High Density Lipoprotein Inhibits Hepatitis C Virus-neutralizing Antibodies by Stimulating Cell Entry via Activation of the Scavenger Receptor BI*

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Hepatitis C virus (HCV), a member of the Flaviviridae family, is transmitted during parenteral exposures to infected material, such as contaminated blood or needles. Its genome encodes a precursor polyprotein of ~3,000 amino acids (1). Cleavage of this polyprotein generates 10 polypeptides, including a core protein, two surface glycoproteins, E1 and E2, and the nonstructural proteins.

HCV is an enveloped virus, implying that specific cell surface molecules mediate the capture of viral particles and their penetration inside the infected cells. Several molecules have been proposed as cell entry receptors of HCV, and most of them have been isolated based on binding studies with soluble recombinant E2 protein or HCV-like particles. Potential receptors include the CD81 tetraspanin (2), the low density lipoproteins (LDL) receptor (3), the scavenger receptor BI (SR-BI) (4) that binds HDL, native, or modified LDL and very low density LDL (vLDL) (5), and several "capture" molecules that induce concentration of viral particles at the cell surface, hence allowing virions to find the cell entry receptors (6). Experimental data using infectious HCV pseudoparticles (HCVpp) harboring authentic E1E2 glycoproteins (7, 8) have substantiated the functional roles of CD81 and SR-BI in HCV entry (6). The requirement for CD81 in cell entry has been confirmed recently with cell culture-grown genuine HCV (HCVcc) (9–11).

Among infected individuals, only 20% recover from infection spontaneously, whereas most patients progress to chronic infection. The viral and host factors that determine HCV persistence or clearance at the acute stage of infection need to be understood in detail to improve antiviral therapy and to develop efficient vaccines. Studies focusing on innate and cellular immune responses have shown that HCV is able to evade or subvert the host defenses. Spontaneous HCV clearance is associated with a strong, early cellular immune response to multiple HCV epitopes, and both CD4+ and CD8+ responses are maintained for several years after viral clearance (12). The recent development of infection assays based on HCVpp and HCVcc provides new opportunities to study the humoral response. There is evidence that neutralizing antibodies could play a role in disease control. Such antibodies have been reported to emerge during the course of acute HCV infection both in patients (13, 14) and in experimentally infected chimpanzees (15). Pre-

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The abbreviations used are: HCV, hepatitis C virus; HDL, high density lipoprotein; HCVpp, HCV pseudo-particles; HCVcc, cell culture-grown genuine HCV; SR-BI, scavenger receptor BI; LDL, low density lipoprotein; mAb, monoclonal antibody; HS, human serum; FCS, fetal calf serum; MLV, murine leukemia virus; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; NOB, neutralization of binding; HA, hemagglutinin.

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**HDLM-mediated Neutralization-Attenuation of HCV**

Various studies have indirectly suggested a role for neutralizing antibodies in the control of viral loads (16, 17). Using HCVpp infection assays, a recent study addressed the kinetics of humoral responses in a cohort of acute phase patients (18). The emergence of a neutralizing response, of seemingly narrow specificity, was correlated with a decrease of high initial viremia, leading to control of viral replication. Of note, high titer, broadly cross-reacting antibodies that neutralize HCVpp are readily detected in chronically infected patients (7, 19–24), suggesting that their effectiveness is limited in patients who do not resolve the disease.

The factors that mitigate the impact of the neutralizing antibody response need to be clarified. Several reports indicate that plasma-derived HCV particles complexed to β-lipoproteins are protected against anti-HCV or anti-E2 antibodies (25–28). On the other hand, a recent study suggested that the interplay of HCVpp with high density lipoprotein (HDL), but not LDL, leads to protection from neutralizing antibodies present in sera of both acute and chronic patients (19). Indeed, HCVpp were more effectively neutralized by purified monoclonal antibodies and immunoglobulins isolated from chronic patients, as compared with the same antibodies in the presence of HDL or human serum (19). Likewise, nonresolving acute phase patients develop low titer neutralizing antibodies that are unable to neutralize HCVpp in infection assays performed in the presence of human serum, as a result of the presence of HDL that completely abrogate their activity (18, 19). Interestingly, deletion or mutation of HVR1, the hypervariable region-1 of the HCV-E2 glycoprotein that is under strong evolution pressure during disease outcome (29), strongly increased sensitivity of HCVpp to cross-neutralizing antibodies present in these sera (19), assigning to HVR1 a critical role in modulation of antibody effectiveness.

These different results suggest that antibody-escape mechanisms operate in vivo and contribute to the inefficiency of the host humoral response against HCV in patients who do not resolve the infection. Despite the high viral variability, it has been possible to isolate monoclonal antibodies derived from either chronic patients or immunized animals that neutralize HCVpp of diverse genotypes and subtypes (30–32), providing novel prospects for antibody-based immunotherapy and vaccine development. By aiming to discover epitopes and/or antibodies as well as drugs that enhance the potency of neutralization in vivo, there is obvious interest to address the mechanisms that counteract HCV neutralization. By using HCVpp and HCVcc in this study, we unravel new features of the mechanism by which HDL induce protection against neutralizing antibodies.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs and Production of HCVpp**—Expression vectors for the E1E2 glycoproteins of HCV strain H77 (AF009606) and for the HVR1 deletion mutant (ΔG384–N411) were described previously (7, 33). The murine leukemia virus (MLV) packaging and GFP transfer vectors and the phCMV-RD114 expression plasmid encoding glycoproteins of cat endogenous virus RD114 were described elsewhere (7). The phCMV-MLV-A and phCMV-HA expression plasmids encoding the glycoprotein of amphotropic MLV and an avian influenza virus hemagglutinin (HA H7N1), respectively, were described previously (34).

HCVpp, RD114pp, MLVpp, and HApp were produced (7, 34) by transfection in 293T cells of vectors encoding viral glycoproteins, packaging proteins, and GFP transfer vector. Viral particles containing supernatants were used to infect Huh-7 hepatoma cells directly or upon purification by ultracentrifugation through a 20% sucrose cushion.

**Expression Constructs and Production of HCVcc**—The pFK-Luc-Jc1 is a chimeric J6CF/JFH1 HCV genome consisting of codons 1–846, derived from J6CF (AF177036), and codons 847–3033, derived from JFH1 (AB047639). The plasmid pFK-Luc-Jc1 also encodes a bicistronic firefly-luciferase reporter with a design analogous to pFK-Luc-JFH1 as described recently (10).

HCVcc were produced (10) by electroporation of Huh-7-Lunet cells. These cells, characterized by high permissiveness for HCV RNA replication (35), originally carried a selectable HCV replicon and were cured by treatment with a specific inhibitor. Culture fluid of electroporated cells was harvested 48 h later and used directly in infection assays using Huh-7-Lunet target cells or after purification, as described above for HCVpp.

**Reagents and Antibodies**—Preparation of HS was described previously (19). The HDL (Calbiochem) preparation (density 1.063–1.2 mg/ml) contained a mixture of HDL2 and HDL3. BLTs (36) were obtained from Chembridge. The JS81 CD81-specific mAb was purchased from Pharmingen. The 9/27, 3/11 (8), AP33 (37), CBH-2, CBH-5, CBH-7 (30), and H35, H48, H53, H54, H57, and H60 (7, 38–40), and the E2mAb-1 were E2-specific mAbs. H111 (30) and A4 (41) are E1-specific mAbs. Polyclonal antibodies against E1 (strain H77) were purified from mouse immune sera (kind gift of G. Verney), obtained by immunization with modified HCVpp harboring E1 glycoproteins only. Antibodies were purified using protein-G-Sepharose (Amersham Biosciences). A pool of HCV immunoglobulins was purified and concentrated from over 30 chronic HCV sera of genotypes 1a, 1b, 2a, 2b, and 3 as described previously (19). The recombinant CD81-LEL fragment (amino-acids 112–202) and a truncated soluble form of E2 glycoprotein (sE2) (amino-acids 384–664) were fused to a His tag, produced in mammalian cells and purified on nickel-nitrilotriacetic acid resin (Qiagen).

**Binding Assays**—Binding of HCVpp was performed as described previously for other types of pseudo-particles (42). Briefly, 50 μl of virus particles purified on a 20% sucrose cushion were incubated to 10^6 target cells in the presence of 0.1% sodium azide for 1 h. Human serum was added or not at the concentration of 2.5%. Cells were then washed twice with PBFA (PBS, 2% fetal bovine serum, and 0.1% sodium azide) and incubated with the H53 anti-HCV-E2 mAb (40 g/ml) of tested antibodies and 2.5% in the presence of 0.1% sodium azide for 1 h at 37 °C. Molt-4 cells (10⁶ cells) were then incubated to the mixture for 1 h at room temperature, and the amount of cell-bound sE2 was determined by FACS analysis.

**Binding of soluble E2 glycoprotein to CD81** was performed as described previously (39) and was used to detect the “neutralization of binding” (NOB) activity of antibodies to CD81. Briefly, 5 μg/ml of sE2 harboring a His tag was mixed with 10 μg/ml of tested antibodies and incubated for 1 h at 37 °C. Molt-4 cells (10⁶ cells) were then incubated to the mixture for 1 h at room temperature, and the amount of cell-bound sE2 was determined by FACS analysis using anti-His tag antibody (penta-His; Qiagen).

**Infection Assays**—Huh-7 and Huh-7-Lunet cells were seeded 24 h prior to inoculation (7, 10). 2 h before infection, target cells were preincubated in Dulbecco’s modified Eagle’s medium containing 0.1% FCS. Medium was then removed, and dilutions of viral supernatants and various compounds were added to the cells as indicated. After 4 h, supernatants were removed, and the infected cells were kept in regular medium (Dulbecco’s modified Eagle’s medium, 10% FCS) for 72 h before analysis.

For infection assays with HCVpp, the infectious titer were deduced from the percentage of GFP-positive cells, as determined by FACS analysis (7). Infections were controlled by pseudo-particles devoid of E1E2 by which HDL induce protection against neutralizing antibodies.

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glycoproteins, which resulted in background titers below $5 \times 10^2$ infectious units/ml. For analyses of HCVcc infectious titers, cells were lysed for luciferase assays, as described previously (10).

**Surface Plasmon Resonance Binding Assays—Biomolecular interactions** were studied using a BIAcore-3000 instrument (BIAcore AB, Uppsal, Sweden), which used surface plasmon resonance as a detection method. The AP33 antibody (100 μg/ml in 10 mM acetate buffer, pH 4.5) was covalently immobilized to the dextran matrix of a CM3 sensor chip (amine coupling kit, BIAcore AB) at a flow rate of 5 μl/min. Activation and blocking steps were performed as described previously (43). Purified HCVpp were injected over AP33 antibody in PBS containing 0.005% P20 surfactant (BIAcore AB) at a flow rate of 5 μl/min at 25 °C. HCVpp capture levels ranged between 200 and 500 resonance units. A control flow cell was prepared by immobilizing irrelevant antibody (mouse anti-IL2) according to the same procedure. Control sensorgrams representing nonspecific binding to the sensor chip surface were automatically subtracted from the sensorgrams obtained with captured HCVpp. Binding assays of HDL, A4 antibody, and CD81-LEL were performed at 25 °C in PBS with 0.005% P20 surfactant at a flow rate of 5 μl/min. The surface was then regenerated with pulse of 0.025% SDS.

**RESULTS**

We reported previously that an interplay between HDL and HCV-E2 leads to infection enhancement of HCVpp (19, 44), an event stimulated by HVR1, and to inhibition of neutralizing antibodies (19). An example of neutralization curves deduced from the results of infection assays obtained with a monoclonal antibody (mAb), carried out in the presence or the absence of HDL or HS, is displayed in Fig. 1. These results allowed estimation of IC$_{50}$ and IC$_{90}$ values for this mAb (i.e. antibody concentrations that reduce HCVpp infectious titers by 2- and 10-fold, respectively) at 1.3 and 8 μg/ml, respectively, in the absence of HS or HDL. This compared with an IC$_{50}$ of 6.6 μg/ml for neutralization assays performed in the presence of HS. The attenuation of neutralization was not affected by the mAb concentration, in the range of values tested, i.e. 0.1–50 μg/ml (data not shown). More importantly, no IC$_{90}$ could be determined from neutralization assays performed in the presence of HS or HDL because the mAb could not neutralize more than 65% HCVpp under these conditions (Fig. 1), even at high antibody concentrations (19). Therefore, such a level of neutralization only reduced the infectious titers by less than 3-fold, which contrasted with the complete inhibition of HCVpp infectivity achieved in serum-free conditions for antibody concentrations of ~50 μg/ml (19, 31). Finally, similar results were obtained for HCVpp carrying E1E2 glycoproteins of other genotypes and/or when HS was added to purified HCVpp/antibody immune complexes (Table 1).

**No Interaction of HDL with HCVpp Can Be Detected—**HDL may interact with HCVpp and hence physically reduce the effectiveness of antibodies either by "shielding" of the glycoproteins or by competing for the neutralizing epitopes. To investigate this possibility, we monitored biomolecular interactions by using surface plasmon resonance assays (BIAcore). HCVpp were captured by an E2 mAb covalently immobilized to a sensorchip (Fig. 2A). In those conditions, HCVpp interacted with E1E2 ligands such as the A4 antibody, which binds E1, or with CD81-LEL, a soluble ectodomain of CD81, which binds E2 (Fig. 2B). However, no interaction could be detected between captured HCVpp and HDL (Fig. 2B). Other experimental setups were designed, including HCVpp capture on the sensorchip via alternative E2 antibodies or via *Galanthus nivalis* lectins, but no HCVpp/HDL interaction could be detected (data not shown). Furthermore, similar levels of HCVpp capture by the E2 mAb could be detected when carried out in the absence versus the presence of HDL (Fig. 2A). Altogether, these results indicated that HDL does not form a stable or a transient complex with HCVpp.

**HDL Stimulates HCVpp Internalization—**We then sought to evaluate whether HS influence cellular uptake of HCVpp by comparing the kinetics of HCVpp internalization in the presence or in the absence of HS (Fig. 3) or HDL (data not shown). After an initial stage of virus-cell

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**TABLE 1**

Desensitization of HCVpp neutralization by human serum

| Sequential treatment of viral particles and Huh-7 target cells | % neutralization$^c$ |
|-------------------------------------------------------------|-----------------------|
| **Step A, treatment of HCVpp**$^a$ | **Step B, infection of Huh-7 cells**$^b$ | |
| NA | HCVpp + mAb | 72.23 ± 10.1 |
| NA | HCVpp + mAb + HS | 19.73 ± 10.7 |
| Preincubation HCVpp + HS | Mix step A + mAb | 21.46 ± 9.4 |
| Preincubation HCVpp + mAb | Mix step A | 76.48 ± 3.8 |
| Preincubation HCVpp + mAb, then purification$^d$ | Mix step A + HS | 38.36 ± 7.5 |
| Preincubation HCVpp + mAb, then purification$^d$ | Purified mix step A | 56.92 ± 10.2 |
| Preincubation HCVpp + mAb, then purification$^d$ | Purified mix step A + HS | 24.90 ± 0.8 |
| Preincubation HCVpp + mAb, then purification$^d$ | Purified mix step A | 64.31 ± 5.5 |
| Preincubation HCVpp + mAb, then purification$^d$ | Purified mix step A + HS | 29.01 ± 0.8 |

$^a$ HCVpp mixed, or not, with 2.5% HS and/or 4 μg/ml of the AP33 mAb, as indicated, were preincubated for 45 min at room temperature. NA indicates not applicable.

$^b$ Huh-7 target cells were simultaneously incubated with the indicated reagents for 4 h at 37 °C.

$^c$ Results are expressed as the mean percentages (mean ± S.D.; n = 6) of neutralization of the infectious titers relative to incubation with medium devoid of antibody. No effect of HS or HDL could be detected on infectivity of control pseudo-particles harboring RD114 glycoproteins (20) (data not shown).
incubation at 4 °C for 1 h in the presence versus the absence of HS, which allowed identical levels of cell surface binding (Fig. 3A) but prevented internalization of virions, followed by two steps of washing to remove unbound viral particles, cell entry was allowed to proceed for various periods of time by shifting cell temperature to 37 °C. Viral particles that had remained bound at the cell surface without undergoing internalization were then inactivated by a 1-min acidic shock at pH 3 (42, 45), and the levels of infection, reflecting internalization rates, were assessed 2 days later. In the presence of HS, internalization increased steadily after the cells were warmed and reached a plateau of infection after over 4 h of incubation (Fig. 3B). In contrast, in the absence of HS, the onset of HCVpp internalization was strongly delayed, by about 70 min. After this lag time, the virions were internalized progressively until reaching a plateau. The infection levels at plateau were 3–4-fold higher in the presence of HS, consistent with the infection enhancement of HCVpp induced by HDL/SR-BI interplay (19, 44), similar results of accelerated HCVpp internalization kinetics were obtained when using HDL, rather than HS (data not shown). Such an enhancement of virion internalization and infection was specific for HCVpp and was not detected with control viral particles harboring alternative glycoproteins such as those from an MLV (Fig. 3C) or from an influenza virus (Fig. 3D).

**Attenuation of Antibody Neutralization Requires Active SR-BI**—Assuming that neutralization attenuation is not likely mediated through HDL association to HCVpp (Fig. 2), the results of HDL-triggered HCVpp internalization prompted us to investigate whether neutralization of HCVpp could be modulated by HDL at the level of the cell surface. Because HDL is a ligand of SR-BI (46), a putative HCV receptor (4, 33), we thought that their mutual interaction could influence neutralization. To address this question, we investigated a potential relation between infection enhancement, which is induced by HDL/SR-BI interplay (19, 44), and neutralization inhibition. We therefore compared the results of infection for different concentrations of HS or HDL in the presence, or in the absence, of a neutralizing mAb (Fig. 4A). As reported previously (19, 44), the fold of infection enhancement steadily increased with the concentration of HS/HDL. Most interestingly, the levels of neutralization inhibition, expressed as percent of attenuation (Fig. 1), increased concomitantly with HS/HDL concentrations and were in pro-

![FIGURE 2. Absence of direct interaction between HDL and HCVpp. A, detection of interaction by surface plasmon resonance analysis. HCVpp or HDL-treated HCVpp, as indicated, were captured by the AP33 monoclonal E2 antibody covalently immobilized to the dextran matrix. The absence of HDL binding to AP33 antibody was demonstrated by the lack of capture of HDL, upon its injection on the AP33-bound sensor chip, as indicated. B, purified CD81-LEL, A4 monoclonal E1 antibody, or HDL were injected over captured HCVpp at 20, 1.33, and 4.61 μmol/liter, respectively, and the binding of either molecule was determined by the number of resonance units (RU). The displayed sensorgrams represent specific binding obtained after online subtraction of the nonspecific binding measured on the control flow cell containing an immobilized irrelevant antibody. The brief positive or negative peaks in responses detected at the start and at the end of injection of HCVpp, HDL, CD81-LEL, or A4 mAb are induced by the change of buffer.](image-url)
portion to the levels of infection enhancement. Concentrations of HS that induced the maximal infection enhancement (~4-fold) resulted in over 60% attenuation of neutralization (Fig. 4A). The statistical analysis of the correlation between neutralization inhibition and infection enhancement raised $R^2$ factors of 0.802 and 0.924 for HDL and HS, respectively, hence establishing a clear connection between the two events. This result therefore suggested that neutralization attenuation depends on the level of SR-BI activation by HDL.

To confirm the involvement of SR-BI, we performed neutralization assays in the presence of specific SR-BI inhibitors that prevent its capacity to mediate lipid transfer with HDL (46) and HDL-mediated infection enhancement of HCVpp (19, 44). The treatment of Huh-7 target cells with BLT-4, one of such inhibitors (36), significantly increased the neutralizing efficiency of a mAb (Fig. 4B) or of patient-derived polyclonal antibodies (data not shown) in infection assays performed in the presence of HS. Indeed, although a less than 1.3-fold decrease of infectious...
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FIGURE 4. Involvement of the scavenger receptor BI in neutralization attenuation. A, the results of infection enhancement (on the x axis), as fold increase of infection, and of neutralization inhibition (on the y axis), as % of attenuation, were calculated for different concentrations of HS or HDL. The fold increase of infection was determined as the ratio between average infectious titers in the presence versus in the absence of HS or HDL. The fold increase was determined by the mean percentage of attenuation of the infectious titers relative to incubation with medium devoid of antibody. The percentage of attenuation was determined by the mean percent attenuation of the infectious titers relative to incubation with medium devoid of antibody. The percentages (mean ± S.D.; n = 3) of neutralization inhibition (on the x axis), as fold increase of infection, and of neutralization inhibition (on the y axis), as % of attenuation, were calculated for different concentrations of HS or HDL relative to the neutralization in the absence of human serum as exemplified in Fig. 1. Neutralization of HCVpp was performed using the AP33 monoclonal antibody at 2 μg/ml. B, restoration of neutralization of HCVpp by the BLT-4 and glyburide SR-BI inhibitors. Neutralizing assays were performed with the AP33 mAb (2 μg/ml) in the absence (−) or in the presence of 50 μM of BLT-4 or 250 μM of glyburide, which were added to Huh-7 target cells during infection with HCVpp. The results are expressed as the percentages (mean ± S.D.; n = 3) of neutralization of the infectious titers relative to incubation with medium devoid of antibody. C, results of infection assays performed in the absence or in the presence of 2.5% HS, 6 μg/ml HDL, 2 μg/ml AP33 antibody and/or the BLT-4 inhibitor, as indicated. The results show the effect of BLT-4 in attenuation of AP33-mediated HCVpp neutralization induced by HS or HDL, expressed as the percentage of attenuation (mean ± S.D.; n = 3). D, restoration of neutralization of HCVpp by the blocking of SR-BI, using a polyclonal antibody against the ectodomain (19, 33). Neutralizing assays were performed with the AP33 mAb (4 μg/ml) in the absence (−) or in the presence of SR-BI antibody, which was added to Huh-7 target cells 30 min before and during infection with HCVpp. The results are expressed as the percentages (mean ± S.D.; n = 3) of neutralization of the infectious titers relative to incubation with medium devoid of antibody. The infection assays were performed in the absence (left panel) or the presence (right panel) of 2.5% HS. No effect of BLT-4 could be detected on infectivity of control pseudo-particles harboring RD114 cat endogenous virus glycoproteins (data not shown).

FIGURE 5. Involvement of the scavenger receptor BI in neutralization attenuation. A, results of infection enhancement (on the x axis), as fold increase of infection, and of neutralization inhibition (on the y axis), as % of attenuation, were calculated for different concentrations of HDL or HDL relative to incubation with medium devoid of antibody. The results of infection enhancement (on the x axis), as fold increase of infection, and of neutralization inhibition (on the y axis), as % of attenuation, were calculated for different concentrations of HDL or HDL relative to incubation with medium devoid of antibody. The fold increase of infection was determined as the ratio between average infectious titers in the presence versus in the absence of HDL. The fold increase was determined by the mean percentage of attenuation of the infectious titers relative to incubation with medium devoid of antibody. The percentage of attenuation was determined as the ratio between average infectious titers in the presence with medium devoid of antibody and/or the BLT-4 inhibitor, as indicated. The results show the effect of HDL in attenuation of AP33-mediated HCVpp neutralization induced by HS or HDL, expressed as the percentage of attenuation (mean ± S.D.; n = 3). B, restoration of neutralization of HCVpp by the blocking of SR-BI, using a polyclonal antibody against the ectodomain (19, 33). Neutralizing assays were performed with the AP33 mAb (4 μg/ml) in the absence (−) or in the presence of SR-BI antibody, which was added to Huh-7 target cells 30 min before and during infection with HCVpp. The results are expressed as the percentages (mean ± S.D.; n = 3) of neutralization of the infectious titers relative to incubation with medium devoid of antibody. The infection assays were performed in the absence (left panel) or the presence (right panel) of 2.5% HS. No effect of HDL could be detected on infectivity of control pseudo-particles harboring RD114 cat endogenous virus glycoproteins (data not shown).

titers was achieved by the mAb (i.e. <28% neutralization), the addition of BLT-4 resulted in over a 12-fold titer decrease (i.e. >92% neutralization). The restoration of the neutralizing property by BLT-4 was dose-dependent (Fig. 4C), yielding at ~40–50 μM the levels of neutralization obtained in the absence of HS/HDL (Fig. 4E). Similar results were obtained using alternative neutralizing mAbs and other SR-BI inhibitors, such as glyburide (Fig. 4B), a molecule used to treat diabetic patients (36, 47). Furthermore, blocking of SR-BI (19), using a polyclonal serum (Fig. 4D), or down-regulation of SR-BI (21), using RNA interference (data not shown), also stimulated neutralizing antibody effectiveness. Finally, as reported previously (19), deletion or mutation of HVR1, a key viral component of HCV-E2 and SR-BI interaction (4, 19, 33), strongly increased the sensitivity of HCVpp to neutralizing antibodies (data not shown). Altogether, these data established that interaction of HCVpp with HDL-activated SR-BI molecules promotes neutralization attenuation.

SR-BI Activation by HDL Modulates E2/CD81 Interactions—Because SR-BI acts as a co-factor of the CD81 tetraspanin (33), a putative HCV receptor (2), we asked whether the role of CD81 could be influenced by HS/HDL-mediated activation of SR-BI. Thus, to address this question, we performed infection assays with polypeptides that prevent the binding of E2 to CD81 as follows: JS-81, a CD81-blocking antibody or, alternatively, a soluble form of the CD81 ectodomain (CD81-LEL). Both polypeptides inhibited cell entry by HCVpp (Fig. 5, A and B), as reported before (7). Interestingly, the inhibitory effect of JS-81 was strongly reduced in the presence of HS (Fig. 5A) or HDL (data not shown). Similarly, inhibition by CD81-LEL added during infection was reduced in the presence of HS (Fig. 5B) or HDL (data not shown). Indeed, although 10 μg/ml of CD81-LEL inhibited HCVpp infection to up to 75% in the absence of HS/HDL, the inhibition was decreased to less than 30% in the presence of HS (Fig. 5B).

Most importantly, the attenuation of inhibition by CD81-LEL was dependent on a functional interplay between HCVpp, HDL, and SR-BI. Indeed, an SR-BI inhibitor, BLT-4, alleviated this attenuation in a dose-dependent manner, restoring a full inhibition by CD81-LEL at ~50 μM of BLT-4 (Fig. 5C). Likewise, attenuation of inhibition by CD81-LEL was not observed for HCVpp harboring a deletion of HVR1 (Fig. 5D). Altogether these results suggested that the role of CD81 in cell entry is diminished under infection conditions that favor activation of SR-BI or,
Alternatively, that compounds that block E2/CD81 interaction are less potent when SR-BI is activated.

Neutralizing Antibodies That Target E2/CD81 Interaction Are Attenuated by SR-BI Activation—Because HS/HDL modulates the role of CD81 during cell entry (Fig. 5), we reasoned that attenuation of neutralization (Figs. 1 and 4) may specifically involve antibodies that block infection at a step relating to HCVpp interaction with CD81. To address this possibility, we performed neutralization assays with a panel of mAbs of known specificity in the presence or in the absence of HS (Fig. 6).

A majority of E2 antibodies, of human and mouse origins, exhibited reduced neutralizing efficiencies in the presence of HS (Fig. 6A) or HDL (data not shown), similar to results obtained with the AP33 mAb (Fig. 1). For antibodies for which this could be calculated, i.e. for E2mAb-1, H35, H54, and H57 mAbs, the differences of IC50, determined in the presence versus the absence of HS in the neutralization assays, were at least 10-fold (Fig. 6A). Furthermore, although most mAbs efficiently neutralized serum-free HCVpp, none of them inhibited HCVpp infectivity by more than 70% in the presence of HS, even at high antibody concentration. This suggested that over 10-fold higher antibody concentrations are required to reduce HCVpp infectious titers by 2-fold in the presence of HS/HDL (i.e. 50% neutralization) and that, in any case, neutralizing antibodies in HS cannot reduce viral infectious titers by more than 3-fold (i.e. 70% neutralization).

Interestingly, we also identified several mAbs whose neutralizing activities were not reduced by HS (Fig. 6B) or HDL (data not shown). This was the case for the 9/27 antibody, which targets an epitope in HVR1 (39) and abrogates E2 interaction with SR-BI (4, 33). Its IC50 of less than 1 μg/ml was unchanged when infection assays were performed in the presence versus the absence of HS. Two other antibodies that were not attenuated by HS were the mouse H60 and human H111 mAbs, targeted to E2 and E1, respectively (39, 48). Although they exhibited much weaker efficiencies as compared with that of the 9/27 mAb, their activity was not altered by HS (Fig. 6B).

We then sought to correlate the properties of these different mAbs to their capacity to inhibit E2/CD81 interaction, as determined by NOB assays with soluble E2 proteins (Table 2). The results indicated a clear relationship between the capacity of the antibodies to inhibit sE2 binding to CD81 and the attenuation of their neutralizing properties by HS or HDL. All mAbs that were attenuated by HS/HDL (Fig. 6A) were those that inhibited the interaction between sE2 and CD81 (Table 2). Conversely, the H111, H60, and 9/27 antibodies, whose neutralizing activity was not impaired by HS/HDL (Fig. 6B), had no CD81-NOB activity (Table 2).

These results suggested that efficient HCV neutralization in the presence of HS could be mediated by antibodies directed against particular epitopes, such as those harbored by the HCV-E1 glycoprotein (e.g. H111 hu-mAb). To substantiate this possibility, we performed neutralization assays with an E1 polyclonal antibody. Although this antibody specifically neutralized HCVpp with an IC50 of 20 μg/ml (Fig. 6B), its neutralizing titer was not altered by the presence of HS (Fig. 6B) or HDL (data not shown).

Neutralization of HCVcc—To confirm these results in a more relevant model of HCV infection, we performed neutralization assays of HCVcc particles (10). Of note, infectivity of purified HCVcc was enhanced by ~4–6-fold in the presence of HS/HDL (Fig. 7A), indicating that, like HCVpp, HCVcc exploits similar features of SR-BI/HDL interaction during cell entry. Some monoclonal and patient polyclonal antibodies that neutralized HCVpp could also neutralize HCVcc (Fig. 7B) yet seemingly less efficiently than HCVpp. Indeed, ~100-fold higher concentrations of the E2mAb-1 antibody were required to neutralize HCVcc as compared with HCVpp (compare IC50: ~50 μg/ml for HCVpp (Fig. 7B) versus 0.5 μg/ml for HCVcc (Fig. 6A)). Likewise, polyclonal antibodies purified from HCV chronic patients were unable to neutralize HCVcc by more than 35%, even at concentrations higher than 1.4 mg/ml (Fig. 7B), although they neutralized over 90% of HCVpp at concentrations of ~100 μg/ml (19).

The weak neutralization of HCVcc by these antibodies, reminiscent of that of HCVpp in the presence of HS/HDL (Figs. 1 and 6A), was induced by the secretion of HDL from HCVcc producer hepatoma cells (49), which was assessed by detection of ApoA-I in these cells (data not shown). Indeed, treatment of HCVcc target cells with the SR-BI inhib-
FIGURE 6. Sensitivity of monoclonal antibodies to neutralization inhibition by human serum. Results of infection assays performed with HCVpp of genotype 1a. The results show the titration curves of a panel of monoclonal antibodies in the absence (—) or in the presence of 2.5% HS and are expressed as the mean percentages (mean ± S.D.; n = 3) of inhibition of the infectious titers relative to incubation with medium devoid of antibody. A, results obtained with monoclonal antibodies that block the interaction between E2 and CD81 (Table 2). B, results obtained with antibodies that do not block the interaction between E2 and CD81.

A

B
Neutralization of cell culture-grown authentic HCV. A, results of infection assays on Huh-7 cells using HCVcc of genotype 2a. HCVcc viral particles were purified by ultracentrifugation on a 20% sucrose cushion and were resuspended in PBS to which 2.5% HS or 6 μg/ml HDL were added during infection assays, as indicated. The results show the percentages of infection (mean ± S.D.; n = 2) determined by the ratio of the average infectious titers in the presence of the indicated components relative to the average infectious titers in the absence of HS or HDL (−). B, restoration of neutralization of HCVcc by the BLT-4 SR-BI inhibitor. Neutralizing assays were performed with the E2mAb-1 antibody (E2 monoclonal Ab), with polyclonal antibodies derived from a pool of chronic patients (patient polyclonal Ab) and with E1 polyclonal antibodies (E1 polyclonal Ab) in the absence (−) or in the presence of 50 μl of BLT-4. The results are expressed as the percentages (mean ± S.D.; n = 3) of inhibition of the infectious titers relative to incubation with medium devoid of antibody.

TABLE 2
Determination of CD81-NOB activity of antibodies

| Antibodies     | HS* | HDL* | LDL* | NOB* |
|----------------|-----|------|------|------|
| CBH2           | +   |      |      | +    |
| CBH5           | +   | +    |      |      |
| CBH7           | +   | +    |      |      |
| E2mAb-1        | +   | +    |      |      |
| 3/11           | +   | +    | +    |      |
| H35            | +   | +    | +    | +    |
| H48            | +   | +    | +    |      |
| H54            | +   | +    | +    |      |
| H57            | +   | +    |      |      |
| AP33           | +   | +    |      | +    |
| JS-81          | +   | +    |      |      |
| 9/27           | −   |      |      |      |
| H60            | −   |      |      |      |
| H111           | −   |      |      |      |
| E1 polyclonal Ab| −   |      |      |      |

* Antibodies for which the neutralizing activity was (+) or was not (−) attenuated by HS, as deduced from the results of Fig. 6.
* Antibodies for which the neutralizing activity was (+) or was not (−) attenuated by HDL and LDL.
* Antibodies that neutralized binding of sE2 to CD81 expressed in Molt-4 cells. The NOB activity of some of the mAbs was reported previously (37, 61, 77, 78).

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BLT-4 (Fig. 7B). Importantly, similar to HCVpp, the E1 polyclonal antibody efficiently neutralized HCVcc (Fig. 7B), resulting in a 10-fold reduction of infectious titers at 20 μg/ml, whether or not HS/HDL was present during infection assays (data not shown) and whether or not SR-BI was inactivated with BLT-4 (Fig. 7B). These results therefore confirmed that association of neutralizing antibodies with SR-BI inhibitors or use of antibodies that target E1 epitopes are valuable strategies to induce efficient HCV neutralization.

DISCUSSION

Like many viruses, HCV has developed several mechanisms to escape from the innate and adaptive host immune responses. As for the humoral response, the interplay or the association of HCV with lipoproteins seems to protect HCV from antibodies or at least attenuate their effect, perhaps via shielding of the viral surface glycoproteins (19, 25–28, 50). HCV has indeed been detected in many heterogeneous forms of diverse buoyant density (d < 1.06 to > 1.25 g/ml) in the blood of infected patients, where it is present either in “free” forms, complexed with immunoglobulins, or associated with high, low, or very low density lipoproteins (25–28, 51–54). Addressing the mechanisms by which interaction of HCV with lipoproteins may counteract the neutralizing response is a particularly complex task because ex vivo characterization of HCV derived from plasma has proven extremely difficult. To overcome these limitations, a significant number of model systems of HCV particles have been developed (6). They include the following: (i) noninfectious HCV-like particles (HCV-LP) (55–57); (ii) “infectious” HCV pseudo-particles derived from vesicular stomatitis virus (58) or from retroviruses (HCVpp) (7, 8, 59); and (iii) HCVcc that produces infectious virus particles (9–11). Recent results suggest that there may be more similarities than anticipated between current in vitro HCV models and virus purified from patients’ plasma. Indeed, HCVcc with the highest specific infectivity was shown to have a low buoyant density (9), similar to clinical HCV isolates with high infectivity in animals (51). Moreover, HCV E1E2-targeted antibodies have been detected in patients’ blood, or induced in immunized animals, that prevented contamination of patients, or infection of chimpanzees, with plasma-derived HCV (24, 60) and that readily neutralized both HCVpp and/or HCVcc (20, 24) (Fig. 7 of this report), indicating some shared serological and antigenic properties.

Neutralizing antibodies can block virus particles at different stages of the cell entry process, i.e., the interaction of viral glycoproteins with cell surface receptors, the conformational changes in the viral envelope, and/or virus uncoating (62). As shown by down-regulation/blocking experiments in permissive cells and by de novo expression in CD81-deficient hepatocarcinoma cells, CD81 appears a critical cell entry molecule of HCV (8–10, 21, 33, 63–65). It is therefore not unexpected that antibodies that block E2/CD81 interaction have a strong impact on infection and significantly reduce infectious titers of both HCVpp or HCVcc (8–10, 21, 33, 63–65). It is therefore not unexpected that antibodies that block E2/CD81 interaction have a strong impact on infection and significantly reduce infectious titers of both HCVpp or HCVcc (8–10, 21, 33, 63–65) and plasma-derived HCV.9 In contrast, the relative importance of SR-BI in the infection process is still being debated because SR-BI blocking by antibodies or its down-regulation by short interfering RNAs results in at most 50–90% inhibition of infectivity (19, 21, 33, 44), which initially suggested that it is dispensable for infection.

Our results shed light on a particularly original feature of the mechanism by which HDL induce protection of HCVpp and HCVcc from neutralizing antibodies (19). In contrast to other mechanisms of protection of plasma-derived HCV by β-lipoproteins (25–28), our results obtained by a highly sensitive BIacore analysis do not support the exist-

* P. Maurel, personal communication.
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tence of a direct physical interaction (stable or transient) between HDL and HCV glycoproteins that could explain the masking of neutralizing epitopes. Rather, we demonstrate that the attenuation of neutralization induced by HDL operates via a novel mechanism involving the biological activity of SR-BI. Recently, SR-BI was shown to influence infection by promoting interaction and cellular uptake of plasma-derived HCV associated with β-lipoproteins (50) and, at least in the HCVpp (19, 23, 44) and HCVcc (Fig. 7 of this report) in vitro infection assays, by stimulating their infectivity. Indeed, HCV appears to use SR-BI during cell entry not merely as an additional docking molecule for the viral particle (4, 33, 66) but also for exploiting its physiological activity, i.e. the capacity to mediate lipid transfer from HDL (46). Remarkably, our results show that BLT-4 and other inhibitors of SR-BI-mediated lipid transfer fully restore the potency of neutralizing antibodies in infection assays conducted in the presence of HS/HDL (Fig. 4), indicating an intriguing link between neutralization efficiency and stimulation of cell entry. Likewise, HDL/SR-BI-dependent neutralization inhibition as well as infection enhancement is under control of HVR1, the amino-terminal region of HCV-E2 that acts as a key viral element coordinating the interplay between HCV, SR-BI, and HDL (19, 44).

Our data suggest that the mechanism by which HDL increases HCVpp or HCVcc infectivity operates via stimulation of cell entry at a post-binding stage, which may involve different functions of SR-BI. On the one hand, the presence of HDL during the initial stage of infection suppresses a time lag of ~60–70 min during which cell-bound virions are not internalized (Fig. 3). It is likely that such HCVpp internalization is mediated or triggered by the interaction of HDL with SR-BI, which itself may induce HDL endocytosis (67). As HCVpp and HDL do not compete for a same binding site on SR-BI (19, 44), it is then possible that HDL/SR-BI interaction will induce the endocytosis of SR-BI-bound viral particles. On the other hand, HCV particles may interact with SR-BI and HDL to specifically target cholesterol-enriched microdomains and to stimulate local cholesterol enrichment, which would thus enhance its entry, perhaps by facilitating membrane fusion events (68) or, alternatively, by inducing conformational changes within the HCV glycoproteins that are required for membrane fusion processes. Finally, the capacity of SR-BI to modulate the lipid composition of cell membranes (69, 70) may render it more permissive to HCV entry by favoring the membrane mobility, recruitment, and/or endocytosis of other HCV receptors, such as CD81. The activity of molecules that block E2-CD81 interactions, i.e. antibodies and/or CD81-LEL polypeptides, is reduced under conditions that stimulate infection (Fig. 5), and this suggests a role for CD81 at an early step of the cell entry process. Indeed, the time lag detected for internalization of cell surface-bound viral particles may reflect the time interval required to assemble a functional HCV receptor complex and may be reduced upon SR-BI activation through modifications of the cell membrane. A possibility is that HDL/SR-BI interaction augments the rate of CD81 recruitment at virion-binding sites and/or internalization of HCV-CD81 complexes via a cholesterol-dependent pathway (71).

Our results have several implications for active and passive antibody-based immunotherapy strategies against hepatitis C. Indeed, although these data were obtained from infection assays performed in vitro, it is likely that HDL-mediated inhibition of neutralization will also operate in vivo. It is worth noting that attenuation differentially affects neutralizing antibodies, depending on their targeted epitopes and hence the cell entry functions they block (Fig. 6). That HDL/SR-BI interaction accelerates the cell entry rate of HCV is likely to have a strong impact on neutralization by antibodies that inhibit the initial virus/cell interactions, because it could indeed reduce the time window during which such antibodies are effective. Antibodies naturally induced in HCV patients are raised against both E1 and E2 glycoproteins in particular (72). Overall, that such antibodies are attenuated by HDL can be attributed to the fact that among the E1 and E2 antigens the latter is involved in interaction with CD81 (73) and hence elicits polyclonal antibodies that target the E2/CD81 interaction. Treatment of patients with drugs that inhibit the HDL/SR-BI interaction (e.g. BLT-4, gliburide) might thus prove a valid option to stimulate the neutralizing activity of antibodies inoculated in patients or naturally induced by HCV.

Interestingly, our data show that some antibodies that resist attenuation can be retrieved from patients (e.g. H111) or from immunized rodents (e.g. 9/27, H60, E1 polyclonal antibodies) and clearly block cell entry functions different from E2/CD81 interactions (Table 2 and Fig. 6). This provides guidance for the screening of neutralizing mAbs and for designing vaccines that induce efficient antibodies in vivo. Because HVR1 coordinates the HDL/SR-BI interplay (19, 44), it is expected that HVR1-targeted antibodies are not attenuated by HDL. Conserved amino acid positions have been suggested to maintain a global conformation of HVR1 (74), perhaps in connection to its role in mediating the HDL/SR-BI interplay (19), and this may be used to design cross-reactive antibodies (75). On the other hand, E1-targeted antibodies may also be valuable to neutralize HCV, as shown here for the first time using infectious HCV (Fig. 7). Little is known about the functions of E1 during cell entry; yet it has been proposed to harbor fusion functions (73, 76). It will be highly interesting to discover the neutralizing epitopes targeted by E1 antibodies and the viral functions suppressed by such antibodies.

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