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Capitalizing on knowledge of hepatitis C virus neutralizing epitopes for rational vaccine design

Leopold Kong¹, Kelli N. Jackson², Ian A. Wilson^{1,3}, and Mansun Law^{2,*}

¹ Department of Integrative Structural and Computational Biology, The Scripps Research Institute, La Jolla, California 92037, USA

² Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California 92037, USA.

³ Skaggs Institute for Chemical Biology The Scripps Research Institute, La Jolla, California 92037, USA.

Abstract

Hepatitis C virus infects nearly 3% of the world population and is often referred as a silent epidemic. It is a leading cause of liver cirrhosis and hepatocellular carcinoma in endemic countries. Although antiviral drugs are now available, they are not readily accessible to marginalized social groups and developing nations that are disproportionately impacted by HCV. To stop the HCV pandemic, a vaccine is needed. Recent advances in HCV research have provided new opportunities for studying HCV neutralizing antibodies and their subsequent use for rational vaccine design. It is now recognized that neutralizing antibodies to conserved antigenic sites of the virus can cross-neutralize diverse HCV genotypes and protect against infection *in vivo*. Structural characterization of the neutralizing epitopes has provided valuable information for design of candidate immunogens.

Introduction

Hepatitis C virus (HCV) was identified to be the causative agent of non-A non-B hepatitis in 1989 [1]. It is estimated that HCV infects nearly 3% of the world population [2-5]. In the US, the national rate of symptomatic HCV infection declined and began to level around 2005 [6]. However, HCV had overtaken HIV as a cause of death between 1999-2007 [7]. Perhaps the most alarming trend is the increasing number of cases in the 15-24 year age group [8,9]. HCV infection is considered a silent killer because it can be transmitted to new hosts unnoticed and can take up to 20-40 years before severe clinical symptoms develop. Potent direct acting antivirals (DAAs) against different viral targets have recently been developed [10-13]. However, access to therapy has historically been low, partly due to poor HCV awareness, diagnosis and high drug cost [14-16]. To solve the global HCV problem,

* Corresponding author: Mansun Law, mlaw@scripps.edu, Tel: +1-(858)784-7033..

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more effective and affordable drugs against HCV, as well as an effective vaccine, are needed.

The extreme genetic diversity of circulating HCV is a major roadblock to an HCV vaccine [17] (Figure 1). To overcome this challenge, a broadly effective vaccine must target conserved B and T cell epitopes. Ongoing research on a T cell-based vaccine showed that prime-boost immunization with heterologous viral vectors presenting non-structural antigens of HCV could elicit high levels of functional T cells in healthy volunteers. The prophylactic efficacy of this vaccine candidate is currently under evaluation in a Staged Phase I/II Clinical Trial ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01436357) Identifier NCT01436357) [18,19]. A subunit vaccine candidate based on the viral envelope glycoprotein complex E1E2 had been investigated in a Phase I Trial ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00500747) Identifier NCT00500747) [20-23]. The majority of the subjects did not produce neutralizing antibodies (nAbs) despite the antigens appearing to perform reasonably well in mouse, guinea pig and chimpanzee models [24,25].

Studying HCV neutralizing antibody responses

Previously, HCV nAbs were difficult to study because of the lack of a laboratory assay to measure antibody neutralization of virus and a small animal model for studying viral infection. Since nAbs to HCV target the viral envelope glycoproteins E1 and E2, anti-viral antibody activity was determined by a “neutralizing of binding (NOB)” assay to measure the ability of an antibody sample to inhibit E2 binding to human cell lines bearing the viral receptor CD81 [26]. This assay was later found not to fully represent the biological activity of nAbs. It is noted that some antibodies can block E2 binding to CD81 but do not neutralize (e.g. mAb AR1A) [27]. The observation implies the presence of antigenic sites accessible on isolated E1E2 but not on the virus. In the last 10-15 years, the development of the HCV pseudotyped virus (HCVpp) [28-30] and cell culture virus (HCVcc) [31-34] systems, and humanized mouse models susceptible to HCV infection [35-40], have greatly enhanced the study of anti-HCV antibodies. Early works of Purcell *et al.* [41-43] and Houghton *et al.* [25,44] established the importance of nAbs in HCV protection. Recently, however, using these viral systems, multiple research groups reported the positive correlation of early induction of nAbs and viral control/clearance in humans [45-47]. The viral systems also help explain the poor results of an early clinical trial of the nAb HCV-Ab^{XTL}-68 for prevention of recurrent HCV in liver transplant patients [48,49]. The nAb was later found to be only moderately neutralizing in the HCVpp assay (~50% virus neutralization at 20 µg/ml nAb) despite showing promising results in multiple surrogate assays [50,51]. Of note, these *in vitro* viral systems do not fully recapitulate the envelope composition of infectious virions from infected humans. HCV is known to be associated with apolipoproteins, particularly ApoE, and this association is speculated to play a role in masking E1E2 neutralizing epitopes [52-57]. Further studies of the subtle differences between the virus particles produced *in vitro* and the exact composition of viral and host proteins on native HCV virions will be crucial for the field [55,58]. Nevertheless, these assays have accelerated identification of broadly nAbs (bnAbs) that cross-neutralize diverse HCV genotypes [27,59-64], protect animal models from HCV challenge in passive transfer experiments [27,65-67], and even delay viral rebound following liver transplantation in HCV patients [68].

HCV neutralizing epitopes for immunogen design

E2 antigenicity—HCV nAbs and viral escape mechanisms have been reviewed recently [69-74]. Here we aim to discuss information that is complementary to these reviews and to introduce concepts that are relevant to rational vaccine design. A collection of monoclonal antibodies (mAbs) to E1 and E2 used by many labs in the field have also been summarized elsewhere [74-76]. Regarding the antigenicity of the HCV enveloped glycoproteins, Keck *et al.* first proposed three immunogenic domains in E2 similar to the antigenic structural and functional domains of other flavivirus envelope E glycoproteins [77-80]. E2 was designated as having Domains A, B and C based on binding of non-competing mAbs isolated from humans, and these domains were later expanded to include antigenic Domains D and E [63,64,81]. Examples of mAbs to the different Domains are CBH4B, CBH5, CBH7, HC84-1 and HC33.1, respectively. The epitopes of some of the mAbs have been mapped by site-directed mutagenesis, selection of escape mutants, mass spectrometry, and protein crystallography [70,81-86]. However, the recently determined structures of E2 from two different HCV genotypes do not support the analogy between *hepacivirus* E2 and *flavivirus* E proteins [87,88].

Our lab has isolated a panel of human mAbs to discontinuous epitopes on E1E2 by phage-display [27,65]. Based on cross-competition between the mAbs binding to E1E2, the epitopes recognized by the mAbs were grouped into 5 clusters, or antigenic regions (ARs) (Figure 2A). Antigenic regions 1, 2 and 3 (AR1-3) are present on E2, and AR4 and AR5 on the E1E2 complex. AR1 is proximal to the CD81 binding site on E2 (E2 CD81bs). However, AR1 is not conserved and is probably not exposed on the viral surface. The mAbs to AR1 only bind genotype 1 HCV and do not have significant neutralizing activity. Interestingly, mAb AR1A competes strongly with mAb AR1B in E2 binding, but only mAb AR1A blocks E2-CD81 interactions [27]. AR2 is distal from the E2 CD81bs and is exposed on E2 because mAb AR2A can neutralize several HCV isolates. AR3 is conserved and overlaps with E2 CD81bs. Multiple mAbs to AR3 can cross-neutralize diverse HCV genotypes. AR4 and AR5 are present only on the E1E2 complex and are adjacent to each other. Mapping data suggest that they may be binding near the stalk region of E2 and interacting with the N-terminal region of E1 [65]. In passive transfer experiments using humanized mouse models, mAbs to AR3 and AR4 offered significant levels of protection against challenge by multiple HCV genotypes [27,65,67].

This antibody panel guided us to systematically truncate the N and C termini, substitute the flexible variable region 2 with a short linker, and remove N448 and N576 glycosylation sites, of the wildtype E2 of the prototypic genotype 1a H77 to generate the E2 core domain (E2c) [87]. E2c in complex with bnAb AR3C was able to produce diffraction-quality protein crystals for determination of the first E2 structure. The structure reveals a novel protein fold of E2c consisting of a central β -sandwich flanked by an N-terminal front layer and a C-terminal back layer. The front layer consists of mostly strands and a short helix that packs against both the central beta-sandwich and the back layer. The back layer contains antiparallel β -sheets and short helices. Importantly, the structure and the antibody panel roughly define four faces of E2c: glycan, occluded, non-neutralizing and neutralizing (Figure 2B). The accessible neutralizing face overlaps with E2 CD81bs and identifies a

relatively large and conserved antigenic surface for immunogen design. The novel fold of E2 was later confirmed by the Marcotrigiano lab using an E2 derived from genotype 2a HCV with different truncations [88].

In addition to the above attempts to describe the antigenicity of HCV envelope glycoproteins, Zhang *et al.* proposed the presence of two “epitopes” at E2 residues 412-419 (“epitope I”) and 434-446 (“epitope II”) that played important roles in regulating antibody neutralization of HCV [89,90]. Strictly speaking, these two E2 regions could be more accurately described as “antigenic sites” instead of “epitopes” since no mAb was generated in their studies to define the epitopes. Using peptides corresponding to these two antigenic sites, the Zhang group isolated or depleted antibody fractions specific to the two antigenic sites from human and chimp antisera. They observed that antibodies to E2 antigenic site 412-419 (named AS412 here) could neutralize HCV but the neutralizing effect was negated by antibodies to E2 antigenic site 434-446 (named AS434 here) [89,90]. The authors suggested the presence of “interfering antibodies” to HCV nAbs and, therefore, for vaccine design, that it was important to avoid eliciting antibodies to AS434. Using mAbs to different E2 antigenic sites, Sautto *et al.* observed mild “interference” of mAb AP33 (to AS412) by mAb e509 (to AS434) [91]. However, the significant levels of “interference” reported by Zhang *et al.* have not been reproduced to date by two other leading labs in HCV antibody research [63,64,92].

AS412—Notably, these two E2 antigenic sites (AS412 and AS434) are now considered prime targets for vaccine design [62,63,87,93-97] (Figure 2B). AS412 is highly conserved, contains residues critical for CD81 receptor binding, and is occasionally targeted by antibodies isolated from human and animal sera [21,62,93,98,99]. Crystal structures of the AS412 peptide bound to bnAbs HCV1, AP33, hu5B3.v3 and MRCT10.v362 all indicate that epitope I folds as a short β -hairpin loop [94-97]. These antibodies recognize the same, hydrophobic face of the hairpin, burying its bulky residues in the antibody heavy and light chain combining sites. Some variation in the angle of approach by the antibodies to the peptide is observed, indicating this epitope is well exposed. Surprisingly, a glycosylation site at Asn417 is situated on the opposite face of the peptide that is recognized by the antibodies, suggesting that this hairpin loop is not packed against the protein but extends into solvent with some level of flexibility. This may explain why AS412 is disordered in the crystal structure of E2 bound to AR3C. Escape mutations to bnAbs targeting AS412 have been noted by different laboratories [66,68,97,100]. One interesting feature in AS412 is the presence of an unusual sequon of a N-linked glycosylation signal (underscored) at Asn417 (-Asn415-Thr416-Asn417-Gly418-Ser419-) [97]. A point mutation to Ser417 or Thr417 can shift the N-linked glycan to Asn415 (-Asn415-Thr416-Ser/Thr417-Gly418-Ser419-). Asn415 is situated in the antibody bound face and the shift of this N-linked glycan effectively masks the epitope from recognition by the above AS412-specific bnAbs. Interestingly, recent structural studies of the rat mAb 3/11 and human mAb HC33.1 to AS412 revealed two different, extended conformations [101,102]. Rat mAb 3/11 was previously reported to be moderately neutralizing [103]. In the new study, it was found to be as potent as mAb AP33 although it is still susceptible to the glycan-shift escape mutation [101]. In contrast, the human mAb HC33.1 disrupts the β -hairpin loop using a long heavy

chain complementarity-determining region 3 (HCDR3) and that the side chain of Asn415 is solvent exposed [102]. As a result, bnAb HC33.1 can tolerate the Asn417 to Asn415 glycan-shift mutation. Nevertheless, escape mutations within and outside AS412 in response to bnAb HC33.1 selection have also been identified [81]. Therefore, though AS412 is a promising target because of its well-defined conformations, it will be necessary to target multiple epitopes to minimize virus escape in vaccine design. Further study is also needed to assess the flexibility of AS412 and whether immunogen design should consider the different conformations.

AS434—AS434 consists of a short helix that is packed against the back layer of E2c [86,87] (see below). Crystal structures have revealed that bnAbs AR3C, HC84-1 and HC84-27 recognize a common face of the helix. Although HC84-1 and HC84-27 were crystallized with short peptides whereas AR3C was crystallized with the E2 protein, the epitope adopts a nearly equivalent helical conformation in these structures. Differences are apparent in the strands extending out from the helix in the peptide structures compared to the E2c structure. However, the strand regions of the free peptides may not represent the native conformation since they are not constrained by the rest of the E2 protein or important points of contact with the antibodies. *In vitro*, it is suggested that HCV cannot easily generate escape mutants to bnAbs to AS434 [63]. Weakly neutralizing mAb #8 has also been isolated that recognizes the AS434 helix from another angle of approach that would result in severe clashes with the rest of E2c [104]. Deng *et al.* suggested that AS434 may shift between open and closed conformations that facilitate immune evasion, although this seems unlikely due to the tight backing between the front layer and the rest of the E2 protein. However, if conformational flexibility does play a role in dampening an effective antibody response, then protein-engineering efforts to stabilize this region may be critical for vaccine development.

E1 antigenicity—E1 is known to be less immunogenic than E2 during infection [105,106] and few mAbs have been reported to E1 [61,75,76,107,108]. Neutralizing activities had been identified for mAbs H-111 recognizing E1 N-terminal antigenic site 192-202 (AS192) [108], and IGH506 and IGH526 recognizing E1 antigenic site 313-327 (AS313) [61]. It has been extremely difficult to study E1 because of the inability to produce stable, folded recombinant E1. Previous studies showed that the folding of E1 and E2 is inter-dependent [109-111] and the non-covalent heterodimer is stabilized by their transmembrane domains [112-114] and ectodomains [65]. Folded E1 can only be produced as an E1E2 complex [114,115]. In contrast, soluble E2 monomers have been produced independent of E1 by us and others [114-121]. Such biochemical properties suggest to us that E2 may serve as a chaperone protein for E1 folding and probably also as an accessory protein for E1 functions. We speculate that, in viral entry, E2 serves as the receptor binding protein while E1 may serve as the fusion protein to facilitate membrane fusion. However, the biochemical data reported on E1 as a fusion protein have not been conclusive [122-131]. Recently, the Stuart group determined the crystal structure of the N-terminal domain of E1 (residues 192-270) revealing a novel fold that is incompatible with class II fusion proteins [132,133]. Surprisingly, the E1 fragments formed oligomers linked by disulfide bonds. It is unclear if unpaired cysteines may play a role in HCV entry [134,135]. In this E1 structure, AS192

targeted by mAb H-111 forms part of a β -hairpin involving in the dimerization of the E1 fragments [133].

Conclusion

A major advance in drug discovery in recent decades has been the application of X-ray crystallography to elucidate the structures of target proteins to guide the design of small molecules that bind tightly with high specificity. Presently, there is much effort to translate this approach to vaccine design, particularly against highly variable viral pathogens such as HIV and influenza that have their surface antigens cloaked by N-linked glycans and variable loops. This effort has been driven by the isolation of exceptionally potent bnAbs that interact with “sites of vulnerability” that are conserved across viral strains, are not immunodominant in the context of the wild-type, full-length antigen. The recent discovery of HCV bnAbs and their corresponding epitope structures can be used to guide protein engineering to optimize the presentation of the epitopes within their correct conformational contexts as vaccine immunogens. Since bnAbs to HCV can be elicited in mice, HCV appears to be an excellent model to evaluate a structure-based vaccine approach and other new concepts in B cell epitope vaccine design [136-140].

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Highlights

- *In vitro*: HCV pseudotyped virus (HCVpp) and cell culture HCV (HCVcc) were developed in 2003 and 2005 for the study of neutralizing antibodies to HCV.
- *In vivo*: The chimpanzee model supports HCV replication but is largely unavailable for research. Several humanized mouse models are being developed as a possible replacement.
- Several structures of linear peptides spanning neutralizing epitopes have been determined.
- An accessible and conserved face for antibody neutralization was identified from the crystal structure of the core domain of the E2 envelope glycoprotein.

sites are underlined. Consequently, HIV-1 can change its glycosylation pattern in many more ways than HCV to escape antibody neutralization.

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FIGURE 2. Antigenicity of HCV Env

(A) Antigenic regions of E1E2 complex. The viral envelope glycoproteins E1 and E2 form non-covalent heterodimers. A panel of human mAbs isolated from an HCV-immune antibody library by phage display defines 5 distinct antigen regions (ARs) on the E1E2 complex [27,65]. AR1, AR2 and AR3 are present on E2, and AR4 and AR5 on the E1E2 complex. AR3 overlaps with CD81bs on E2. Several mAbs to AR3 and AR4 cross-neutralize diverse HCV genotypes and protect against HCV infection in small animal models. **(B)** Antigenic surface of E2c. The molecular surface of the E2c structure is colored according to known properties: green, glycan face; grey, occluded face; yellow, non-neutralizing face; and red, neutralizing face. The conserved, accessible neutralizing face contains the E2 CD81bs, AS412 and AS434, and is the prime target for structure-based immunogen design [86,87,94-97,101].