact with cellular receptor(s). These findings suggest that it is possible to induce a broadly reactive clonal immune response to multiple HCV variants and that this mechanism could be used in principle to induce protective immunity for a large repertoire of HCV variants.

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1. Introduction

Infection with hepatitis C virus (HCV) is a leading cause of chronic liver disease and it is now known to infect approximately 3% of the world's population. The HCV genome is translated as a single polyprotein which undergoes processing by host and viral proteases to produce structural and nonstructural proteins, respectively (Lindbach and Rice 2001). The virus displays a high mutation rate, and at least 6 major genotypes have been recognized

based on nucleotide sequencing of conserved and non conserved regions (Simmonds 1999). The HCV-specific immune response is generally unable to clear the virus and, therefore, spontaneous resolution is extremely rare, while over 70% of acute infections eventually become persistent. Most infections remain asymptomatic for several years resulting in late recognition of the disease (Everhart *et al* 1997). The high proportion of chronic HCV infections may be due to active escape mechanisms which eludes the host immune response during the acute infection

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Abbreviations used: Ab, Antibodies; HCV, hepatitis C virus; HVR1, hypervariable region 1; mAb, monoclonal antibodies; SRBI, scavenger receptor class B type I.

through early selection of phylogenetically divergent mutants (Farci *et al* 2000) most likely generated by an inappropriate selective pressure exerted by the host immune response, or to the inability of HCV to induce an efficient protective immunity. Interestingly, a dramatic reduction in genetic diversity leading to an increasingly homogeneous viral population has been recently shown to be a consistent feature associated with viral clearance in sustained responders to interferon treatment, independently of viral genotype (Farci *et al* 2002). While most HCV infections do elicit cellular and humoral immune responses (Missale *et al* 2001), there is little conclusive evidence in support of the existence of protective immunity. A vaccine containing proteins of the viral envelope will soon be evaluated in phase I clinical trials.

2. What role for hypervariable region 1?

In contrast with hepatitis B virus infection in which envelope-specific neutralizing antibodies (Ab) closely correlate with clinical recovery, patients chronically infected with HCV invariably have envelope specific Ab detectable in their serum, indicating an ongoing B cell response

(Cerino *et al* 1997). The significance and utility of such circulating Ab are currently uncertain, since re-challenge of experimentally infected chimpanzees with high levels of circulating anti-HCV immunoglobulin still results in the reappearance of viremia (Farci *et al* 1992). Our current understanding of the possible functions of B-cell responses in HCV infection is depicted in figure 1.

Passive immunization studies and *in vitro* neutralization of HCV isolates with hyperimmune serum-specific for the hypervariable region 1 (HVR1) would suggest a protective role for anti-HVR1 Ab (Farci *et al* 1994, 1996). It is known, however, that such Ab appear to co-exist with the HVR1 variants they recognize and are frequently cross-reactive with unrelated HVR1 sequences isolated from different patients (Lesniewsky *et al* 1993; da Silva-Cardoso *et al* 1995; Scarselli *et al* 1995; Zibert *et al* 1995, 1997; Cerino *et al* 1997; Jackson *et al* 1997; Yoshioka *et al* 1997; Hattori *et al* 1998; Mondelli *et al* 1999; Shang *et al* 1999). Thus, the significance of HVR1-specific humoral immune responses, and their relationship to HVR1 sequence variation, are still incompletely defined.

Available evidence would suggest that HVR1 variation has an adaptive significance and results from a conti-

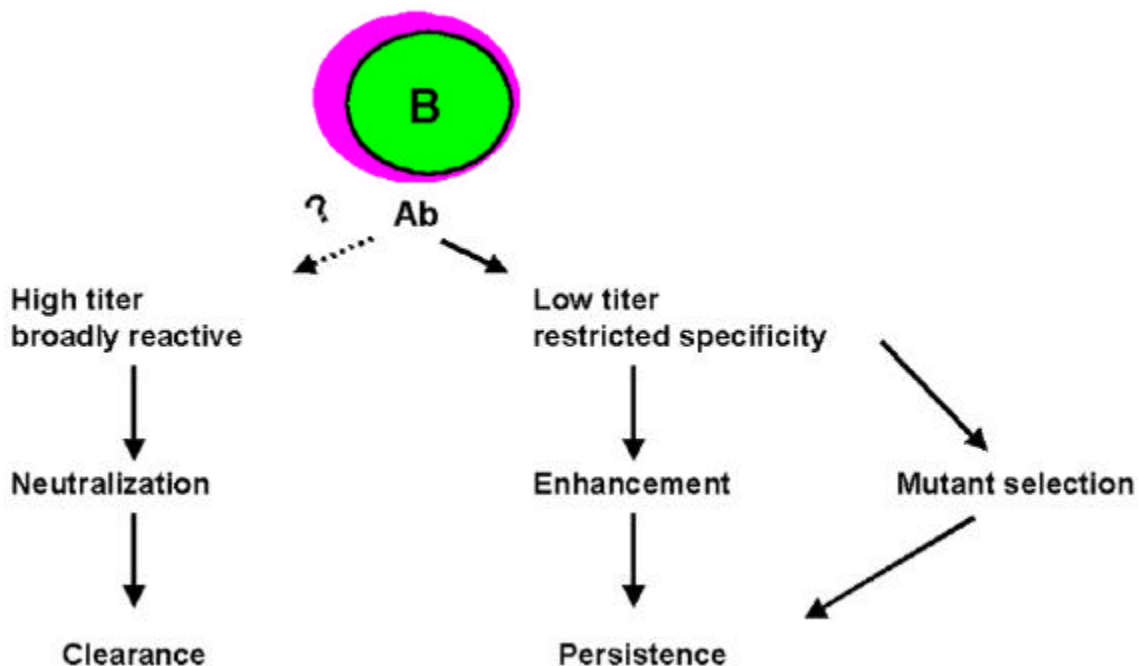


Figure 1. Proposed functions of B-cell responses in HCV infection. Production of highly efficient neutralizing antibodies to HCV is currently uncertain, although it is thought to occur only in certain circumstances. Such antibodies should be produced at high concentration and possibly have broad specificity, though this is currently unknown. However, in most circumstances neutralizing antibodies would be produced at low concentrations and with restricted specificity; this would on the one hand favour selection of viral mutants and on the other hand it could generate an enhancement mechanism which would increase the spread of the infection to other susceptible cells. These phenomena may have a deleterious effect on the evolution of HCV infection.

nuous selection process which is likely controlled by humoral immune responses, as suggested by a minimal or absent sequence mutation rate in subjects with congenital immunoglobulin defects (Kumar *et al* 1994; Booth *et al* 1998). Moreover, appearance of circulating anti-HVR1 Ab in chimpanzees inoculated with an identical HCV strain was associated with HVR1 sequence variation, whereas no sequence mutations were observed in the absence of detectable HVR1-specific humoral immune responses (van Doorn *et al* 1995). Evidence in support of Ab-driven HVR1-variant selection has also been provided by others. For instance, Ray *et al* (1999) showed that persistent viremia was associated with higher inter-sample antonymous vs synonymous substitutions (Ka/Ks), suggesting that HVR1 can function as an immunological decoy, stimulating a strong, immune response which would be responsible for variant selection, but would be ineffective to clear HCV. Recent additional data obtained from a prospective study of viral evolution during perinatal infection argues in favour of a dominant role of positive selection for amino acid changes in driving the pattern of HCV genetic diversification (Manzin *et al* 2000).

In contrast with these studies, others failed to find evidence of strong selective pressure driving the emergence of viral variants. Immune responses to HVR1 were generally weak and not correlated with nucleotide or amino acid substitutions (Bassett *et al* 1999; Major *et al* 1999). However, it is important to emphasize that those data were generated in chimpanzees, the only available model of HCV infection, although largely inadequate for pathogenetic studies on HCV infection, because of distinct disease features which differentiate human from primate infection. The role of the host immune response in selecting HVR1 variants has also been recently questioned by Allain *et al* (2000) who failed to detect a correlation between the evolutionary rate or the heterogeneity of the viral quasispecies in the patients studied and the strength of immune responses to HVR1 epitopes, suggesting that genetic drift is independent on the host immune pressure. However, in view of the overwhelming evidence in support of an active selection process, mechanisms of HCV variation, such as random genetic drift, are unlikely to be operative in this setting.

In a previous study we prospectively followed serological responses to synthetic oligopeptides derived from HVR1 sequences of patients with acute and chronic HCV infection obtained at baseline and after a defined follow-up period (Mondelli *et al* 1999). Extensive serological cross-reactivity for unrelated HVR1 peptides was observed in the majority of the patients. Ab responses were restricted to the IgG1 subclass and were focused on the carboxy-terminal end of the HVR1 region. Cross-reactive Ab could also be readily elicited following immunization of mice with multiple antigenic peptides carrying HVR1

sequences derived from our patients. Consistent with the inter-isolate Ab cross-reactivity is the recently reported HLA class II-restricted CD4+ T-cell response to conserved motifs located at the C terminus of the HVR1 sequence (Shirai *et al* 1999). Indeed, the predominant T-cell response to the HVR1 region was directed against the C terminus, similarly to findings obtained by others who also showed stronger HVR1-specific CD4 T-cell responses in patients who cleared the infection after successful antiviral therapy (Del Porto *et al* 2000).

3. HVR1 is conformationally conserved and is expressed on HCV particles

To investigate further the molecular basis for Ab cross-reactivity for unrelated HVR1 sequences, we generated a panel of murine monoclonal antibodies (mAb) from mice immunized with HVR1 surrogate peptides (mimotopes), affinity-selected with sera from HCV-infected patients from a phage-display library (Puntoriero *et al* 1998). A significant number of antigen-specific hybridomas was obtained after immunization with a pool of nine mimotopes. The mAbs were shown to recognize a number of 16-mer and 27-mer peptides derived from natural HVR1 sequences isolated from patients with acute and chronic HCV infection, and a major binding site was mapped at amino acid position 390–405, akin to our previous findings using human sera (Cerino *et al* 2001). HVR1 mimotope-specific mAb were also able to efficiently compete with sera from HCV-infected patients for binding to peptides derived from natural HVR1 sequences, thus confirming previous data obtained with polyclonal Ab, showing that HVR1 peptide mimotopes are efficient antigenic and immunogenic mimics of naturally occurring HCV variants.

A plausible explanation accounting for the promiscuous binding characteristics of HVR1-specific antibodies comes from a recent theoretical study on the structural conformation of HVR1 which showed either a broad amino acid repertoire at each position despite a remarkable residue conservation in specific sites or replacements with amino acids with similar physicochemical properties, usually positively charged basic residues, in the most variable segments (Penin *et al* 2001). The very similar hydrophathy and antigenicity profiles of HVR1 variants revealed a substantial conformational conservation, thus providing a plausible explanation for the extensive cross-reactivity demonstrated by Ab and confirming the existence of an active selection process. Interestingly, two sites with a high antigenicity score were identified at positions 1–13 and 19–24, a pattern which is predicted also with phylogenetically distant variants (Penin *et al* 2001). The latter

site contains the immunodominant B-cell epitope(s) described previously (Scarselli *et al* 1995; Mondelli *et al* 1999) and is compatible with B-cell epitope mapping data (Zibert *et al* 1995, 1997; Mondelli *et al* 1999).

The possibility to generate broadly reactive antibodies may represent a useful approach to overcome the natural diversity of a virus such as HCV, suggesting that mimotope-based vaccines can be used as potentially effective HCV immunogens. This assumption is based on evidence indicating that antibodies to HVR1 can prevent HCV infection in the chimpanzee model by both *in vitro* and *in vivo* neutralization of a pedigreed virus inoculum of HCV strain H (Farci *et al* 1994, 1996). However, exposure of HVR1 on complete viral particles has not been formally proven and, in principle, the role of this sequence in binding neutralizing antibodies is far from being established. A recent preliminary study suggested that mAbs obtained by immunization with peptides derived from natural HVR1 isolates were able to capture *bona fide* viral particles only from homologous HCV isolates and could also prevent infection of an allegedly susceptible cell line *in vitro* (Zhou *et al* 2000). These findings are in partial agreement with our data in that one of our mAbs

was also able to capture *bona fide* and recombinant viral particles, although there was no apparent genotype or isolate-specific recognition (Cerino *et al* 2001). Similar findings were reported by Li *et al* (2001), who were able to show that mAbs generated by immunization with HVR1 peptides, specific for conserved motifs, could capture HCV RNA from plasmas of patients and were also able to block HCV binding to Molt-4 cells which, in previous experiments (Hamaia *et al* 2001), were shown to efficiently bind HCV. These observations fit with the idea of a significant structural conservation of HVR1, as discussed above.

It may be argued that mAbs raised against HVR1 peptides are unable to recognize the same sequence when expressed in the context of a correctly-folded complete E2 glycoprotein which included HVR1. However, our mAbs were shown to recognize correctly-folded E2 polypeptides expressing the same HVR1 sequences synthesized as linear peptide, providing additional evidence supporting the existence of immunodominant, conformation-independent epitope(s) in the C-terminal sub-region which, under certain circumstances, may be exposed on integral viral particles.

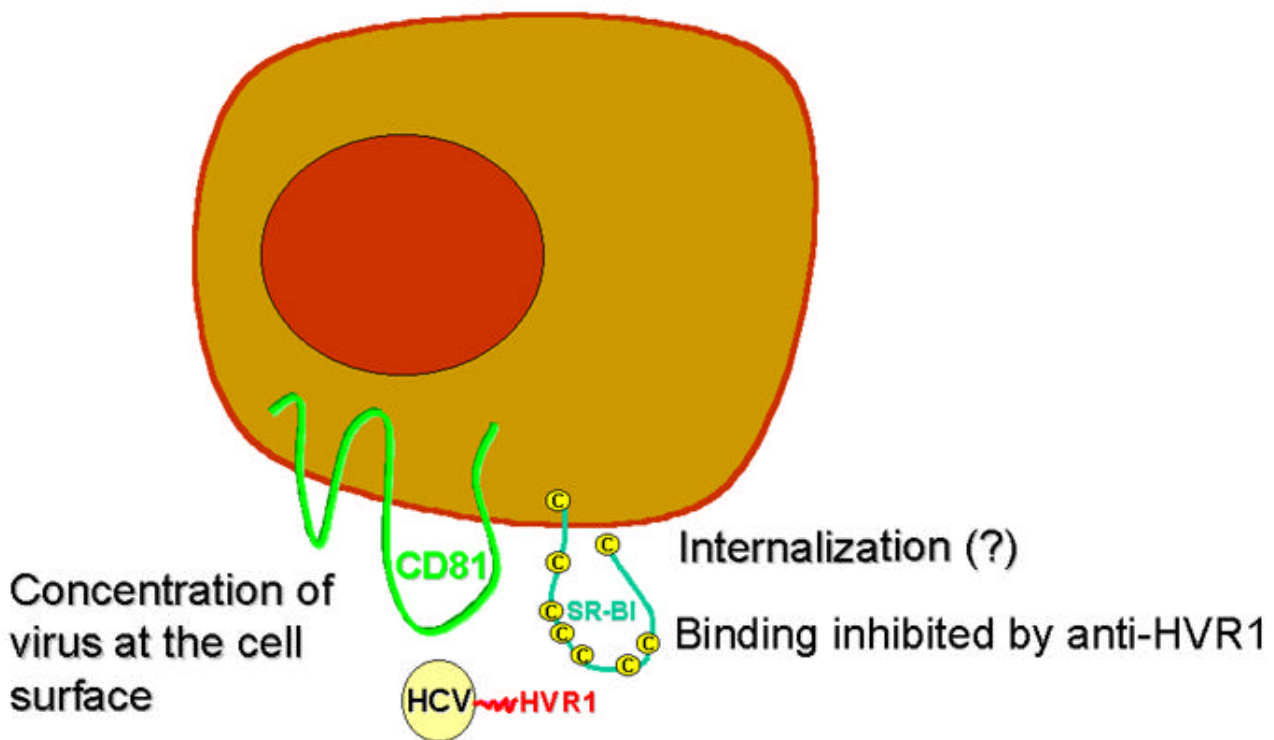


Figure 2. Possible mechanisms of HCV-receptor(s) interaction on the hepatocyte. CD81, a tetraspanin expressed on hepatocytes and B cells (among many other cells), binds HCV via HVR1-independent ligand(s) and concentrates viral particles on the cell surface. However, the recently identified HCV co-receptor, the scavenger receptor class B type I (SRBI), would bind to viral particles which would then be internalized. The role of lipoproteins, which normally coat circulating viral particles, in the viral uptake process is still undefined, although the LDL receptor has been proposed as an additional candidate co-receptor molecule for HCV (Agnello *et al* 1999).

4. Conclusions and outlook

The role, if any, played by HVR1 in host-virus interactions at the protein level is still unknown. Current evidence suggests that mammalian cell-derived E2 glycoproteins (Petracca *et al* 2000) and, to a much lesser extent, HCV-LP 1a (M Triyatni and T J Liang, unpublished observations) can bind human CD81, a candidate receptor molecule for HCV. Yet HCV-LP can penetrate into HepG2 cells via alternative receptor(s) which are constitutively expressed on hepatocytes (M Triyatni and T J Liang, unpublished observations), supporting the hypothesis that HCV requires a second receptor molecule for internalization (Petracca *et al* 2000) (figure 2). Preliminary evidence suggests that HVR1 is not involved in binding to CD81 (Flint *et al* 1999; Patel *et al* 2000). Recently, a novel HCV candidate receptor has been identified on human hepatocellular carcinoma cell lines. Such a putative receptor, the scavenger receptor class B type I (SRBI), binds E2 via HVR1 and is species-specific (Scarselli *et al* 2002). Thus, high-affinity anti-HVR1 Ab elicited by immunization could modulate HCV infection by inhibiting binding of viral particles to SRBI. This approach may have important implications for immunotherapy or prophylaxis of HCV infection.

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