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Incomplete Humoral Immunity against Hepatitis C Virus Is Linked with Distinct Recognition of Putative Multiple Receptors by E2 Envelope Glycoprotein

Tae-Hwe Heo,* Jae-Hoon Chang,* Jae-Woo Lee,* Steven K. H. Foung, † Jean Dubuisson,‡ and Chang-Yuil Kang2*

Little is known about the role of the humoral immune response to hepatitis C virus (HCV). This study provides molecular evidence for the mechanism by which neutralizing Abs from the sera of chronic HCV patients have lower inhibitory activities against the binding of HCV E2 envelope protein to human hepatoma cell lines than to a lymphoma cell line. E2 binds to several putative receptors, specifically human CD81; human scavenger receptor, class B, type 1; and heparan sulfate. We have shown that E2 binds to target cells via these receptors in a noncompetitive manner. Thus, incomplete inhibition of one of the receptors leads to only a partial E2 blockade and, possibly, evasion of the host immune response. We demonstrated that the difference in and reduction of inhibition was closely related to impaired blockade of E2 binding to scavenger receptor, class B, type 1, and heparan sulfate. We have also shown that soluble E2 protein binds to multiple soluble receptors via separate binding domains on E2, providing further evidence for the distinct recognition of multiple cellular receptors by E2. This report suggests a novel finding that biased humoral immune responses to HCV E2 might provide an alternative mechanism for viral escape without the involvement of mutation. Additionally, our data give crucial consideration to the development of HCV vaccines that stimulate protective humoral immune responses. The Journal of Immunology, 2004, 173: 446–455.

Hepatitis C virus (HCV)3 is a small, enveloped, positive-strand RNA virus belonging to the genus Hepacivirus in the Flaviviridae family. The genome of HCV is ~9000 nt and contains a single large open reading frame encoding a single polyprotein of ~3010 aa. This polyprotein is subsequently processed co- and posttranslationally, generating the structural proteins Core, E1, E2, and p7, and the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B, and most likely their larger precursors (1, 2). The structural proteins are located in the N-terminal region of the polyprotein and are cleaved by cellular proteases. The nonstructural proteins are cleaved by internal viral proteases.

HCV infects ~170–200 million people worldwide (3), and in most cases, the infections develop into chronic hepatitis. Chronic HCV infection is one of the most prevalent causes of liver cirrhosis and hepatocellular carcinoma and, furthermore, represents the most frequent indication for liver transplantation. Currently available therapies are IFN-α treatment alone or in combination with ribavirin (4, 5). Such treatments are expensive, show low response rates, and carry the risk of significant side effects. Given the huge reservoir of HCV worldwide, the development of an effective vaccine will be the proper way to control the spread of disease associated with HCV infection.

Despite the elicitation of humoral and cellular immune responses against HCV, only 15–30% of the individuals infected with HCV resolve their infection (6). Additionally, it has been reported that HCV can cause more than one episode of acute hepatitis in the same individual (7). Thus, infection with HCV apparently does not provide complete protection against subsequent exposures. One of the explanations for the lack of protective immunity is the genetic and serological heterogeneity of HCV (8). The most variable region of the genome is the hypervariable region 1 (HVR1) located at the N terminus of the E2 glycoprotein (9). This region is a target of anti-HCV neutralizing Abs (Nt Abs) and undergoes diversification of sequence probably resulting from humoral immune response pressure (10). However, the mechanisms by which HCV escapes the humoral immune responses are not well defined.

The lack of a popular tissue culture system for propagating HCV and testing Nt Abs adds further complexity to the task of understanding the humoral immune response to HCV and vaccine development. Although the role of Nt Abs is still controversial, it has been shown that the early development of Abs to the HCV E2 protein, especially to the HVR1, correlated with recovery from the infection, suggesting that such Abs may play a role in viral clearance (11–13). To determine the significance and role of Nt Abs, several assays have been described (14–19). Although such assays described the neutralization of HCV (E2) interaction with target cells by Nt Abs, it is still necessary to investigate which cellular molecule(s) is related with the neutralization.
HCV encodes two putative envelope glycoproteins, E1 and E2, which are believed to play a role in viral attachment and entry into host cells. Many groups have made considerable efforts toward the identification of HCV receptor(s). Low-density lipoprotein receptor (LDLR) has been suggested as the HCV receptor due to viral association with plasma lipoprotein (20). Truncated soluble E2 was reported to bind to CD81 in a lymphoma cell line (21); scavenger receptor, class B, type 1 (SR-B1) in a hepatoma cell line (22); liver-specific ICAM-3-grabbing integrin in liver sinusoidal endothelial cells (23–25); and highly sulfated heparan sulfate (HS) in another hepatoma cell line (26). Except for the E2-CD81 interaction study, detailed follow-up studies have not been reported yet. Needless to say, the complex interactions between E2 and such putative multiple receptors require further investigation. Verifying the complex interactions between E2 and a group of receptor candidates would lead to a better understanding of the HCV infection process.

In this article, we examined the molecular basis of the impaired ability of serum from chronically infected HCV patients to inhibit E2 binding to human hepatoma cells. We found that, in addition to CD81, use of SR-B1 and HS induced incomplete blockade of E2 binding to hepatoma cells. This was most likely due to naturally insufficient humoral immunogenicity. Evidence of distinct interactions of E2 with CD81, SR-B1, and HS at the molecular level clearly support our model of impaired humoral immunity. In total, these findings provide detailed evidence that CD81, SR-B1, and HS could serve as separate viral attachment receptors, as well as present novel implications in the development of Ab-based passive and active immunoprophylaxis.

Materials and Methods

**Proteins, Abs, and cell lines**

Type 1a HCV E2 sequence (aa 370–661) from pCV-H77C (a gift of J. Butk, National Institutes of Health, Bethesda, MD) was cloned into pCMVdhr expression vector with 6×His tag and transfected into CHO (American Type Culture Collection, Manassas, VA) cells by lipofectamine (Invitrogen Life Technologies, Carlsbad, CA). After G418 (Invitrogen Life Technologies) selection and methotretate (Sigma-Aldrich, St. Louis, MO) induction, resistant single clones were adapted to a spinner culture (Belco, Vineland, NJ). ProCHO serum-free medium (Cambrex, East Rutherford, NJ) was used for maintenance of the cells. The supernatant was loaded into the GNA-agarose column (Sigma-Aldrich; or Vector Laboratories, Burlingame, CA). After G418 (Invitrogen Life Technologies) selection and methotretate (Sigma-Aldrich, St. Louis, MO) induction, resistant single clones were adapted to a spinner culture (Belco, Vineland, NJ). ProCHO serum-free medium (Cambrex, East Rutherford, NJ) was used for maintenance of the cells. After determining the saturating concentration of E2 protein for 1 h at RT, cells were washed and then incubated with penta-His mAb for 1 h at RT, followed by incubation with anti-mouse IgG-RPE secondary Ab (Southern Biotechnology Associates, Birmingham, AL). Samples were analyzed on a FACSII flow cytometer (Partec, Münster, Germany). For inhibition assays, subsaturating concentrations of E2 protein were mixed with serially diluted inhibitors and incubated with cells. Cells were washed and then incubated with penta-His mAb for 1 h at RT, followed by goat anti-mouse IgG-RPE or streptavidin-RPE secondary Abs (Pierce). Samples were analyzed on a FACSII or FACS-Calibur flow cytometer (BD Biosciences).

**Colocalization assay using confocal microscopy**

Huh-7 and HEPG2 cells that were grown on chamber slides (Nunc, Naperville, IL) and Molt-4 cells were fixed with 4% paraformaldehyde in PBS buffer. After washing in FACS buffer, blocking was performed with 5% goat serum in PBS for 15 min, followed by incubation with 5 μg/ml E2 protein for 1 h at RT. Bound E2 protein and putative receptors were detected by simultaneous incubation with human polyclonal anti-HCV serum (1/30 dilution) and one of the anti-receptor Ab for 1 h at RT, followed by incubation with either Cy5-conjugated goat anti-human IgG (H + L) (Jackson ImmunoResearch Laboratories, PA), or biotin-conjugated goat anti-mouse IgG (Sigma-Aldrich) or Cy3-conjugated monoclonal anti-vesicular stomatitis virus (VSV) glycoprotein clone P54 (Sigma-Aldrich) secondary Abs. The stained cells were analyzed using DM IRB/E confocal microscopy (Leica, Deerfield, IL).

**Results**

Sera from chronically infected HCV patients show weaker IOD activities against E2 binding to hepatoma cell lines than a lymphoma cell line

The existence of anti-HCV Nt Abs in some chronic HCV patients has been described previously (16). To examine the effect of Nt Abs against HCV E2 envelope protein, sera from nine chronically infected HCV patients and one uninfected individual were assessed for anti-E2 titers by ELISA using purified soluble E2 (Fig. 1A). To estimate the correlation of anti-E2 Ab titers with inhibitory activities of Nt Abs in sera, the IOD assay (16), which evaluates inhibition of E2 binding to human cells, was performed. In addition to Molt-4, a lymphoma cell line, which is commonly used as a target cell in this assay, we also included two well-known hepatoma cell lines (Huh-7 and HepG2), because HCV mainly targets hepatocytes. After determining the saturating concentration of E2 per cell, subsaturating quantities of E2 were blocked with serum
that was diluted 1/20. This was the dilution at which complete inhibition of E2 binding to Molt-4 cells was achieved, and that showed the highest IOB activity. In general, higher anti-E2 titers of sera led to stronger IOB potencies against binding of E2 to cells (Fig. 1). Interestingly, the inhibition data (Fig. 1B) demonstrate that HCV infection greatly impairs E2 binding to Molt-4 cells, but only moderately or weakly impairs E2 binding to Huh-7 or HepG2 cells, respectively. However, DK3 serum selectively reduced E2 binding to HepG2 cells to levels comparable to those of other sera, even though it had a low anti-E2 titer.

**HCV E2 binding to target cell lines depends upon the expression level and type of putative cellular receptor**

To clarify the mechanism of impaired inhibition activities against E2 binding to hepatoma cells (Fig. 1B), it was necessary to first examine the binding of E2 to target cells. To reveal these cellular components involved in E2 binding, we performed three experiments: a quantitative and kinetic binding assay between E2 and target cells, an inhibition assay of E2 binding to cells with recombinant soluble receptors, and a colocalization assay between E2 and putative cellular receptors.

As hepatocellular counterparts of HCV, several candidate receptors have been identified: CD81 (21), LDLR (20), SR-B1 (22), and HS (26). LDLR did not bind to E2 and was excluded in our assays. First, the expression levels and types for each of the three putative receptors from three different target cell lines were estimated. Molt-4 cells expressed one of the three putative receptors, CD81. Huh-7 cells expressed all of the putative receptors, and HepG2 cells expressed two of the three receptors, SR-B1 and HS. On the basis of the existence of multiple receptor candidates for HCV, we expected that the binding profiles of E2 to human cells vary depending on the expression levels of CD81, SR-B1, and HS (Fig. 2A). However, it was also possible that E2 binding occurred via a single, potent cellular receptor. To address these possibilities, quantitative binding of E2 to each cell line was performed. An increasing concentration of E2 was applied to Molt-4 cells and allowed to reach binding equilibrium. As shown in Fig. 2B, left, the binding was dose dependent and saturable. Scatchard plot analysis of the binding data revealed a single component binding curve, with a $K_d$ of $1.4 \times 10^{-8}$ M (Fig. 2C, left, inset), very similar to the previous data (16). We concluded that the main cellular receptor of E2 binding to Molt-4 cells was CD81. Identical procedures were performed with Huh-7 cells. The binding of E2 to Huh-7 cells was also dose dependent and saturable (Fig. 2B, center). However, E2 binding to Huh-7 cells was consistent with a two-component binding curve, with $K_d$ values of $1.2 \times 10^{-8}$ and $3.8 \times 10^{-9}$ M (Fig. 2C, center, inset). The former value is comparable to the $K_d$ of E2-CD81 binding. The latter value represents the $K_d$ for interaction between E2 and either SR-B1 or HS. HepG2 cells did not express the CD81 receptor; thus we postulated that E2 binding would be mediated by SR-B1, HS, or both. E2 bound to HepG2 cells via two components, but its binding was insaturable (Fig. 2C, right, inset). Because HepG2 binding was not saturable under accessible experimental circumstances, the number of possible E2 binding sites on the HepG2 cell surface had to be considered as extremely high. The most likely structures to provide an abundant number of binding sites were HS (28). Based on this fact, unidentified binding component in Huh-7 binding seems to be not HS but SR-B1. Thus, comparing with the $K_d$ values of E2-Huh-7 interaction, the high affinity $K_d$ may correspond to SR-B1 binding, and low affinity $K_d$ to HS binding.

To gain more direct evidence for E2-cellular receptor binding, it was necessary to perform the inhibition assays using each purified soluble putative receptor individually. Inhibition assays for both CD81-EC2 and heparin-BSA have been described in several reports (21, 26, 27, 29, 30). There have been no previous reports on soluble human SR-B1 expression in tissue culture cells. In this study, we have expressed a recombinant, soluble form of SR-B1 protein for the first time in mammalian cells.

Human SR-B1 is a 509-aa glycoprotein with a large extracellular loop anchored to the plasma membrane at both the N and C termini by transmembrane domains that have short extensions into the cytoplasm (Fig. 3A) (31). To investigate the interaction between E2 and SR-B1, as well as the direct inhibition of E2 binding, we constructed a plasmid that expresses the SR-B1-EC with a C-terminal Flag tag, downstream of the tissue plasminogen activator leader (Fig. 3A). This SR-B1-EC protein was obtained as
Expression of CD81, SR-B1 described in Materials and Methods.

**FIGURE 2.** Kinetic analysis of HCV E2 binding to human cell lines. A. Expression of CD81 (top), SR-B1 (middle), and HS (bottom) molecules on three human cell lines, Molt-4, Huh-7, and HepG2. Each cell line was incubated with anti-CD81 mAb, anti-SR-B1 mAb, or isotype control mAb, followed by anti-mouse IgG-RPE. In the case of HS expression, each cell was stained with single-chain anti-HS Ab HS4C3 or single-chain control Ab MPB49. Cells were incubated with Cy3-labeled anti-VSV Abs and then analyzed by flow cytometry. B. E2-cell binding assay. Serially diluted E2 protein was incubated with each cell line. Cells were stained with penta-His mAb, followed by anti-mouse IgG-RPE, and analyzed by flow cytometry. C. Graph of binding data and Scatchard plot analysis. Nonspecific fluorescence was measured by adding primary and secondary Abs to cells in the absence of E2. The net MFI was determined after subtracting the nonspecific fluorescence value. The saturation curve was created by plotting the net MFI (specific binding, y-axis) vs E2 concentration (x-axis). Then, Scatchard analysis was performed to determine the number of components involved in binding. We created a secondary plot (inset) of net MFI/E2 concentration (y-axis) vs net MFI (x-axis), Regression line, straight line for each receptor (inset), and $K_a$ value were determined using Sigmaplot software.

Of both SR-B1-EC and heparin-BSA showed enhanced inhibition of E2 binding. Additionally, some inhibitory effect of CD81-EC2 is presumably due to physically proximal binding domains between E2-CD81 and E2-SR-B1 or E2-HS binding.

Although confocal microscopy experiments for detecting E2/HCV-cell interaction have been performed (32, 33), the direct visualization of both E2 protein and the putative cellular receptors has not been clearly demonstrated. In this study, we present the colocalization of E2 with cell surface CD81, SR-B1, or HS (Fig. 4). On Molt-4 cells, E2 completely colocalized with CD81 (Fig. 4A) but not with HLA Ag (control; data not shown). On Huh-7 cells, E2 colocalized with CD81 as well as SR-B1, but not with HS. On HepG2 cells, E2 partially colocalized with SR-B1 as well as HS.

Taken together, the data suggest that E2 binds to Molt-4 via CD81, Huh-7 via CD81 and SR-B1, and HepG2 via SR-B1 and HS.

**Reduced inhibitory activity of HCV sera against E2-hepatoma cell binding correlates with impaired inhibition of E2 binding to SR-B1 and HS.**

We have provided evidence that E2 can bind to multiple receptors, depending upon the cell type. Based on these observations, we investigated the molecular mechanism of impaired inhibitory activities of HCV sera described in Fig. 1. Protein-protein interactions between E2 and soluble receptors were inhibited by serially...
files of CD81, SR-B1, and HS are taken into account (Figs. 1 and 5). Namely, lower inhibitory Abs against E2-Huh-7 binding than E2-Molt-4 are due to SR-B1 involvement in binding, and also much lower inhibitory Abs against E2-HepG2 are due to SR-B1 as well as HS. These results prompted us to reason that E2 possesses distinct and noncompetitive binding domains to CD81, SR-B1, and HS, which elicit differential humoral immune responses in chronic HCV patients.

**HCV E2 binds to each soluble receptor in a dose-dependent and specific manner via distinct binding domains**

Although many reports have shown the direct binding of E2 to the soluble CD81 protein (21, 29, 34–36) and one report has shown the binding of E2 to heparin (26), the E2-SR-B1 interaction at the soluble protein level has not been determined yet. Using purified E2 and putative receptors, we examined their direct interaction (Fig. 6). First, we examined the interaction of E2 protein with CD81-EC2 and heparin-BSA. Heparin is not a constituent of cell membranes but is a close structural homolog of highly sulfated HS. Similar to previous results (21, 26, 29, 34–36), we found that E2 protein binds specifically to CD81-EC2 and heparin-BSA in a dose-dependent manner (Fig. 6, B and C). In contrast, E2 did not react with human LDLR. Next, we examined whether SR-B1-EC indeed binds to E2 protein. E2 protein does bind to SR-B1-EC, and this binding was highly specific and dose dependent (Fig. 6A). Specificity was further confirmed by the fact that E2 binding to plate-coated SR-B1-EC was quantitatively competed with the self SR-B1-EC protein (Fig. 7B). This is the first indication that the extracellular domain spanning aa 33–439 of SR-B1 may be useful in studies of the interaction between E2 protein and SR-B1.

The binding domains involved in these interactions are still unknown. Based on the results in Fig. 5, we hypothesized that E2 possesses distinct binding domains to cellular CD81, SR-B1, and HS. To test this hypothesis in a more direct fashion and to investigate whether or not E2 could bind these three putative receptors simultaneously, we compared the inhibition profile of E2 binding to plate-coated SR-B1-EC, CD81-EC2, or heparin-BSA by three soluble receptors (Fig. 7). Subsaturating levels of E2 binding to CD81-EC2 was inhibited significantly by soluble CD81-EC2, but not by soluble SR-B1-EC or heparin-BSA (Fig. 7A). Likewise, the E2 binding to SR-B1-EC was inhibited by soluble SR-B1-EC, but not by the other soluble receptors (Fig. 7B). E2-heparin-BSA binding exhibited the same pattern of inhibition. These data suggest that CD81, SR-B1, and HS all bind to E2 in a noncompetitive manner.

**Simultaneous blockade of multiple receptors is required for efficient inhibition of E2 binding to target cells**

We have shown that putative multiple receptors did not compete for binding to E2, which led us to hypothesize that a mAb that blocks E2-CD81 binding would not be able to block the E2-SR-B1 and E2-HS binding. To test this idea, we screened several panels of mAbs (Fig. 8A). Consequently, CBH-5, one of the anti-E2 human mAbs and an inhibitor of HCV binding to CD81 (37), displayed an inhibitory effect on the E2-CD81-EC2 interaction but no effect on the E2-SR-B1-EC and E2-heparin-BSA interactions (Fig. 8B). Another human mAb, CBH-7 (37), possesses higher inhibitory activity than CBH-5, but showed no inhibitory effect on E2-SR-B1-EC and E2-heparin-BSA binding (Fig. 8B). Interestingly, H33 (38), one of the anti-E2 mouse mAbs, inhibited E2-CD81-EC2 binding significantly (IC50 ≈ 0.7 μg/ml), while inhibiting the E2-SR-B1-EC interaction only moderately (IC50 ≈ 18.8 μg/ml) (Fig. 8C). This suggests that the H33-binding site of E2 may be located near both the CD81-EC2 and SR-B1-EC binding sites. We
have been unable to find an inhibitor of E2 binding to heparin-BSA (Fig. 8A), possibly as a result of its low immunogenicity (Fig. 5).

Because of the double blocking properties (against CD81 and SR-B1) of H33, we postulated that H33 mAb would be more potent than CBH-5 or CBH-7 in blocking E2 binding to target cells expressing both CD81 and SR-B1. To test this hypothesis, we attempted an inhibition of E2 binding to Molt-4, Huh-7, and HepG2 cells (Fig. 8D). H33 mAb quantitatively inhibited E2 binding to Huh-7 cells, expressing both CD81 and SR-B1 on their surfaces, and reached complete inhibition at a concentration of 200 μg/ml. A strong CD81 blocker, CBH-7 mAb could not completely inhibit E2-Huh-7 binding and reached a plateau. A weak blocker, CBH-5 mAb inhibited the binding inefficiently and RO4 (control mAb) had no effect (Fig. 8D, middle). This discrepancy of the IOB activities was consistent with E2-Molt-4 and E2-HepG2 IOB assays despite differences in the extent of binding (Fig. 8D, left, right).

Discussion

This study provides the molecular basis for the experimental observation that chronic HCV sera show significantly weaker IOB activities against HCV E2 binding to hepatoma cell lines than to a lymphoma cell line (Fig. 1). This reduction in IOB ability is closely correlated with impaired blockade of E2 binding to SR-B1 and HS. We have demonstrated that HCV E2 envelope protein could bind to target cells via multiple receptors. Therefore, incomplete inhibition of one of these receptors may result in partial E2 blockade, leading to evasion of the host immune response. In this study, we present an alternate view on the mechanism of viral escape against humoral immunity and also suggest important points for consideration in blockade of viral attachment.

Although HCV infection is resolved in 15% of cases, it becomes chronic in up to 85% of infected individuals. The mechanisms for the high rate of viral persistence are unknown, but are thought to represent a complex interplay between viral diversity and host humoral and cellular immunity (39, 40). Selective pressure from the host’s humoral immune response is thought to be one of the mechanisms of viral persistence despite evidence for Nt Ab response directed to the E2 HVR1 region, perhaps due to rapid selection of escape variants in the chronic state (40). However, definitive evidence in support of Ab-driven HVR1 variant selection is lacking and, in principle, other mechanisms of variation, such as random drift, could be envisioned (10).

Apart from Ab-driven escape mutants, reinfection by homologous virus still occurs in HCV-infected chimpanzees with high levels of circulating anti-HCV Abs (41). Furthermore, chimpanzees infected with rHCV lacking the E2 HVR1 region became chronically infected, demonstrating that the most variable region of HCV was not the only determinant of progression to chronic infection (42). HVR1 has been the primary candidate in the search for neutralizing epitopes, as well as for the mechanism of viral persistence. In a multiple-receptor point of view, HVR1 seems to be essential in the E2-SR-B1 interaction (22, 43). This binding occurred in both HCV 1a and 1b; however, anti-HCV 1a HVR1 mAb could inhibit only the HCV 1a E2 binding, but not HCV 1b E2 binding (22), similar to the natural situation of impaired humoral immunity against heterologous virus infection (41). However, this situation could not explain the unsuccessful immunity against reinfection by a homologous virus. Based on our data, it is likely that the virus could still attach to the target cell via the distinct recognition of alternate receptors, such as CD81 or HS. This hypothesis could easily be supported by the evidence that HVR1-deleted HCV is still infectious, as mentioned above, implying another route for binding and entry (42). Therefore, we suggest that impaired humoral immunity is not only due to viral variability but also due to discrete recognition of multiple receptors by the HCV E2 envelope protein.

Although substantial progress has been made toward understanding the HCV infection cycle, the initial binding process still remains obscure, especially concerning the multiple interactions and spatial relationships between E2 and its putative receptors.
This study provides direct evidence that the HCV E2 protein contains distinct domains to which human CD81, SR-B1, and HS bind. E2-CD81 binding has been well characterized at the molecular and cellular levels (21, 26, 27, 29, 30), and the binding domains of E2 were presumed to be located outside of the HVR1 region (29).

Recently, a cell-based E2-SR-B1 interaction was discovered (22). To examine the interaction of E2 and SR-B1 more closely, we expressed SR-B1-EC in 293T and CHO cell lines. Binding of E2 to purified SR-B1-EC was highly specific and could not be disrupted with increasing concentrations of CD81-EC2 or heparin (Fig. 7). It has been speculated that the CD81 molecule would play a role as the primary receptor for E2 (HCV) binding, and this binding subsequently elicits the conformational change(s) in E2 (44). Recently, with the HCV pseudoparticle model, Bartosch et al. (38, 43) have suggested that the cell entry of HCV requires a set of coreceptors that includes CD81 and SR-B1. In this study, we have provided evidence that soluble SR-B1-EC protein can potentially bind to the E2-CD81-EC2 complex as well as to E2 protein alone, suggesting the possibility of the formation of a heterotrimERIC E2-two-receptor complex. This hypothesis is further supported by the colocalization data on Huh-7 cells, which indirectly show colocalization of CD81 and SR-B1 through E2 (Fig. 4). Therefore, based on these data, we favor a model in which HCV E2 forms a complex with both CD81 and SR-B1 in vivo. Further study of the receptor complex formation will be necessary to gain a more detailed view of the complex HCV binding process.

Very recently, E2-HS binding was shown (26), but the precise binding domain has not been determined yet. Visual inspection of HCV E2 sequences reveals a number of arginine, lysine, and histidine residues scattered across the protein, but none clustered in such a manner to suggest a conventional heparin-binding motif. Experimental and computational data have shown that the HCV HVR1 (26) and aa 559–614 (45) are candidate regions for heparin binding. We favor the latter region as the HS binding domain, because we have shown that the heparin does not compete with the CD81 molecule with E2 (Fig. 7). Furthermore, as shown in Fig. 5, although HVR1 elicited a moderate Ab response, only extremely low humoral immunogenicity was observed against E2-heparin binding. We could not demonstrate why the heparin binding domain (HBD) of HCV E2 did not elicit a strong humoral immune response. There are two possible explanations. One is that the HBD of E2 is inaccessible to Ab. The other is that HBD of E2 is prone to induction tolerance, because it is regarded as self by the immune systems of infected human patients. The former hypothesis is based on the fact that the aa 559–614 region of E2 has relatively high hydrophobicity (data not shown), thus forming a hidden structure. The latter hypothesis is based on the rationale that viral proteins, as well as self-proteins, use a HBD to promote target cell interaction. For instance, several chemokines, growth factors, and adhesion molecules use HS for attachment to target cells (46). Viral HBDs can therefore escape the host immune response if they are structurally similar to self-HBDs used by the host. As a result of tolerance induction, viral HBD-reactive B cells can be deleted or anergized.

Initially, we were curious as to which molecule among the candidate receptors was the actual HCV receptor. Taken together, the results from our kinetic, colocalization, and soluble receptor inhibition assays suggest that CD81 is the primary molecule for E2 cell binding. In the absence of CD81 molecules (HepG2), an augmented role for SR-B1 and HS in E2 binding was observed. It has been suggested that more than two neutralization epitopes may exist on HCV (14, 18), and as well as CD81 and SR-B1, additional hepatocyte-specific cofactor(s) are necessary for HCV entry (38, 43). However, we could not find evidence for another cofactor identical with HS in E2-Huh-7 binding (Figs. 2–4). Nevertheless, it has been demonstrated previously that highly sulfated HS is required for binding of E2 and HCV-like particles (26). We have also observed that E2 binding is decreased on heparinase-treated Huh-7 cells (data not shown). In contrast, the contribution of HS in E2 binding to HepG2 is clearly shown. The contribution of HS in E2 binding might be hidden in the Huh-7 assays due to the two high affinity receptors, CD81 and SR-B1. Although HS seems to play a minor role in E2 binding in tissue culture cell lines, it may play a greater role in the host, based upon the data concerning HCV fusion activity (17), cell tropism (26), and low humoral immunogenicity (Fig. 5).

Although some assays were devised for analyzing the Nt Abs, little is known about the role of the humoral immune response to HCV infection. Hence, there is no Reliable HCV culture system; surrogate HCV models have been used: HCV in serum (19), recombinant E2 envelope protein (16), pseudotyped VSV (15), HIV-HCV pseudotyped virus (14), CHO cells expressing HCV envelope proteins (17), and HCV-pseudotyped particles (18). It is believed that E2 plays a role in the viral binding process and E1 in

![Figure 7](http://www.jimmunol.org/)
the internalization process; however, the role of E1 is still theoretical. Thus, clear demonstration of an E2-putative receptor interaction does not implicate the direct relevance of internalization, despite several plausible reports (17, 43, 47). Alternatively, pseudotyped particles displaying mature oligomeric glycoproteins seem to be reliable for testing Nt Abs and studying the viral entry process. As target cell lines, primary hepatocytes (19), Molt-4 (16), Huh-7 (14, 18), and HepG2 (15, 17) cells have been used. As our data indicate, a single target cell line for neutralization experiments should be used cautiously because of the possibility of missing the E2-multiple receptor interaction. With respect to receptor expression, the Huh-7 cell line expresses all of the putative receptors and thus has merits in estimating the complex interactions between E2 and its putative multiple receptors. Actually, Huh-7 is highly infectable by pseudotyped particles (38) and the only possible cell line in the replicon system. Very recently, a new neutralization assay was described based on a pseudotyped particle-Huh-7 system (18). This system is invaluable for estimating Nt Ab in terms of viral entry events and characterization of the humoral immune response. However, we suggest that an Ab characterized as a Nt Ab in this system should be confirmed by additional tests. For instance, the impact process among binding, fusion, and penetration needs to be elucidated. Furthermore, if the binding process is the target, it will also be necessary to elucidate the exact target receptor using the molecular-based assay.

According to our study, a single mAb seems to be unable to block all the multiple receptors, implicating the requirement for a combination of Abs for complete inhibition. In our experiments,
H33 mouse mAb was defined as a double (CD81 and SR-B1) blocker and showed significant inhibitory activities at the molecular and cellular level (Fig. 8). Owing to the nature of recognizing conformational epitopes in E2, the epitope has not been characterized yet. As a single (CD81-E2C) blocker, CBH-5 and CBH-7 human mAbs were defined (Fig. 8). These mAbs were obtained from B cells of an HCV-infected patient with a 2-year history of normal alanine aminotransferase values but exhibition of plasma viremia (37). Among the CBH series of mAbs, CBH-5 and CBH-7 showed outstanding inhibitory activities against E2-CD81-E2C protein binding and E2-Molt-4 cell binding. No mAbs, including CBH-5 and CBH-7, however, were able to block E2-SR-B1-EC and E2-HS binding. This result was connected to incomplete neutralization activities against E2-Huh-7 and HepG2 cell binding. This B cell donor’s continual viremia, despite a high titer of IOB Abs, was probably caused by a low titer of inhibitory Abs against HCV-SR-B1 or HCV-HS interaction in terms of humoral immunity. The results may provide a clue to understand the strong antiviral Ab response. Moreover, we have found a direct correlation between the molecular and cellular inhibition assays in vitro. Consequently, for the purposes of analyzing and estimating the humoral immune response of patients to HCV, IOB activities against SR-B1 and HS should be included as well as CD81. It is worth considering double or triple blocking in the development of novel inhibitors or vaccines against HCV infection.

In summary, this study elucidates the mechanism by which a single viral but multiple cellular proteins mediate the binding of HCV to permissive cells, as well as the involvement of multiple sites or factors on both the virus and the host cells. The concept of multiple-receptor use by HCV may provide insight into the novel escape mechanism of HCV from the humoral immune response. In addition, it would be of interest to determine whether there are any correlations between biased humoral immunity against E2 and disease progression. Also, the data from studies of the mechanism of E2 binding and epitope mapping will aid in the development of novel vaccines and inhibitors against HCV. Additional studies will be needed to find any clinical correlations, as well as to determine the fine mapping of the E2 epitopes, which will offer insights into the unique mechanisms of viral control and facilitate the understanding of the E2-p pz receptor interactions.

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