High-density lipoproteins reduce the neutralizing effect of hepatitis C virus (HCV)-infected patient antibodies by promoting HCV entry

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The neutralizing activity of anti-hepatitis C virus (HCV) antibodies is attenuated by a factor present in human sera, which has been proposed to be high-density lipoproteins (HDLs). HDLs have also been shown to facilitate the entry of HCV pseudoparticles (HCVpp) into target cells. Here, the aim of the study was to determine whether HDL-mediated facilitation of HCVpp and infectious HCV (HCVcc) entry and attenuation of neutralization are two related phenomena. The data indicated that HDLs attenuate neutralization at a constant rate. In addition, as for HDL-mediated facilitation of HCVpp entry, attenuation of neutralization depended on the expression of the scavenger receptor BI (SR-BI) and its selective lipid-uptake function. Finally, kinetic experiments showed that HDL-mediated facilitation of HCVpp entry is more rapid than virus neutralization. Altogether, these observations indicate that HCV is exploiting the physiological activity of SR-BI for promoting its entry into target cells, which consequently also protects the virus against neutralizing antibodies.

HCVpp containing subtype 1a envelope glycoproteins (strain H) and infectious JFH1 virus (HCVcc) were generated as described previously (Bartosch et al., 2003b; Rouillé et al., 2006; Wakita et al., 2005). Infection experiments were performed on Huh-7 cells (Nakabayashi et al., 1982). HCVpp and HCVcc were produced in lipoprotein-free medium (2% fetal calf lipoprotein-depleted serum, LPDS) and neutralization experiments were also performed in medium supplemented with 2% LPDS. Human HDL (density, 1.13–1.18 g ml⁻¹) fractions from fresh human plasma were isolated as described by Hatch (1968). A series of sera from patients infected chronically with various HCV genotypes was used.

To evaluate the impact of HDLs from HCV-positive sera on HCVpp neutralization, sera containing HDLs and antibodies were co-incubated with HCVpp and target cells.
Classically, neutralization assays are performed by pre-incubating virus and sera from infected patients before contact with target cells. However, as HDLs potentially have an effect on virus entry by acting directly on the target cell, we considered that it would be more relevant to study the effect of HDLs on neutralization by co-incubating sera, HCVpp and target cells. In these conditions, we observed that only two of the 10 HCV-positive sera tested were able to neutralize HCVpp entry by >50% (Fig. 1a). In contrast, all of the sera displayed a neutralizing activity in a classical neutralization assay based on a pre-incubation of HCVpp and serum before contact with target cells (Fig. 1a). These data show that, in most cases, HCV-positive sera are not able to neutralize HCVpp when antibodies and HDLs are incubated simultaneously with HCVpp and the cells.

As HCV-positive sera contain various concentrations of antibodies and HDLs, we used antibodies purified from HCV-positive sera and purified HDLs in our neutralization assay. The neutralizing activity of anti-HCV antibodies was analysed in the presence or absence of HDLs. As shown in Fig. 1(b, c), the neutralizing activity of anti-HCV antibodies on HCVpp and HCVcc entry was reduced strongly in the presence of HDLs. Interestingly, the presence of HDLs always enhanced the infectivity levels of HCVpp by approximately three times (Fig. 1d). In addition, this enhancement of infectivity was very similar for all of the antibodies tested, whether they were neutralizing or not (Fig. 1d). Similar data were obtained by using HCVpp bearing E1E2 from different genotypes (data not shown). These data suggest that the constant enhancement obtained in the presence of HDLs may be related to a constitutive action of HDLs on target cells, independently of HCVpp neutralization by antibodies. This is in agreement with the absence of interaction between HDLs and HCVpp (Voisset et al., 2005).

To further investigate the mechanism of HDL-mediated attenuation of neutralization, we knocked down SR-BI

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**Fig. 1.** HDLs attenuate HCVpp neutralization at a constant rate. (a) Neutralization experiments were performed by either pre-incubating HCVpp with 2% human sera from HCV-infected patients for 2 h at 37°C before contact with Huh-7 cells (empty bars) or by simultaneous co-incubation of HCVpp, 2% serum from HCV patients and Huh-7 cells for 3 h at 37°C (shaded bars). HCV” corresponds to a representative healthy individual. Control experiments (ctrl) were performed by co-incubating HCVpp in medium supplemented with 2% LPDS in the absence of human serum. (b) Neutralization experiments were performed by simultaneous co-incubation of HCVpp, antibodies purified from human sera (15 μg ml⁻¹) and Huh-7 cells for 3 h at 37°C in the absence (empty bars) or the presence (shaded bars) of 30 μg HDLs ml⁻¹. (c) Neutralization experiments were performed by simultaneous co-incubation of HCVcc, antibodies purified from human sera (60 μg ml⁻¹) and Huh-7 cells for 2 h at 37°C in the absence or in the presence of 60 μg HDLs ml⁻¹. Data obtained with patient P8 are representative of the data obtained by using antibodies from other patients that are able to neutralize HCVcc entry (data not shown). Infectivity was measured by quantification of the expression of E2 and is presented as percentage of infectivity of the untreated virus. (d) Each dot represents the ratio between the level of HCVpp infectivity obtained in the presence of both HDLs and antibodies, or in the presence of antibodies purified from the serum of HCV-infected patients. The results of (a) and (b) are presented as percentages of the infectivity relative to infectivity of HCVpp in 2% LPDS medium (ctrl) in the absence of antibodies. Results are reported as the mean±SD of three independent experiments. Pseudoparticles bearing no envelope glycoproteins displayed an infectivity of <2%.
expression. HDLs are indeed a physiological ligand of SR-BI (Rhainds & Brissette, 2004). RNA-interference experiments were done as described previously (Voisset et al., 2005). SR-BI-specific small interfering RNA (siRNA) downregulated SR-BI expression to approximately 70% compared with the negative-control siRNA (data not shown). As observed previously (Voisset et al., 2005), when a specific siRNA against SR-BI was used, HCVpp infectivity was not affected in the absence of HDLs, whereas HDL-mediated enhancement of infectivity was reduced strongly (Fig. 2a). This contrasts with another study showing that silencing of SR-BI expression reduces HCVpp infectivity (Lavillette et al., 2005b). The discrepancy between these data may potentially be explained by differences in the levels of downregulation of expression of SR-BI in these experiments. Indeed, one cannot exclude the possibility that HCV requires only very small amounts of SR-BI to enter target cells. Interestingly, when SR-BI expression was downregulated, the efficiency of neutralization of sera was increased markedly and reached neutralization levels similar to those obtained with purified antibodies in the absence of HDLs (Fig. 2a). To further investigate the role of SR-BI in HDL-mediated attenuation of neutralization, we also analysed the effect of BLT-4, a drug known to block SR-BI-mediated selective uptake of HDL lipids (Nieland et al., 2002). This compound has also been shown to strongly reduce the HDL-mediated enhancement of HCVpp entry (Bartosch et al., 2005; Voisset et al., 2005). Interestingly, when the cells were treated by BLT-4, HCVpp were neutralized more efficiently by antibodies from HCV-positive patients, despite the presence of HDLs (Fig. 2b). These data indicate that HDL-mediated attenuation of HCVpp neutralization is dependent on SR-BI and its selective lipid-uptake property. In addition, they also show that HDL-mediated facilitation of HCVpp entry and attenuation of neutralization are two related phenomena.

We then hypothesized that HDLs accelerate HCVpp entry and thus permit HCVpp to escape the immune response. Indeed, as we described previously, HDL facilitation of HCVpp entry is a post-binding event (Voisset et al., 2005), which indicates that HDLs may favour internalization of virions and thus escape from neutralization. Therefore, we analysed the kinetics of HCVpp entry in the presence of either HDLs or antibodies. It is important to note that the kinetic experiments were performed at early time points and, under these conditions, HCVpp infectivity had not yet reached saturation (data not shown). As shown in Fig. 3(a), HDL facilitation of HCVpp entry was already efficient after 15 min incubation, whereas neutralization by antibodies from HCV-infected patients was only observed after longer incubation times (Fig. 3a; data not shown). Thus, the kinetics of HDL facilitation of HCVpp entry were more rapid than the kinetics of neutralization by antibodies from HCV-infected patients. These data indicate that HDLs may overcome antibody neutralization by reducing the time window during which the antibodies can bind to and neutralize HCVpp.

Antibodies purified from HCV-positive patients are a pool of antibodies potentially directed against various epitopes. In addition, the proportion of neutralizing antibodies in patient sera is unknown. We therefore also analysed the effect of HDLs on the behaviour of some well-characterized neutralizing mAbs. For the majority of neutralizing mAbs tested, the presence of HDLs attenuated their neutralizing
activity, as shown for H48 (Fig. 3b). However, the neutralizing activity of mAb 9/27 was not affected by HDLs (Fig. 3c). Comparison of the kinetics of HCVpp entry in the presence of either HDLs or mAbs showed that, as observed for antibodies from HCV-infected patients, neutralization by mAb H48 was rather slow (Fig. 3d). In contrast to mAb H48, the kinetics of neutralization by mAb 9/27 were very rapid (Fig. 3d). Therefore, the absence of HDL effect on the neutralizing activity of mAb 9/27 is probably due to its rapid kinetics of neutralization, suggesting that mAb 9/27 might work slightly before HDLs can provide a signal to facilitate HCVpp entry. As neutralization by antibodies isolated from HCV-infected patients was attenuated by HDLs, neutralizing antibodies behaving like mAb 9/27 are probably induced poorly in a natural infection. Together, these data indicate that the rapid kinetics of HDL facilitation of HCVpp entry may allow HCVpp to escape partially from some neutralizing antibodies, like mAb H48, but not all of them, as shown for mAb 9/27. The 9/27 epitope is located in HVR1 (Hsu et al., 2003), whereas the H48 epitope is conformation-dependent (J. Dubuisson, unpublished data) and is located in the CD81-binding region of E2 (J. Ball & A. Patel, personal communication). Our data indicate that the difference of neutralization between 9/27 and H48 in the presence of HDLs is probably related to their relative affinity for their respective epitope and/or to the accessibility of these epitopes. However, we cannot exclude the possibility that mAbs 9/27 and H48 recognize different epitopes, which may be required for interactions with receptor(s) that are influenced differentially by HDLs.

The exact role of SR-BI in HCV entry is far from understood. SR-BI has been shown to modulate the plasma-membrane composition (Huang et al., 2003; Peng et al., 2004). The relationship between SR-BI and HCV infection may thus be based on the capacity of SR-BI to modulate the lipid composition of the plasma membrane to render the membrane permissive to HCV entry. In agreement with this hypothesis, the SR-BI-mediated selective uptake of lipids from HDLs has been shown to enhance HCVpp entry markedly (Bartosch et al., 2005; Voisset et al., 2005). It is thus tempting to speculate that SR-BI is involved at some
stage of HCV entry by modulating the plasma-membrane composition.

In conclusion, our data demonstrate that HDLs reduce HCVpp neutralization by accelerating HCVpp entry, through the lipid-transfer property of SR-BI. The rapid kinetics of HDL facilitation of HCVpp entry may allow HCVpp to escape some neutralizing antibodies. While antibodies bind to HCVpp and neutralize their entry, HDLs accelerate the entry of HCVpp that are not yet neutralized.

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