Title: OPTIMIZATION OF A TCP CONNECTION

Abstract: The present invention relates to a method for selecting an HCV epitope which is not sensitive to HDL-mediated decrease of the neutralizing capacity of anti-HCV antibodies, as well as to methods for treating an HCV-infected patient or a subject who has been exposed to HCV, comprising administering to the patient a therapeutically effective amount of a compound inhibiting HCV/HDL/SR-BI interplay, or comprising reducing the plasma loads of HDL.
METHODS FOR ENHANCING THE POTENCY OF HCV NEUTRALIZING ANTIBODIES

FIELD OF THE INVENTION

The present invention is in the field of hepatitis virology. More specifically, the present invention relates to methods for treating HCV-infected patients and to pharmaceutical compositions useful for the treatment of HCV-infected patients.

BACKGROUND OF THE INVENTION

Hepatitis C poses a major public health problem with nearly 3% of the world population infected and approximately 3 to 4 million new infections each year (20). The prevalence of Hepatitis C virus (HCV) infection is currently about 5 million cases in Europe, 4 million in the US and 2 million in Japan, and the disease burden and costs due to complications are estimated to continue to increase in these countries until 2015-2020. In other parts of the world, the prevalence is on average higher and the peak disease burden will likely appear later. In the US, HCV infection is the most common chronic blood-borne infection and HCV-associated chronic liver disease is the principal cause of liver transplantation and the tenth leading cause of death among adults (25). At present, there exists no vaccine against HCV infection and the only authorized treatments, Interferon-alpha and Ribavirin, have shown limited effect against HCV in general and against genotype 1 in particular and have significant side effects (26).

HCV is transmitted by blood and progresses slowly, causing no or only mild symptoms in the acute phase of infection. However, only 20% of infected individuals clear the virus spontaneously while 80% develop chronic disease which in one out of five cases leads to various severe hepatic pathologies (cirrhosis and hepatocarcinoma) in the long term. The factors leading to spontaneous clearance of HCV or to viral persistence are elusive. Understanding virus-host interactions that enable acute viral clearance or that favor HCV persistence is key to the development of more effective therapeutic and prophylactic strategies. Although the HCV genome was cloned 15 years ago and abundant studies were devoted to its description, investigations of HCV biology have been difficult for several reasons (23). HCV is genetically highly diverse, comprising six principal genotypes and numerous subtypes. No cell culture system supports propagation of HCV in
vitro and the development of small animal model systems for the analysis of HCV pathology has been unsuccessful.

In human patients, HCV has been described to exist in heterogeneous forms within sera. By density equilibrium centrifugation, HCV genomes are detected in high-density fractions where they are thought to represent virions bound to immunoglobulins. In addition HCV can also be detected in fractions of low density, within the range occupied by plasma lipoproteins. Indeed, several lines of evidence suggest that HCV associates with lipoprotein particles of very low, low and high densities (1, 16, 18, 27, 28, 37, 45). Furthermore several studies have shown a correlation between acute or persistent liver damage and detection of lipoprotein-associated, rather than immunoglobulin-associated HCV (16, 18).

Therefore, novel therapeutic strategies are urgently required as the health costs for HCV infected people are predicted to spiral dramatically in the next few decades. Passive immunotherapy by means of inoculation of HCV-neutralizing antibodies is an option for treating patients chronically infected with HCV. Although such monoclonal antibodies or HCV Ig derived from chronically-infected patients are efficient to inhibit cell entry of HCV in vitro, they are likely to be poorly effective in vivo.

**SUMMARY OF THE INVENTION**

The present invention provides new prophylactic and/or therapeutic treatments of HCV based on modulation of HCV/HDL/SR-BI (scavenger receptor B1) interplay in a subject. The invention is based on the discovery of the involvement of HDL (High Density Lipoprotein) in the infectivity of HCV and in the protection of HCV against HCV-neutralizing antibodies. The inventors contemplated various mechanisms to account for these observations. They first thought of a HDL-mediated shielding. By investigating the mechanism that modulates neutralisation desensitisation by HDL, they demonstrated that HDL-mediated activation of the scavenger receptor B1 strongly reduces neutralisation of HCV by antibodies that target the interaction of E2 with CD81. They further showed that the inhibition of HDL/SR-BI interaction restores the neutralising activity of antibodies in human serum.

On this basis, the present invention relates to methods for selecting an HCV epitope that is not sensitive to HDL-mediated desensitisation, as well as to new prophylactic and/or therapeutic treatments of HCV based on the circumvention of the protection of HCV by HDL against HCV-neutralizing antibodies.
Accordingly, the present invention concerns a method for selecting an HCV epitope which is not sensitive to HDL-mediated decrease of the neutralizing capacity of anti-HCV antibodies, said method comprising:

- testing an antibody against an HCV epitope for its capacity to neutralise HCV or HCV pseudoparticles in the presence of HDL and/or human serum and in the absence of HDL and/or human serum;
- comparing the capacity to neutralise HCV or HCV pseudoparticles in the presence of HDL and/or human serum and in the absence of HDL and/or human serum;
- selecting the HCV epitope if the capacity of an antibody to neutralise HCV or HCV pseudoparticles is not substantially decreased in the presence of HDL and/or human serum.

The present invention also concerns a method for treating HCV infection in a patient, comprising administering to the patient a therapeutically effective amount of a compound inhibiting HCV/HDL/SR-BI interplay, e.g. HDL/SR-BI interaction, or HCV-HDL complexes. A possible mechanism is that the therapeutically effective amount of a compound inhibiting HCV/HDL/SR-BI interplay will decrease the infectivity of HCV and increase the sensibility of HCV to HCV-neutralizing antibodies. Preferably, the method further comprises the administration of at least one HCV-neutralizing antibody to said patient. Alternatively, the method can also comprise the administration of an HCV antigen for eliciting HCV-neutralizing antibodies in said patient. In a particular embodiment, the compound inhibiting HCV/HDL/SR-BI interplay is a compound which decreases the plasma loads of HDL in said patient. In another particular embodiment, the compound inhibiting HCV/HDL/SR-BI interplay is a compound which inhibits the interaction between HDL and HCV, directly or indirectly, i.e. more particularly through HDL binding to SR-BI. Indeed, in the most preferred embodiment, the compound inhibits the HDL-SR-BI interaction.

The term "HCV/HDL/SR-BI interplay" refers to the direct or indirect interactions between the three components HCV, HDL, and SR-BI.

The present invention further concerns a method for treating HCV infection in a patient, comprising administering to the patient a therapeutically effective amount of at least one HCV-neutralizing antibody or an HCV antigen for eliciting HCV-neutralizing antibodies, wherein said HCV-neutralizing antibody is specific of an epitope which is not
sensitive to HDL-mediated decrease of the neutralizing capacity of anti-HCV antibodies. In a preferred embodiment, said epitope is selected from E1 fragment or HVR1. For example, a particular epitope from E1 is E1 (192-202).

Additionally, the present invention concerns a pharmaceutical composition comprising a compound inhibiting HCV/HDL/SR-BI interplay and at least one HCV-neutralizing antibody, for simultaneous, separate or sequential administration. Alternatively, a pharmaceutical composition according to the present invention comprises a pharmaceutical composition comprising a compound inhibiting HCV/HDL/SR-BI interplay and an HCV antigen for eliciting HCV-neutralizing antibody, for simultaneous, separate or sequential administration.

The present invention further relates to a method for treating HCV infection in a patient, comprising reducing the plasma loads of HDL in said patient. The method is useful for treating a patient infected by HCV or a subject who has been exposed to HCV. As shown in the present invention, a reduction of the plasma loads of HDL in said patient will decrease the infectivity of HCV and increase the sensibility of HCV to HCV-neutralizing antibodies. Preferably, the method further comprises the administration of at least one HCV-neutralizing antibody to said patient. Alternatively, the method can also comprise the administration of an HCV antigen for eliciting HCV-neutralizing antibodies in said patient. In a particular embodiment, a reduction of the plasma loads of HDL in said patient is performed by depletion of HDL by plasmapheresis. In an alternative particular embodiment, a reduction of the plasma loads of HDL in said patient is performed by administering to the patient a compound which decreases the plasma loads of HDL.

**LEGEND OF THE FIGURES**

**Fig. 1. Enhancement of HCVpp infectivity by HS.** Results of infection assays on Huh-7 cells using HCVpp of genotype 1a or, as control, RD114pp. The infectious titters of the HCVpp and RD114pp were ca. 5x10⁴ and 10⁷ i.u./ml, respectively. The concentration of either virus was therefore adjusted to obtain infection of ca. 5-10% target cells. **Figure 1A.** Production of virions in cell culture media containing the indicated quantities of normal human serum (HS) or fetal calf serum (FCS). Results are expressed as percentages of the average infectious titters ± standard deviations (n=5) relative to titers determined in the absence of serum. The inset shows a western-blot of viral particles pelleted through 20% sucrose-cushions that were produced in the absence or in the presence of 1% HS. The
glycoproteins of HCVpp were revealed using the A4 and H52 monoclonal antibodies against E1 and E2. The glycoproteins of RD114pp were detected using an anti-SU antiserum (ViroMed Biosafety Laboratories, USA). The MLV capsid (MLV CA) proteins of either pseudo-particle were detected with an anti-capsid antiserum (ViroMed Biosafety Laboratories, USA). Figure 1B. Viral supernatants produced in low serum medium (0.1% FCS) were used in infection reactions to which sera from the indicated species were added to 1%. The results are expressed as percentages of the average infectious titers ± standard deviations (n=3) relative to titers determined in 0.1% FCS. Figure 1C. Viral supernatants produced in low serum medium (0.1% FCS) were incubated with 2.5% normal HS, 2.5% immunoglobulins-depleted HS (HS Ig depl), 1.4 µg/µl purified Ig from a pool of HBV-positive patients (HBV Ig), or 2.5% heat-inactivated HS (HS decomp). The results are expressed as percentages of the average infectious titers ± standard deviations (n=3) relative to titers determined in 0.1% FCS (no HS). Virions harboring the RD114 glycoprotein (not sensitive to human complement) or the VSV-G glycoprotein (sensitive to human complement) (40) were used as control. Figure 1D. Pseudo-particles harboring the glycoproteins derived from the indicated viruses (vesicular stomatitis virus (VSV), RD114, fowl plague virus (FPV), amphotropic murine leukemia virus (MLV-A), Lassa virus (LV), lymphocytic chorio-meningitis virus (LCMV), human immunodeficiency virus (HIV) and west Nile virus (WNV)) were produced in low serum medium (0.1% FCS) and used in infection assays on Huh-7 target cells and, in the case of HIVpp, on CD4-transfected HOS (human osteosarcoma) cells, which express SR-BI (4). Infections were performed in the presence of 2.5% FCS or normal HS, 6 µg/ml HDL or 39 µg/ml LDL, as indicated. The results are expressed as percentages of the average infectious titers ± standard deviations (n=3) relative to titers determined in 0.1% FCS.

Fig. 2. Enhancement of HCVpp infectivity by lipoproteins. Results of infection assays on Huh-7 cells using HCVpp (genotype 1a) and RD114pp. Similar inputs of viral particles were used in the experiments, allowing infection of ca. 5-10% target cells. Figure 2A. Production of virions in cell culture media containing 0.1% FCS and defined quantities of purified vLDL, LDL and HDL, as indicated in µg/ml of cholesterol-lipoprotein. The results are expressed as percentages of the average infectious titers ± standard deviations (n=4) relative to titers determined in the absence of lipoproteins. The inset shows a western-blot of viral particles pelleted through 20% sucrose-cushions that were produced in low serum medium supplemented, or not, with 6 µg/ml LDL and HDL, as indicated. The
glycoproteins of the HCVpp were revealed using the A4 and H52 monoclonal antibodies against E1 and E2. The glycoproteins of RD114pp were detected using an anti-SU antiserum (ViroMed Biosafety Laboratories, USA). The MLV capsid (MLV CA) proteins of either pseudo-particles were detected with an anti-capsid antiserum (ViroMed Biosafety Laboratories, USA). Figure 2B. HCVpp produced in cell culture media containing 0.1% FCS and defined quantities of purified vLDL, LDL and HDL, as indicated in μg/ml, were used in infection reactions in Huh-7 target cells to which normal HS was added to 1% (left). HCVpp produced in cell culture media containing the indicated amounts of normal HS (%) were used in infection reactions in Huh-7 target cells to which HDL were added to 6 μg/ml (right). The results are expressed as percentages of the average infectious titers ± standard deviations (n=3) relative to titers determined in 0.1% FCS. Figure 2C. A viral supernatant produced in low serum medium (0.1% FCS) was incubated with defined quantities of vLDL, LDL and HDL, as indicated in μg/ml (left) or with defined quantities of an HDL-deficient serum from a Tangier disease patient (HDL-def. HS), as compared to normal HS (right). The results are expressed as percentages of the average infectious titers ± standard deviations relative to titers determined in the absence of lipoproteins (left panel) or HS (right panel).

Fig. 3. Role of SR-BI in facilitation of infection. Figure 3A. Results of infection assays on Huh-7, PLC/PRF/5, HepG2, HepG2-CD81, SW-13 or SK-Hep1 cells that express, or not, SR-BI receptors. HCVpp and RD114pp were produced in cell culture media containing 0.1% FCS. The infectious titers of HCVpp on these cells were: SK-Hep1 1.2x10^3 i.u./ml, HepG2 0 i.u./ml, HepG2-CD81 10^3 i.u./ml, PLC/PRF/5 5.7x10^4 i.u./ml, SW-13 1.3x10^4 i.u./ml and Huh-7 2.4x10^4 i.u./ml. Infection assays were performed in the presence of 1% normal HS added during infection. The results are expressed as percentages of the average infectious titers ± standard deviations (n=3) relative to titers determined in the absence of serum. The inset shows the expression levels of SR-BI in these cells analyzed by Western-blot analysis of cell lysates using an SR-BI rabbit antiserum (ab396, Abcam; 1/1,500), as described previously (22). Figure 3B. HCVpp or RD114pp were produced in cell culture media containing 0.1% FCS and used in infection assays on Huh-7 cells in the absence (no HS) or in the presence of 1% HS or 6 μg/ml HDL. The same set of infections was performed in the presence of a 1/50-diluted polyclonal anti-SR-BI mouse serum (4). Figure 3C. Huh-7 cells expressing a control or an anti-SR-BI siRNA, as described previously (22), were tested as target cells of HCVpp or
RD114pp produced in 0.1% FCS, in the absence (no HS) or in the presence of HS, as indicated. **Figure 3D.** Huh-7 target cells were treated with 16.7 μM or with 50 μM of BLT compounds diluted in DMSO before and during infection with HCVpp or RD114pp that were produced in 0.1% FCS. Infection assays were performed in the absence (no HS/HDL) or in the presence of 2.5% normal HS or 6 μg/ml HDL added during infection. The results show the ratios of infectivity (means ± standard deviations; n=3) relative to titers determined in the absence of serum.

**Fig. 4. Role of HVR1 in facilitation of infection.** Figure 4A. Immunoblots of HCVpp generated with E1E2 wild type or mutant glycoproteins (genotype 1a) pelleted through 20% sucrose-cushions are shown. E2 point mutations G389L, L399R, G406R, G406L, are located in the HVR1 region. Y276F, an E1 point mutant, served as control. E1, E2 and the MLV capsid (MLV CA) proteins were revealed with A4 and H52 monoclonal antibodies against E1 and E2 or with an anti-MLV capsid antiserum (ViroMed Biosafety Laboratories, USA). **Figure 4B.** Infectivity of HCVpp harboring point mutations. The results are expressed as the average infectious titers ± standard deviations (n=3) determined on Huh-7 cells in the absence of serum or lipoproteins. **Figure 4C.** Results of infection on Huh-7 cells with HCVpp or RD114pp produced in 0.1% FCS (no HS/HDL), in the absence or in the presence of 2.5% normal HS or 6 μg/ml HDL added during infection. To compare the effect of HS or lipoproteins on similar viral titers, the concentrations of the virions were adjusted, allowing infection of ca. 5-10% target cells. The results are expressed as percentages of the average infectious titers ± standard deviations (n=3) relative to titers determined in the absence of serum or lipoproteins.

**Fig. 5. Human serum or HDL protects HCVpp from neutralization.** Figure 5A.

Effect of sera from a cohort of acutely infected HCV patients (21) on infectivity of the indicated HCVpp. The individual kinetics of 4 patients are shown. Patients Pt-3 and Pt-4 are representative of a first group (7/13 patients) who exhibited significant HCV RNA fluctuations leading to control of viral replication (21). Patients Pt-8 and Pt-9 representative of a second group of patients (6/13 patients) who exhibited sustained high replication levels throughout the entire study period (21). The effect of each serum sample on the infectivity of HCVpp of genotype 1b (strain CG1b; dotted lines, open squares), 1a (strain H77; dotted lines, open circles), or of their HVR1-deleted counterparts (plain lines, closed squares and circles, respectively) was analyzed by incubating identical ratios of
viral particles (10^4 i.u.) and sera (1/50 dilution) for 30 min at room temperature before infection of Huh-7 target cells. The results are expressed as the mean percentages (mean±SD, n=3) of inhibition of the infectious titers relative to incubation with medium devoid of patient sera. Note that the neutralization response was hardly detected with HCVpp-1a for patients of group 1 (Pt-3 and Pt-4) and was not detected with both HCVpp-1a and HCVpp-1b for patients of group 2 (Pt-8 and Pt-9), as detailed in Table 2. The specificity of neutralization was controlled by using RD114pp as control pseudo-particles, against which no antibodies are detected in HS (3). As expected, non-specific inhibition of RD114pp was never detected over a value of ±20%. Individual HCV RNA kinetics (thin lines, as IU/ml) were measured on a weekly basis following inclusion of the patients in the cohort. Figure 5B. Titration of neutralizing antibodies in total IgGs purified from chronically infected HCV patients. The neutralization assays were performed in the absence (no HS) or in the presence of 2.5% HS, of 39 µg/ml LDL or 6 µg/ml HDL. The results are expressed as the mean percentages (mean±SD, n=4) of inhibition of the infectious titers relative to incubation with medium devoid of antibodies. C. Neutralization curves of the AP33 monoclonal HCV E2 antibody in the absence (no HS) or in the presence of 2.5% HS using HCVpp harboring the indicated point mutations in HVR1. The results are expressed as the mean percentages (mean±SD, n=3) of inhibition of the infectious titers relative to incubation with medium devoid of antibodies. The influence of HS and HVR1 in protection from neutralization is shown by the variations of antibody concentrations required to inhibit e.g., 50% of the infectivity of HCVpp (dotted lines).

Fig. 6. Detection of neutralising antibodies in immunised mice. Balb/c mice were IP inoculated with concentrated HCVpp harbouring the E1 glycoprotein of genotype 1a (strain H77). For the primo-injection as well as for the subsequent boosts (4 boosts in total), each mouse received about 1e7 particles of HCVpp, conditioned in 100 ul of a PBS suspension. The inoculation were separated by about 20 days. The mouse sera were harvested from the eye veins and were used in neutralisation assays using HCVpp of genotype 1 to 6, as described in Lavillette et al., 2005. The figure shows the results obtained with the serum of one of these mice, yet similar results could be generated with the other sera, in neutralisation of a selected panel of genotyped HCVpp, as indicated. The results are provided as the residual infectivity of these HCVpp following serum treatment and subsequent infection of Huh-7 target cells. Infectivity was measured by FACS analysis, using aa GFP marker gene inserted in the pseudoparticles, as described previously.
(3). Control pseudo-particles (Control pp) were generated with the RD114 glycoprotein. The control serum was made by pooling the sera of mice immunised with control pseudo-particles harbouring the RD114 glycoprotein. The positive control serum was a serum from a chronic HCV-infected patient, as described previously (3, 22).

Figure 7. Infection enhancement and neutralisation desensitisation of HCVpp by human serum. (A) Results of infection assays on Huh-7 cells using HCVpp of genotype 1a. HCVpp were produced in low serum medium (0.1% FCS) to which human serum (HS), HDL and/or polyclonal antibodies derived from a pool of chronic patients (patient polyclonal Ab), the AP33 monoclonal E2 antibody (monoclonal Ab) were added during infection as indicated (2.5%, 6 μg/ml, 35 μg/ml, 2 μg/ml respectively). The results show the percentages of infection determined by calculating the ratio between average infectious titers (n=6) determined in the presence of human serum, HDL, neutralising antibodies or in the absence of human serum and neutralising antibody. (B) Titration curves of the AP33 monoclonal E2 antibody deduced from the results of infection assays in the absence (-) or in the presence of 2.5% HS or 6 μg/ml HDL. The results are expressed as the mean percentages (mean ± SD, n=6) of inhibition of the infectious titres relative to incubation with the relevant medium devoid of antibodies (in the presence or in the absence or in the presence of human serum and HDL). The percentage of desensitisation is determined by calculating the mean percentage of inhibition of the neutralisation in the presence of human serum relative to the neutralisation in the absence of human serum.

Figure 8. Absence of direct interaction between HDL and HCVpp. (A) Infection assays before or after removal of HDL from viral particles. HCVpp preincubated with or without 2.5% HS and/or 4 μg/ml of AP33 antibody (mAb) were purified by ultracentrifugation through a 20%-sucrose cushion. Infection assays were performed with non-purified or purified viral. The results show the percentages of infection determined by calculating the mean percentages (mean ± SD, n=4) of infectious titres relatives to the infectious titres in the absence of human serum and antibody (B) Detection of interaction of HCVpp ligands by surface plasmon resonance (Biacore) analysis. HCVpp or HDL-treated HCVpp, as indicated, were captured by the AP33 monoclonal E2 antibody covalently immobilized to the dextran matrix. Purified CD81-LEL, A4 monoclonal E1 antibody or HDL, as indicated, were injected over captured HCVpp at 20 μmol/l, 1.33
μmol/l and 4.61 μmol/l, respectively and the binding of either molecule was determined. All curves represent specific binding after subtraction of non-specific binding to a flow cell with immobilized irrelevant antibody.

**Figure 9. Involvement of the scavenger receptor BI in neutralisation desensitisation.** Results of infection assays on Huh-7 cells using HCVpp of genotype 1a (A, B) and RD114pp as control (B). (A) The results of infection enhancement (fold increase of infection on the x-axis) and neutralisation inhibition (% desensitisation on the y-axis) were calculated for different concentrations of HS or HDL and using the AP33 monoclonal neutralising antibody at 2 μg/ml. The percentage of desensitisation is determined by the mean percentages (n=4) of inhibition of the neutralisation in the presence of different concentrations of HS or HDL relative to the neutralisation in the absence of human serum. (B) Results of infection assays performed in the absence or in the presence of 2.5% HS, 6 μg/ml of HDL, 2 μg/ml of the AP33 antibody and 16.7 or 50 μM of the BLT-4 compound that inhibits SR-BI-mediated cholesterol uptake from HDL, as indicated. The results of the left panel show the fold increase of infection in the absence or in the presence of 16.7 or 50 μM of the BLT-4 compound determined by calculating the ratio between average infectious titers (mean ± SD, n=3) determined in the presence or in the absence of HS or HDL. The results of the right panel show the neutralisation assays by the AP33 antibody in the presence or in the absence of 16.7 or 50 μM of the BLT-4 compound and HS or HDL. The results are expressed as the percentage of desensitisation determined by calculating the mean percentages (mean ± SD, n=3) of inhibition of the neutralisation in the presence of human serum or HDL relative to the neutralisation in the absence of HDL and HS.

**Figure 10. Human serum reduces the role of CD-81 in infection.** Results of infection assays performed with HCVpp of genotype 1a (A, B, C) or HVR1-deleted HCVpp (D) in the absence or presence of 2.5% human serum (HS), and varying concentration of CD-81-blocking antibody (A) or CD81-LEL (B, C, D) and BLT-4 compound that inhibits SR-BI-mediated cholesterol uptake from HDL (C). The results show the inhibition curves in the absence (-) or in the presence of 2.5% HS and are expressed as the mean percentages (mean ± SD, n=4) of inhibition of the infectious titers relative to incubation with medium devoid of inhibitors (A, B, D). In panel (C), the results show the percentage of desensitisation determined by calculating the mean percentages
(mean ± SD, n=4) of inhibition of the competition by CD81-LEL in the presence of human serum relative to the competition in the absence of human serum.

**Figure 11. Sensitivity of monoclonal antibodies to neutralisation 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 ± SD, n=3) of inhibition of the infectious titres relative to incubation with medium devoid of antibody. **(A)** Results obtained with monoclonal antibodies that block the interaction between E2 and CD81 (Table 5). **(B)** Results obtained with monoclonal antibodies that do not block the interaction between E2 and CD81.

**Figure 12. Neutralisation of HCVpp and cell culture-grown authentic HCV.** (A) Titration curves of HCVpp neutralisation by a polyclonal E1 antibody in the absence (---) or in the presence of 2.5% HS. The results are expressed as the percentages (mean ± SD, n=3) of inhibition of the infectious titres relative to incubation with medium devoid of antibody. No neutralisation by polyclonal E1 antibody of control pseudo-particles harbouring RD114 glycoproteins could be detected (not shown). **(B)** Results of infection assays on Huh7-Lunet cells using cell culture-grown authentic HCV (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% human serum (HS) or 6 μg/ml HDL were added during infection assays, as indicated. The results show the percentages of infection (mean ± SD, n=2) determined by the ratio of the average infectious titres in the presence of the indicated components relative to the average infectious titres in the absence of human serum or HDL. **(C)** Restoration of neutralisation of HCVcc by the BLT-4 SR-BI inhibitor. Neutralising assays were performed with the E2mAb-1 E2 antibody (Monoclonal Ab) and with polyclonal antibodies derived from a pool of chronic patients (Patient polyclonal Ab) in the absence (---) or in the presence of 50 μM of BLT-4. The results are expressed as the percentages (mean ± SD, n=3) of inhibition of the infectious titres relative to incubation with medium devoid of antibody. **(D)** Titration curves of HCVcc neutralisation by a polyclonal E1 antibody in the absence (---) or in the presence of 2.5% HS. The results are expressed as the percentages (mean ± SD, n=2) of inhibition of the infectious titres relative to incubation with medium devoid of antibody.
DETAILLED DESCRIPTION OF THE INVENTION

The inventors have demonstrated that, unexpectedly human serum, and more particularly HDL, enhances the infectivity of hepatic cells by HCV. This infection enhancement requires SR-B1, a receptor for HDL, and involves conserved amino-acid positions in the hyper-variable region-1 (HVR1) of the E2 glycoprotein. Additionnally, the inventors have shown that the presence of human serum or HDL, but not LDL, leads to protection of HCV from neutralizing antibodies.

Furthermore the inventors have identified monoclonal antibodies targeted against neutralising epitopes in both E1 and E2 regions of the HCV envelope complex that are not desensitised by HDL. By incorporating such epitopes in an immunogenic formulation, they have induced potent and broadly neutralizing antibodies against HCV.

The term “broadly neutralizing” refers to the capacity to neutralize more than one of the six principal genotypes and/or more than one of the numerous subtypes of HCV.

Selection of HCV epitopes which are not sensitive to HDL-mediated desensitization:

The present invention concerns a method for selecting an HCV epitope which is not sensitive to HDL-mediated desensitization, said method comprising:

- testing an antibody against an HCV epitope for its capacity to neutralise HCV or HCV pseudoparticles in the presence of HDL and/or human serum and in the absence of HDL and/or human serum;
- comparing the capacity to neutralise HCV or HCV pseudoparticles in the presence of HDL and/or human serum and in the absence of HDL and/or human serum;
- selecting the HCV epitope if the capacity of an antibody to neutralise HCV or HCV pseudoparticles is not substantially decreased in the presence of HDL and/or human serum.

Preferably, said HCV epitope is from E1 or E2 protein of HCV or E1/E2.

In a preferred embodiment, the tested antibody is directed against the HVR1 region of E2.

In another preferred embodiment, the tested antibody (i) is directed against E2 and (ii) does not neutralise HCV by inhibiting E2/CD81 interaction. The latter property is
designated as “non-nob” toward CD81. CD81 belongs to the transmembrane 4 superfamily (TM4SF) which includes CD9, CD53, CD63 and CD82. CD81 is broadly expressed on hematopoietic cells (T and B lymphocytes, granulocytes, monocytes) and on some non-lymphoid tumors. The function of CD81 (or other TM4SF proteins) is incompletely understood, although CD81 appears to modulate the signaling of other membrane receptors.

In a further embodiment, the tested antibody is directed against E1. E1-targeted antibodies may be highly valuable first because E1 does not seem to be involved in interaction with CD81 and second because the inventors demonstrate the possibility to generate high-titre antibodies from mice immunised with E1-based antigens that are not desensitised by HDL/SR-BI interaction.

The neutralizing capacity of an antibody can be evaluated by methods well-known by the man skilled in the art. More particularly, the capacity to neutralise HCV or HCV pseudoparticles can be tested as described in the examples.

The present invention concerns the selected HCV epitope which is not sensitive to HDL-mediated desensitization and the antibody directed against this epitope. In a preferred embodiment, the selected HCV epitope is from E1 or HVR1. For example, a particular epitope from E1 is E1 (192-202). The selected HCV epitope can be used as antigen in a vaccinal composition or in a method for treating a patient infected by HCV or a subject who has been exposed to HCV.

Consequently, the present invention concerns a method for treating a patient infected by HCV or a subject who has been exposed to HCV, comprising administering at least one HCV-neutralizing antibody or at least one HCV antigen for eliciting HCV-neutralizing antibodies, wherein said HCV-neutralizing antibody is directed against an HCV epitope which is not sensitive to HDL-mediated desensitization. By “is not sensitive to HDL-mediated desensitization” is intended that the neutralizing capacity of the antibody is not substantially decreased in the presence of HDL or human serum in comparison to its neutralizing capacity in the absence of HDL or human serum.

The present invention concerns a pharmaceutical composition comprising either at least one HCV-neutralizing antibody or at least one HCV antigen for eliciting HCV-neutralizing antibodies, wherein said HCV-neutralizing antibody is specific of an epitope which is not sensitive to HDL-mediated desensitization.
The present invention also concerns a vaccinal composition comprising at least one HCV antigen for eliciting HCV-neutralizing antibodies, wherein said HCV-neutralizing antibody is specific of an epitope which is not sensitive to HDL-mediated desensitization. This vaccinal composition can further comprise an adjuvant.

In addition, the present invention concerns the use of at least one HCV-neutralizing antibody or at least one HCV antigen for eliciting HCV-neutralizing antibodies, wherein said HCV-neutralizing antibody is specific of an epitope which is not sensitive to HDL-mediated desensitization, for preparing a medicament for treating a patient infected by HCV or a subject who has been exposed to HCV.

Inhibition of HCV/HDL/SR-BI interplay

The present invention further concerns a method for treating a patient infected by HCV (therapeutic treatment) or a subject who has been exposed to HCV (prophylactic treatment), comprising administering a therapeutically efficient amount of a compound inhibiting HCV/HDL/SR-BI interplay, thereby decreasing the infectivity of HCV and/or increasing the sensitivity of HCV to HCV-neutralizing antibodies. Optimally, the treatment leads to HCV clearance.

Alternatively, the present invention concerns a method for treating a patient infected by HCV or a subject who has been exposed to HCV, comprising reducing the plasma loads of HDL, hereby decreasing the infectivity of HCV and increasing the sensitivity of HCV to HCV-neutralizing antibodies.

Furthermore, the present invention also concerns a pharmaceutical composition comprising a therapeutically efficient amount of a compound inhibiting HCV/HDL/SR-BI interplay. In a preferred embodiment, said pharmaceutical composition comprises at least one HCV-neutralizing antibody and a therapeutically efficient amount of a compound inhibiting HCV/HDL/SR-BI interplay. In an alternative preferred embodiment, said pharmaceutical composition comprises an HCV antigen for eliciting HCV-neutralizing antibodies and a therapeutically efficient amount of a compound inhibiting HCV/HDL/SR-BI interplay.

The present invention further concerns the use of at least one HCV-neutralizing antibody and a compound inhibiting HCV/HDL/SR-BI interplay for the preparation of a pharmaceutical composition for treating an HCV infection in a patient. Alternatively, the present invention concerns the use of at least one HCV antigen for eliciting HCV-
neutralizing antibodies and a compound inhibiting HCV/HDL/SR-BI interplay for the preparation of a pharmaceutical composition for treating an HCV infection in a patient.

The present invention additionally concerns a method for increasing the therapeutic activity of an HCV-neutralizing antibody in a subject, the method comprising administering to the subject, prior to, together with or after said HCV-neutralizing antibody, a compound that inhibits HCV/HDL/SR-BI interplay.

The present invention further concerns a method for treating a patient infected by HCV or a subject who has been exposed to HCV, comprising administering at least one HCV-neutralizing antibody or at least one HCV antigen for eliciting HCV-neutralizing antibodies, wherein said HCV-neutralizing antibody is specific of an epitope which is not sensitive to to HDL-mediated decrease of the neutralizing capacity of anti-HCV antibodies. In a preferred embodiment, said epitope is selected from E1 fragment or HVR1. For example, a particular epitope from E1 is E1 (192-202).

In another preferred embodiment, the method according to the present invention comprises the administration of a compound inhibiting HCV/HDL/SR-BI interplay. Such compounds can be any compound which decreases the plasma loads of HDL in the patient. Alternatively, such compound can be a compound which inhibits the interaction between HDL and HCV, more particularly which inhibits the formation of the HDL-HCV complexes, reduces their stability and/or disrupts the complexes. More particularly, the compound inhibits the interaction between the HCV hyper-variable region 1 (HVR1) of the E2 glycoprotein, which comprises the first 27 aa at the N-terminus of E2 and HDL.

In a particular embodiment, by a compound inhibiting HCV/HDL/SR-BI interplay is intended that the compound decreases the number of HCV/HDL/SR-BI complexes by at least 20%, preferably at least 40%, more preferably at least 60, and still more preferably at least 80% in comparison with the number of complexes in the patient who has not been treated with a compound inhibiting HCV/HDL/SR-BI interplay.

The compound inhibiting HCV/HDL/SR-BI interplay may be of various origin, nature and composition. It can be any organic or inorganic substance. It can be selected from the group consisting of a peptide; a protein; an antibody, a derivative or a fragment thereof; a lipid; a lipoprotein; and a chemical compound. For instance, such compound can be a peptide derived from HVR1 which can act as a decoy for HDL. Alternatively, such compound can be an antibody directed against HDL. In a particular embodiment, the
antibody directed against HDL is directed against an apoliprotein contained in HDL such as Apo-A1, Apo-A2, Apo-C1, Apo-C2 and Apo-C3.

In a most preferred embodiment the compound that inhibits the HCV/HDL/SR-BI interplay is an inhibitor of HDL/SR-BI interaction. Said compound can be identified by screening methods, using classical binding assays. Scavenger receptor activities can further be measured by uptake and degradation assays as described by Krieger, 1983 (19a); and Freeman et al., 1991 (11b).

Nieland et al identified small molecule inhibitors of SR-BI-mediated lipid transfer in intact cells. These compounds are in the low nanomolar to micromolar range block lipid transport (BLTs), both selective uptake and efflux. The effects of these compounds are highly specific to the SR-BI pathway. Nieland et al, 2002 (30a). WO04/032716 describes such SRB-I inhibitors that may be useful in the present invention. Preferably, BLT-1, BLT-2, BLT-3, BLT-4 or BLT-5 are preferred, respectively of the following formula.

BLT-1 (MIT 9952-53) is 2-hexyl-1-cyclopentanone thiosemicarbazone:

\[
\text{S} \quad \text{N} \quad \text{N} \quad \text{N} \\
\text{R} \quad \text{R} \quad \text{R} \quad \text{R}
\]

BLT-2 (MIT 9952-61) is 2-(2-butoxyethyl)-1-cyclopentanone thiosemicarbazone:

\[
\text{S} \quad \text{N} \quad \text{N} \quad \text{N} \\
\text{R} \quad \text{R} \quad \text{R} \quad \text{R}
\]

BLT-3 (MIT 9952-19):

BLT-4 (MIT 9952-29) is 1-(2-methoxyphenyl)-3-naphtalen-2-yl-urea:
and BLT-5 (MIT 9952-6) is N'-[1-(4-aminophenyl)ethylidene]-2-iodobenzohydrazide.

BLT-1, BLT-2, BLT-3 and BLT-4 are produced by Chembrige corporation.

Other SRB-1 inhibitors are described in WO04/041179 or US20050019751.

Glyburide (also called glybenclamide) may be a further inhibitor of interest, as well as Fucoidan from fucus vesiculosus (Sigma).

Anti-SR-BI antibodies may further be inhibitors of interest, by blocking the HDL-mediated activity.

The method according to the present invention comprises, in another embodiment, the reduction of plasma loads of HDL in the patient. In a preferred embodiment, the step of reducing the plasma loads of HDL is performed by depletion of HDL by plasmapheresis.

In an alternative embodiment, the reduction of the plasma load of HDL is performed by administering a compound which decreases the plasma loads of HDL. Such compound can be for example any drug that interferes with the lipid metabolism and reduces HDL synthesis.

The pharmaceutical compositions according to the present invention are generally formulated with a pharmaceutically acceptable carrier and may be administered by any desired route. In particular, intravenous, intraperitoneal, intramuscular, subcutaneous, rectal or vaginal administration may be used.

The carrier for the pharmaceutical composition includes any pharmaceutically acceptable agent suitable for delivery by any one of the foregoing routes and techniques of administration. Diluants, stabilizers, buffers, adjuvants, surfactants, fungicides,
bactericides, and the like may also optionally be included. Such additives will be pharmaceutically acceptable and compatible with the HCV-neutralizing antibodies and the compounds inhibiting the HCV/HDL/SR-BI interplay, or lowering of HDL levels. Carriers include aqueous media, buffers such as bicarbonate, phosphate and the like; ringers solution, Ficol solution, BSA solution, EDTA solution, glycerols, oils of natural origin such as almond, corn, arachnis, caster or olive oil; wool fat or its derivatives, propylene glycol, ethylene glycol, ethanol, macrogols, sorbitan esters, polyoxyethylene derivatives, natural gums, and the like.

The pharmaceutical composition can be formulated as liquids, gels and suspensions. The formulations are preferably suitable for injection, insertion or inhalation.

A preferred formulation involves lyophilized HCV-neutralizing antibodies and/or compound active at lowering serum HDL or inhibiting the HCV/HDL/SR-BI interplay, and separate pharmaceutical carrier. Immediately prior to administration, the formulation is constituted by combining the lyophilized HCV-neutralizing antibody and/or compound inhibiting the HCV/HDL/SR-BI interplay and pharmaceutical carrier.

The compound inhibiting the HCV/HDL/SR-BI interplay or lowering serum HDL levels can be administered to the patient before, simultaneously or after the HCV-neutralizing antibodies. In another embodiment, the compound inhibiting the HCV/HDL/SR-BI interplay can be administered to the patient before, simultaneously or after the HCV antigen for eliciting HCV-neutralizing antibodies in said patient.

The dosage and route of administration will generally follow the judgment of the patient's attending physician. In particular, intravenous, intraperitoneal, intramuscular, subcutaneous, oral, rectal or vaginal administration may be used. The dosage of the therapeutically efficient amount of HCV-neutralizing antibody and/or compound inhibiting the HCV/HDL/SR-BI interplay can be determined by therapeutic techniques well known to those of ordinary skill in the art.

An object of the present invention is a screening method to identify a drug useful for treating or preventing a HCV infection comprising the incubation of HDL and HCV (or a pseudoparticle thereof) with a candidate drug and the evaluation of the effect of the candidate drug on the the HCV/HDL/SR-BI interplay or serum HDL levels. Alternatively, the method can comprise the incubation of HDL and HVR1 with a candidate drug and the evaluation of the effect of the candidate drug on the the HCV/HDL/SR-BI interplay. If the candidate decreases or interferes with HCV/HDL/SR-BI interplay, this drug can be useful for treating or preventing a HCV infection.
Neutralizing antibodies

The methods according to the present invention may comprise the administration of either at least one HCV-neutralizing antibody or at least one HCV antigen for eliciting HCV-neutralizing antibodies.

The HCV-neutralizing antibodies according to the present invention can be endogenous antibodies developed by the patient or the subject during its immune response against HCV. The method according to the present invention can comprise the stimulation of an immune response by the patient or the subject for eliciting HCV-neutralizing antibodies. The term “active” immunotherapy refers to this strategy. The method for eliciting HCV-neutralizing antibodies are known by the man skilled in the art. For example, such methods are disclosed in WO0147551 and WO9966033. In a particular embodiment, the method for eliciting HCV-neutralizing antibodies comprises the administration to the patient or the subject of an HCV antigen, preferably an HCV antigen derived from E1 or E2 protein of HCV or E1/E2. Optionally, the HCV antigens for eliciting HCV-neutralizing antibodies are administered before, simultaneously, or after the administration of the compound inhibiting the HCV/HDL/SR-BI interplay (e.g. the complex between HCV and HDL or more preferably the interaction between HDL and SR-BI), or the reduction of the plasma loads of HDL.

Alternatively, the HCV-neutralizing antibodies according to the present invention can be exogenous antibodies which are able to neutralize HCV and which are administered to the patient or the subject. The term “passive” immunotherapy refers to this strategy. Preferably, the method according to the present invention further comprises the administration of a therapeutically efficient amount of at least one HCV-neutralizing antibody. Optionally, the HCV-neutralizing antibody is administered before, simultaneously, or after the administration of the compound the HCV/HDL/SR-BI interplay (e.g. the complex between HCV and HDL or more preferably the interaction between HDL and SR-BI), or the reduction of the plasma loads of HDL.

The HCV-neutralizing antibodies can be polyclonal or monoclonal. For example, polyclonal HCV antibodies can be prepared from anti-HCV positive blood units (US 6,372,216; EP 896545). The HCV-neutralizing antibodies can be human-derived, non-human-derived, humanized, single-chain, polyfunctional or chimeric antibodies. The present invention also contemplates derivatives of HCV-neutralizing antibodies such as fragments (i.e., Fab, Fab', F (ab')2, Fd, Fv or CDR, etc...). For example, HCV-neutralizing

The HCV-neutralizing antibodies are often directed against E1 or E2 protein of HCV or against the complex E1/E2.

By “HCV-neutralizing antibody” is intended an antibody directed against HCV and able to interfere with any of the biological activities of HCV. For example, it can interfere with the capacity of HCV to bind target cells, with the HCV replication, with the HCV infection, or with the survival of HCV. A neutralizing antibody may reduce the ability of HCV to carry out its specific biological activity, preferably by about 50%, more preferably by about 70%, and most preferably by about 90% or more.

The neutralizing capacity of an antibody can be evaluated by methods well-known by the man skilled in the art, for example by examining the binding of HCV or HCV pseudoparticles to target cells, the infection of target cells by HCV or HCV pseudoparticles, etc. (For instance, see Example section). Alternatively, the neutralizing activity of an antibody can be evaluated in a system of viral pseudotypes which are able to produce a protein that produces fluorescence after entering the target cells (48). By revealing the presence or absence of fluorescence in the cells, the method provides a direct measure of the in vivo neutralizing activity of antibodies directed against different epitopes.

Further aspects and advantages of the present invention will be disclosed in the following experimental section, which should be regarded as illustrative and not limiting the scope of the present application.

**EXAMPLES**

To address the significance of lipoprotein particles in HCV biology, the inventors studied the effect of human serum and lipoprotein particles on the infectivity of HCV pseudo-particles (HCVpp), a recently described model of HCV cell entry and its inhibition (2-4, 17).

The inventors have demonstrated that infection of hepato-carcinoma cells by HCVpp is increased over 10-fold by human serum factors, of which HDL appears a major
component. Infection enhancement requires SR-BI, a molecule known to mediate HDL uptake into cells as well as HCVpp entry, and involves conserved amino-acid positions in the hyper-variable region-1 (HVR1) of the E2 glycoprotein. Additionally, the inventors have shown that the presence of human serum or HDL, but not LDL, leads to protection of HCVpp from neutralizing antibodies including monoclonals and antibodies present in patients' sera. Finally, deletion or mutation of HVR1 in HCVpp abolished infection enhancement and lead to increased sensitivity to neutralizing antibodies/sera compared to parental HCVpp. Altogether, these results assign to HVR1 different roles which are complementary in helping HCV to survive within its host: enhancement of cell entry, protection of virions from neutralizing antibodies and immune escape by mutation. By preserving a balance between these functions, HVR1 may be essential for viral persistence of HCV.

**Materials and Methods**

**Expression constructs and production of HCV pseudo-particles.** Expression vectors for E1E2 glycoproteins of genotypes 1a of strain H77 (AF009606), 1b of strain CG1b (AF333324) and the HVR1 deletion mutant (del G384-N411, strain H77) have been described previously (3, 4) and were used to construct point mutations within HVR1 (G389L, L399R, G406L, G406R) and E1 (Y276F) (one-letter amino-acid code, numbering according to the sequence of the polyprotein precursor (AF009606)) by site-directed mutagenesis. The murine leukemia virus (MLV) packaging and GFP-transfer vectors, the phCMV-RD114, phCMV-G, phCMV-HA/NA, phCMV-Lassa, phCMV-LCMV, phCMV-HIV and phCMV-WN expression plasmids encoding glycoproteins of feline endogenous virus RD114, vesicular stomatitis virus, influenza virus, Lassa virus, lymphocytic choriomeningitis virus, human immunodeficiency virus, and west Nile virus, respectively, have been described previously (3, 40). 293T cells were transfected with expression vectors encoding viral glycoproteins, retroviral core proteins, and GFP-transfer vector using a calcium-phosphate transfection kit (Clontech, France). 24 hours after transfection the medium was replaced with DMEM/10% FCS for standard particle production. To produce viruses in varying amounts of serum, DMEM containing the specified amounts of human or other sera/ lipoproteins was used. Supernatants were harvested 24 hrs after medium change, filtered (0.45 μm-pore size), and used to infect Huh-7, PLC/PRF/5 (ATCC CRL-8024), SW-13 (CCL-105), SK-Hep-1 (HTB-52), CD4-transfected HOS (CRL-1543), HepG2 (HB-8065) and HepG2-CD81 (4) cells. 2 hrs prior to infection, target
cells were pre-incubated in DMEM containing 0.1% FCS or no serum at all. Then medium was removed and dilutions of viral supernatants were added to the cells and incubated for 3 hours. As indicated, additional serum or lipoproteins (HDL, LDL and vLDL purchased from Calbiochem containing 2.17 mg/ml, 9.46 mg/ml and 4.39 mg/ml of cholesterol, respectively) were added to the infection reactions at the indicated concentrations. The HDL preparation (density 1.063-1.2 mg/ml) contained a mixture of HDL_2 and HDL_3. After three hours incubation, supernatants were removed and the infected cells were kept in regular medium (DMEM, 10% FCS) for 72 hrs before analysis of the percentage of GFP-positive cells by FACS analysis (3). The down-regulation of SR-BI in Huh-7 cells was achieved via siRNA-expressing lentiviral vectors, as previously described (22).

Reagents and Antibodies. For preparation of sera, blood was incubated on ice for 2 hrs, centrifuged at 4,000 rpm for 20 min, the supernatant harvested and stored in aliquots at -80°C. The human sera used in this study contained on average 1.40±0.38 mg/ml HDL and 3.27±1.48 mg/ml LDL (n=12). Normal human serum contains ca. 1.2-2.98 mg/ml HDL (i.e., 0.37-0.92 mg/ml of cholesterol-HDL) (www.doctissimo.fr/html/dossiers/cholesterol.htm). BLTs were obtained from Chembridge, USA and re-suspended in DMSO. The 9/27 and AP33 (31) and the E2mAb-1 are E2-specific monoclonal antibodies. A pool of HCV IgGs (70 mg/ml) was concentrated and purified from a set of 25 different chronic HCV sera of genotypes 1a, 1b and 3 using DEAE Affi-Gel Blue Gel (BioRad) according to manufacturer's instructions. Anti-RD polyclonal serum was previously described (3). Western-blot analysis of purified pseudoparticles was performed as previously described (3).

Surface plasmon resonance (SRP) binding assays using BIAcore technology. The SPR measurements were performed on a BIAcore 3000 instruments (BIAcore AB, Upssala, Sweden). The AP33 antibody (100 mg/ml in 10 mM acetate buffer, pH4.5) was covalently immobilised to the dextran matrix of a CM3 sensor chip via its primary amine groups (amine coupling kit, BIAcore AB) at a flow rate of 5 ml/min. Activation and blocking steps were performed as described previously (39a). The AP33 antibody was used to capture 20%-sucrose cushion purified HCVpp. HCVpp were injected over AP33 antibody in PBS 0,005% P20 surfactant (BIAcore AB) at flow rate of 5 ml/min at 25°C. An HCVpp capture level ranging between 200 and 500 resonance units (RU) was obtained. A control flow cell was prepared by immobilizing irrelevant HCV antibody (mouse anti-
IL2) in the same procedure. Control sensograms were automatically subtracted from the sensograms obtained with captured HCVpp. Binding assays of purified HDL, A4 antibody and CD81-LEL were performed at 25°C in PBS 0.005% P20 surfactant at flow rate of 5 μl/min over captured HCVpp. The surface was then regenerated with pulse of 0.025% Sodium Dodecyl Sulfate.

**Neutralisation of binding (NOB) assays.** Flow cytometric analyses of soluble E2 glycoprotein binding to CD81 were performed using method previously described (11a). Briefly, 5 mg/ml of the soluble E2 glycoprotein was mixed with 10 mg/ml of antibodies and incubated for 1 hr at 37°C. Molt-4 cells (105) were incubated to the mixture at room temperature for 1 hr and the amount of soluble E2 glycoproteins bound to Molt-4 cells was assessed by detection of tag-His.

**Results**

**Human serum stimulates infectivity of HCVpp.** HCV pseudo-particles (HCVpp) have previously been shown to reproduce the cell entry steps of HCV and its neutralization (2-4, 17). The inventors generated HCVpp by transfecting 293T cells with three plasmids encoding the retroviral core proteins, the full length, unmodified E1E2 glycoproteins of HCV of genotype 1a and a recombinant retroviral genome harboring a green fluorescent protein (GFP) marker (3). Infectivity of the HCVpp recovered in the supernatant of transfected 293T cells was determined on Huh-7 hepatocarcinoma cells by measuring the number of GFP-positive cells. Compared to HCVpp produced in culture medium devoid of serum, addition of normal human serum (HS) to the culture medium of transfected 293T cells strongly stimulated infection of Huh-7 cells in a dose dependent manner, with a maximum increase of 10-20-fold (Fig. 1A). An enhancement of infection was not observed when fetal calf serum (FCS), rather than HS, was used to produce HCVpp (Fig. 1A). Also, for control pseudo-particles generated with the RD114 cat endogenous virus glycoprotein (RD114pp) a less than 3-fold increase of infection was detected (2) (Fig. 1A). This finding correlated well with an up to two-fold increase in particle production in the presence of human serum, as shown by immunoblotting of purified viral particles (Fig. 1A, inset). Altogether, these results suggested that specific component(s) of HS enhance infectivity of HCVpp. To find out whether these component(s) change the composition of HCVpp during viral assembly and thus indirectly influenced infection, or whether they acted directly on HCVpp by increasing their infectivity, the inventors tested the effect of HS...
added in trans to HCVpp produced in serum-free medium. Again, addition of normal HS, but not FCS, increased infectivity (Fig. 1B), though ca. 2-4 fold less efficient compared to production of HCVpp in similar amounts of HS. Enhancement of infection was also detected for other HCV genotypes/subtypes as well as for all normal HS tested (over 12 HS, Fig. 1B). Facilitation of infection was specifically mediated by primate sera, since incubation of HCVpp with normal sera from other vertebrate species resulted in significantly reduced levels of enhancement (Fig. 1B). Enhancement of infection has previously been described for other viruses to be mediated either by antibodies and/or by complement (12, 14, 44). However, IgG-depleted normal HS stimulated infection of HCVpp as efficiently as untreated HS. Secondly, incubation of HCVpp with immunoglobulins purified from HCV-negative HS was not found to facilitate infection. Thirdly, serum de-complementation by heat-treatment did not abrogate the facilitation of HCVpp infectivity (Fig. 1C). Finally, only infectivity of HCVpp, but not of pseudo-particles generated with the glycoproteins from alternative enveloped viruses was stimulated by normal HS (Fig. 1D). These data excluded a role of antibodies or complement in HCVpp facilitation, and showed serum facilitation to be highly specific for HCV.

**HDL and SR-BI are infection-facilitating components.** Since HCV isolated from plasma of HCV patients is often associated to lipoproteins (1, 16, 18, 27, 28, 37, 45), the inventors asked whether lipoprotein interactions could enhance infection of HCVpp. As shown in Fig. 2A, HCVpp produced in the presence of purified HDL had a strongly increased infectivity compared to control RD114pp. Average concentrations of HDL in normal human serum are in the range of 0.37-0.92 mg/ml of cholesterol-HDL and maximal infection enhancement by HS is observed at 0.5-2.5% (Fig. 1A) which amounts to 1.85 to 23 μg/ml of cholesterol-HDL. This number corresponds well to the amounts of purified HDL (4-9 μg/ml cholesterol-HDL) required for maximal infection enhancement. Furthermore, infectivity of HCVpp produced in optimal concentrations of HDL could not be further enhanced by addition of HS (Fig. 2B) and vice-versa (Fig. 2B), suggesting that HDL is the predominant enhancing factor in HS. Consistently, no or less than 2-fold infection enhancement was detected when the HCVpp were produced with LDL or vLDL, respectively (Fig. 2A).

To exclude possible indirect effects of lipoproteins on HCVpp infectivity via modification of the membrane lipid composition during viral production, all further experiments were performed in trans, by adding HS or lipoproteins to HCVpp produced in
low serum medium. Consistent with previous experiments performed in cis, infection of HCVpp was significantly increased when adding in trans purified HDL, but not LDL or vLDL, to HCVpp just before infection of Huh-7 cells (Fig. 2C). Also an HDL-deficient HS from a patient with Tangier disease did not stimulate infection in trans (Fig. 2C), further demonstrating that HDL is an essential infection-enhancing factor in normal HS. Interestingly, the pre-treatment of HS with several polyclonal antibodies targeted against Apo-A1, -A2, -C1, -C2 and -C3 which are expressed on HDL did not abrogate infection enhancement, suggesting that the HDL component involved in the effect may not be a protein alone, but perhaps a lipid or protein/lipid complex. Finally, enhancement of infection by normal HS or by HDL was restricted to pseudo-particles harboring glycoproteins derived from HCV but not those from alternative enveloped viruses (Fig. 1D), suggesting a specific interplay of the former glycoproteins with HDL and their cell surface receptors.

Indeed, HDL is a ligand of the scavenger receptor SR-BI, an HCV receptor candidate (4, 41), and its high affinity binding to SR-BI mediates the selective lipid uptake of cholesteryl esters from lipid-rich HDL to cells (19). The inventors therefore asked whether HDL-mediated facilitation of HCVpp requires SR-BI. While infection enhancement of HCVpp incubated with HS was detected in Huh-7 cells as well as in other SR-BI-expressing cells such as PLC/PRF/5 hepatocarcinoma cells, or in SW-13 adrenocortical cells, no facilitation of infection could be detected in SK-Hep1 hepatoma target cells that express undetectable SR-BI levels (Fig. 3A). Additionally, no infection enhancement could be detected when SR-BI was blocked using a polyclonal SR-BI blocking antibody. Indeed while such antibody reduced infection of HCVpp in the absence of HS, as previously reported (4), it completely inhibited the stimulation of infection mediated by both HS and HDL (Fig. 3B). Likewise, downregulation of SR-BI with lentiviral vectors carrying a siRNA targeted against SR-BI (22) was found to significantly reduce infection enhancement by HS (Fig. 3C). Altogether, these results suggested that enhancement of infection involves an interplay between HCVpp, serum component(s) including HDL and SR-BI. Interestingly, infection enhancement by HS was detected in HepG2 cells transfected with CD81, another HCV co-receptor (33), but not in parental HepG2 cells (Fig. 3A). Furthermore, no infection could be detected in CHO cells transfected with both SR-BI and CD81 in the presence of HS. Altogether, these results suggested that cell entry mediated by SR-BI and HDL remains dependent on other HCV co-receptors that include CD81.
To investigate which stages of cell entry HS or HDL enhance, the inventors performed experiments where HS/HDL and virus were added consecutively to target cells. Pre-incubation of target cells with HS/HDL for varying amounts of time followed by washing the cells two times with PBS before infection with the virus did not stimulate infection of HCVpp added subsequently, as compared to when the washing step was omitted (Table 1). Reciprocally, HS or HDL added to target cell-bound HCVpp enhanced infection (Table 1). This indicated that HDL needs to either interact with the virus particles, or to be present at the same time as HCVpp, to exert a stimulatory effect. Importantly, the inventors were unable to detect a physical interaction between HDL and HCVpp using sucrose-gradients and immuno-precipitation assays performed with apolipoprotein or E1E2 antibodies, which suggested an unstable or transient HCVpp/HDL association. Finally, they were not able to detect an increased binding of HCVpp to SR-BI-positive target cells in the presence of HS or HDL, confirming that the enhancement of infection may occur at a post-binding stage.

To further examine the role of SR-BI in HDL facilitation of HCVpp, the inventors pre-incubated target cells with BLT-4, a small molecule inhibitor that blocks SR-BI-mediated selective cholesterol uptake from HDL (30), prior to infection. While the BLT-4 compound did not reduce the infectivity of HCVpp in the absence of HS or HDL, it abrogated the enhancement of infection by HS or HDL in a dose dependent manner (Fig. 3D). These results were confirmed by using a second inhibitory molecule, BLT-3 (Fig. 3D). Importantly, the BLT compounds acted on HCVpp only but not on RD144pp (Fig. 3D), suggesting that the cholesterol-uptake function of SR-BI mediates infection-enhancement by HS/HDL.

**HVR1 is a viral component involved in HDL-mediated enhancement of infection.** The hypervariable region 1 (HVR1), the 27 N-terminal amino acids of the E2 HCV glycoprotein, has previously been implicated in mediating interactions of HCVpp with SR-BI during cell entry (4, 41). Removal of HVR1 still allows efficient incorporation of E1E2 glycoproteins (Fig. 4A) but results in 10 to 40 fold reduced cell entry (4). Interestingly, the inventors found that the infectivity of HCVpp harboring HVR1-deleted glycoproteins was not stimulated by HS or HDL (Fig. 4C), indicating that HVR1 is required for enhancement of infectivity, perhaps by interacting with the facilitating serum component(s) or HDL. They confirmed this result with HVR1-deletion mutants generated from HCVpp harboring E1E2 glycoproteins from alternative HCV genotypes.
To further analyze the role of HVR1 in infection, the inventors introduced changes in amino-acid positions of HVR1 (G389L, L399R, G406L, G406R) that are conserved and that are thought to be essential for the conformation of HVR1 and perhaps cell interaction (32). None of the mutations significantly altered the levels of viral incorporation of the HCV glycoproteins (Fig. 4A) and the mutant HCVpp retained similar levels of infectivity in the absence of HS, as compared to parental HCVpp (Fig. 4B). Interestingly, some mutations, i.e., L399R and G406L, abrogated enhancement of HCVpp infection mediated by HS and HDL (Fig. 4C). The G406L mutation resulted in an intermediate phenotype. In contrast, the G389L mutation enhanced infectivity in the presence of HS or HDL, underlining the importance of HVR1 conformation in virus entry. Altogether, these results confirmed the important role played by HVR1 in cell entry and its enhancement by HS or HDL and pointed out key amino-acid residues mediating these functions.

**The HVR1 suppresses a neutralizing immune response in HCV-infected patients.** The HVR1 is an important target of neutralizing antibodies *in vivo* and its variability is thought to allow HCV to adapt to its host or to persist *in vivo* (9). Since HVR1 also appeared to be the target of the enhancing serum factor (Fig. 4), the inventors asked to what extent its interplay with HS or HDL could modulate HCVpp inhibition by neutralizing antibodies.

First, the inventors investigated the effect of facilitating serum components on neutralization of HCVpp in the sera of a cohort of acute phase patients contaminated during a nosocomial outbreak with a single HCV virus of 1b subtype (21). A first group of patients (7/13 patients) developed a neutralizing antibody response of narrow specificity, which correlated with control of viremia (see Table 2 and results of patients Pt-3 and Pt-4 from this first group in figure 5A). Indeed, as previously reported (21), the emergence of neutralizing antibodies could be readily detected using HCVpp harboring autologous – patient-derived – or highly homologous E1E2 glycoproteins of a 1b subtype, but no or poor neutralization was detected when using HCVpp of slightly divergent strains or of different subtypes/genotypes. In contrast, HVR1-deleted HCVpp of genotypes 1a and 1b were cross-neutralized more efficiently than the parental wild-type HCVpp (Fig. 5A and Table 2). Conversely, a second group of acute-phase patients (6/13 patients) was characterized by sustained and high viral loads and did not develop a neutralizing response detectable with HCVpp harboring autologous E1E2 glycoproteins (21) (see Table 2 and patients Pt-8 and Pt-9 in Fig. 5A). Again, the inventors found that these sera could efficiently cross-neutralize HVR1-deleted HCVpp of both 1a and 1b subtypes (Fig. 5A and
Table 2). Altogether, these results suggested that the neutralizing activity of antibodies from sera of HCV-infected patients is inhibited or not detectable in the presence of HVR1 on HCVpp.

The presence of HVR1 and HDL protects HCV from neutralizing antibodies.

To investigate this issue further, the inventors performed neutralization assays of HCVpp with purified antibodies in the presence or absence of HS or lipoproteins. In the absence of HS or in the presence of LDL, the infectivity of HCVpp could be readily neutralized by over 95% by antibodies purified from HCV patients (Fig. 5B, Table 3) or by some E2 monoclonal antibodies (Table 3). In contrast, in the presence of HS or HDL, the neutralization of HCVpp was much less efficient. Indeed, the polyclonal and some monoclonal HCV antibodies could even at high concentrations hardly neutralize the HCVpp by more than 80% and 60%, respectively (Fig. 5B and Table 3). Interestingly, the 9/27 monoclonal antibody, raised against HVR1, formed an exception. By performing serial dilutions of neutralizing antibodies, the inventors calculated that, depending on the antibody, 4 to 20 fold more antibody, at least, was required to neutralize HCVpp in the presence of HS or HDL compared to neutralizations in the presence of LDL or in the absence of HS (Fig. 5B and Table 3). These findings were specific to HCVpp as RD114pp were neutralized with the same efficiency by an anti-RD114 polyclonal antibody in the absence or in the presence of HS (Table 3). These results suggested that the interaction of HVR1 with component(s) of normal HS triggers or provides a mechanism to protect HCV from neutralization by antibodies targeted outside the HVR1. These findings are consistent with the increased capacity of patient sera to neutralize HVR1-deleted HCVpp (Fig. 5A).

Furthermore, the capacity of HVR1 to mediate both infection enhancement and protection from neutralization was confirmed using HCVpp harboring point-mutated HVR1. Indeed, the mutations that inactivated HVR1-mediated infection enhancement (e.g., L399R and G406L; Fig. 4C) also abolished the protection by HS from inhibition by neutralizing antibodies (Fig. 5C).

Selection of neutralising antibodies

Since high density lipoproteins or serum impair the capacity of antibodies to neutralise HCV in infection assays in vitro, it is likely that antibody-mediated therapy may have a limited impact in patients. It is therefore essential to identify the neutralising epitopes that either are or are not sensitive to HDL-mediated desensitization. Indeed the identification of epitopes that are optimally recognised by the corresponding antibodies
even when HDL or serum is present will be of particular value. Furthermore, the definition
of such epitopes will be useful in vaccine preparations aiming to elicit the B cell responses
that are the most potent for neutralisation in vivo.

The inventors therefore tested a series of antibodies directed against different
domains of the E1E2 glycoprotein complex for their capacity to neutralise HCVpp in the
presence of HDL, LDL, human serum, bovine serum or in the absence of either component
(Table 4). The results indicate that while all the antibodies directed against epitopes located
in the E2 glycoprotein (outside HVR1) were highly sensitive to masking by HDL or human
serum, the neutralising activity by antibodies directed against HVR1 or against epitopes
located in the E1 glycoprotein was not impaired by HDL, LDL or human serum. Indeed
while it was possible to reach over 95% neutralisation of HCVpp for all antibodies in the
absence of lipoproteins or serum, the E2-directed neutralising antibodies could not
neutralise HCVpp by more than 60% at the highest concentration in the presence of HDL
or of human serum. Furthermore, at least 10-fold higher concentration of these E2-directed
antibodies were required to reach these low values of neutralisation (Fig. 5B and 5C).
Altogether, this suggested that neutralising antibodies against E1 or against HVR1 are
likely to be highly important in “passive” immunotherapy strategies and, likewise, that E1
and/or HVR1 are likely to be highly important in “active” immunotherapy strategies.

The inventors therefore attempted to generate HCVpp able to elicit E1-targeted
antibodies. HCVpp harbouring E1 glycoproteins were therefore produced in vitro, as
previously described (3) and used to immunise Balb/c mice. After repeated immunisations
(one primo-injection and 3 boosts separated by 2-3 weeks), the sera of the immunised mice
were found to harbour neutralising activity that could efficiently inhibit the infectivity of
HCVpp carrying functional E1E2 glycoproteins (Fig. 6). Furthermore, the inventors found
that the neutralising activity of these sera was not impaired in the presence of HDL or
human serum (Table 4). Finally, the inventors demonstrated that HCVpp harbouring E1E2
glycoproteins of genotypes 1 to 6 and of diverse subtypes or strains, as described in
Lavillette, et al, 2005. were readily neutralised by the sera of such immunised mice as
shown in Fig. 6 in infection assays using HCVpp of genotypes 1a, 1b, 2a and 2b.

**Confirmation of the results and investigation of the mechanism of action:**

The above data show an interplay between high-density lipoproteins (HDL) and
HCV-E2 leads to infection enhancement of HCV pseudo-particles (HCVpp), an event
modulated by HVR1, the hypervariable region 1 of E2, and to inhibition of neutralisation by antibodies derived from chronic patients.

Indeed, addition of human serum (HS) or HDL to HCVpp during infection induced a 3-4 fold enhancement of HCVpp infectivity (Fig. 7A; “no Ab” bars). While polyclonal antibodies derived from a pool of chronic patients neutralised more than 75% of HCVpp infectivity at a concentration of 35 μg/ml, the antibodies neutralised less than 25% of HCVpp at the same concentration in the presence of HS (Fig. 7A; “patient polyclonal Ab” bars). The inventors obtained similar results with a monoclonal antibody (mAb) directed against E2 (Fig. 7A). The neutralisation curves deduced from the raw results of infection assays obtained with this mAb are displayed in Fig. 7B. They allowed determining IC50 and IC90 values of 1.3 μg/ml and 8 μg/ml, respectively, in the absence of HS or HDL. This compared to an IC50 of 6.5 μg/ml for neutralisation assays performed in the presence of human serum (Fig. 7B). No IC90 could be determined from neutralisation assays performed in the latter conditions since the mAb could not neutralise the HCVpp in the presence of HS to levels higher than 65% (Fig 7), even at high antibody concentrations (data not shown). The level of desensitisation was not affected by the concentration of HS and mAb, in the range of values tested here (data not shown). While the results shown in Fig. 7 were obtained upon simultaneous addition of HCVpp, antibodies and HDL to target cells, inhibition of neutralisation by human serum was also obtained when HS was added to HCVpp complexed to neutralising antibodies (Table 5). This suggested that in vivo HDL may not only inhibit neutralisation of the newly cell-produced viral particles, but also of viral immune complexes formed. Finally, similar results were obtained with HCVpp carrying E1E2 glycoproteins of other genotypes and/or using other target cell types (data not shown). These findings were specific of HCVpp, as control pseudoparticles harbouring glycoproteins from alternative enveloped viruses like e.g., RD114 cat endogenous virus were neutralised with the same efficiency by a RD114 antibody in the absence or in the presence of HS. Of note, while infection enhancement clearly involved the biological activity of the scavenger receptor BI (SR-BI), a receptor for both HCV-E2 and HDL, the mechanisms of neutralisation desensitisation remained undefined. The inventors therefore sought to investigate this further.

No interaction of HDL with HCVpp can be detected by Biacore analysis. HDL may interact with HCVpp and hence physically reduce the effectiveness of antibodies either by “shielding” of the glycoproteins or by competing for the neutralising epitopes. To
distinguish between these two possibilities, the inventors pre-incubated HCVpp with HS and subsequently separated the two components by ultracentrifugation methods. The inventors then compared the fold of HCVpp infection enhancement by HS, before or following their separation (Fig. 8A). The inventors found that the stimulation of infection was lost after purification of the HCVpp. This result indicated that both HCVpp and HDL need to be present simultaneously to enhance infection. Consistently, no HDL apolipoproteins could be detected in the purified viral particles (Fig. 8A), indicating the absence of a stable HCVpp/HDL complex.

The inventors then investigated the possibility that HDL may transiently interact with HCVpp, using surface plasmon resonance (Biacore) analysis (Fig. 8B). HCVpp were immobilised on a sensorchip coupled with carboxy-methylated dextran to which an E2 monoclonal antibody was covalently attached (Fig. 8B, upper panel). The immobilised HCVpp could readily interact with E1E2 ligands such as the A4 antibody, which binds E1, or with CD81-LEL, a soluble ectodomain of CD81 which binds E2 (Fig. 8B, lower panel). However, no interaction could be detected with HDL for all different experimental conditions tested. Furthermore, similar levels of HCVpp capture could be detected, whether immobilisation was carried out in the absence or in the presence of HDL (Fig. 8, upper panel). Similar results were obtained for HCVpp immobilised on the sensorchip via alternative E2 antibodies or via Galanthus Nivalis lectins (data not shown). Altogether, these results indicated that HDL does not form a stable or a transient complex with HCVpp.

Inhibition of antibody neutralisation is correlated with infection enhancement and requires active SR-BI. Since HDL is a ligand of SR-BI, we thought that their mutual interaction could influence neutralisation. To address this possibility, we investigated a potential relation between infection enhancement, which is induced by HDL/SR-BI interplay, and neutralisation inhibition. We therefore compared the results of infection for different concentrations of human serum or HDL in the presence, or in the absence, of a neutralising mAb (Fig. 9A). The fold of infection enhancement steadily increased with the concentration of HS or HDL. Interestingly, the levels of neutralisation inhibition, expressed as % of desensitisation, increased concomitantly with HS/HDL concentrations and were well proportionate to the levels of infection enhancement. Concentrations of HS that induced the maximal infection enhancement – of ca. 4-fold – resulted in over 60% desensitisation of neutralisation (Fig. 9A). The statistical analysis of the correlation
between neutralisation inhibition and infection enhancement raised R2 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 SR-BI is involved in neutralisation desensitisation.

To confirm this assumption, the inventors performed neutralisation assays in the presence of a specific inhibitor of a major function of SR-BI, its capacity to mediate cholesteryl ester uptake from HDL (19). The treatment of Huh-7 target cells with BLT-4, one of such inhibitors (30), blocked HS/HDL-induced infection enhancement (Fig. 9B, left panel). Importantly, BLT-4-mediated inhibition of SR-BI activity fully restored the potency of a neutralising mAb (Fig. 9B, right panel) or of polyclonal patient-derived antibodies (data not shown) whose activity had been abrogated by HS or HDL. Indeed, while HS reduced neutralisation of HCVpp to up to 70%, the neutralising property of the mAb was restored by BLT-4, in a dose-dependent manner, yielding at ca. 40-50 µM the levels of neutralisation obtained in the absence of HS or HDL (Fig. 9B). Similar results were obtained using higher mAb concentrations (data not shown). These data established that active SR-BI molecules are required to promote both infection enhancement and neutralisation desensitisation, thus raising the possibility of a common mechanism.

Reduced importance of CD81 in infection upon SR-BI activation. Since SR-BI acts as a co-receptor of CD81 during cell entry (4), the inventors asked whether the role of CD81 could be influenced by HS/HDL-mediated activation of SR-BI. Thus, to address this question, the inventors performed infection assays with polypeptides that block the binding of E2 to CD81: JS-81, an antibody that blocks CD81/E2 interaction, or, alternatively, a soluble form of the CD81 ectodomain (CD81-LEL). Both polypeptides inhibited cell entry by HCVpp, as reported before (3) (Fig. 10A and B). Interestingly, the inhibitory effect of JS-81 was strongly reduced in the presence of HS (Fig. 10A) or HDL (not shown). Similarly, inhibition by CD81-LEL added during infection was reduced in the presence of HS (Fig. 4B) or HDL (not shown). Indeed, while 10 µg/ml of CD81-LEL inhibited HCVpp infection to up to 75% in the absence of HS/HDL, its efficacy was decreased to less than 30% in the presence of HS (Fig. 10B). At higher CD81-LEL concentrations, the level of desensitisation was reduced, suggesting that while the role of CD81 in cell entry was modulated by HDL/SR-BI interaction, CD81 still remains an essential component of infection.

Importantly, the desensitisation of inhibition by CD81-LEL was dependent on a functional interplay between HCVpp, HDL and SR-BI. Indeed, BLT-4 alleviated this
desensitisation in a dose-dependent manner, restoring a full inhibition by CD81-LEL at ca. 50 μM of BLT-4 (Fig. 10C). Similar findings were obtained both with HS and HDL (data not shown). Likewise, desensitisation of inhibition by CD81-LEL was not observed for HCVpp harbouring a deletion of HVR1 (Fig. 10D), a key viral component of both infection enhancement and neutralisation desensitisation. Altogether these results suggested that the role of CD81 in cell entry is diminished under conditions of infection that favour activation of SR-BI.

Neutralising antibodies that target E2/CD81 interaction are desensitised by SR-BI activation. Since HS or HDL alleviate the requirement of CD81 (Fig. 10), the inventors reasoned that desensitisation of neutralisation (Figs. 7 and 9) may involve antibodies that block infection at a step involving HCVpp interaction with CD81. To address this possibility, the inventors performed neutralisation assays with a panel of monoclonal antibodies of known specificity in the presence or in the absence of HS (Fig. 11).

A majority of antibodies (CBH2, CBH5, CBH7, E2mAb-1, 3/11, H35, H48, H54, H57) exhibited reduced neutralising efficiencies in the presence of HS (Fig. 11A), similar to results obtained with the AP33 mAb (Fig. 7). Indeed, although they could efficiently neutralise HCVpp in the absence of HS, none of these mAbs could neutralise HCVpp infectivity by more than 70% in the presence of human serum. For antibodies for which this could be calculated, i.e., for the E2mAb-1, H35, H48 and H54 mAbs, the differences of IC60 determined in the presence vs. the absence of HS in the neutralisation assays were at least 10-fold (Fig. 11A).

Interestingly, the inventors also found some monoclonal antibodies whose neutralising activity was not reduced by HS (Fig. 11B). This was the case for the 9/27 antibody, which targets an epitope in HVR1 (11a) and abrogates E2 interaction with SR-BI (4). The IC90 of this mAb, of less than 1 μg/ml, was unchanged when infection assays were performed in the presence vs. the absence of HS. Two other antibodies that were not desensitised by HS were the H60 and H111 mAbs, targeted to E2 and E1, respectively (11a;18a). Although they exhibited much weaker efficiencies as compared to that of the 9/27 mAb, i.e., with IC50 differences over 30-fold, their activity was not altered by human serum (Fig. 11B).

The inventors then sought to correlate the properties of these different mAbs to their capacity to inhibit E2/CD81 interaction, as determined by neutralisation of binding (NOB) assays with soluble E2 proteins (Table 6). The results indicated a clear relationship
between the capacity of the antibodies to inhibit sE2-binding to CD81 and the
desensitisation of their neutralising properties by HS or HDL. All the mAbs that were
desensitised by HS/HDL (Fig. 5A) were those that inhibited the interaction between sE2
and CD81 (Table 6). Conversely, the H111, H60 and 9/27 antibodies, whose neutralising
activity was not impaired by HS/HDL (Fig. 11B), had no CD81-NOB activity (Table 6).

Design of antibodies that neutralise HCVpp and HCVcc in the presence of HS.
These results suggested that it is advantageous to induce neutralising antibodies against E1,
owing to the finding that the H111 E1-targeted monoclonal antibody was not desensitised
by HS (Fig. 5B). To further explore this possibility, the inventors generated E1 antibodies
by immunising mice with modified HCVpp harbouring E1 glycoproteins only
(3;40a). Strong HCVpp-neutralising titres were recovered from the sera of these mice. The
polyclonal antibody purified from mouse sera displayed an IC90 of 10 µg/ml (Fig. 12A),
i.e., 13-fold lower than that obtained with a polyclonal antibody purified from HCV
chronic patients. Importantly, in sharp contrast to the latter antibodies (Fig. 7A), the
neutralising titres of the polyclonal E1 antibody were not changed when the infection
assays were performed in the presence of HS (Fig. 12A) or HDL (data not shown).

To confirm the advantage of neutralising antibodies against E1 over E2-targeted
antibodies, the inventors performed neutralisation assays of cell culture-grown authentic
HCV particles (HCVcc) (Fig. 12). Of note, like for HCVpp (Fig. 7A), infectivity of
purified HCVcc was enhanced by ca. 4-fold in the presence of human serum or HDL (Fig.
12B), confirming that either HCV infection model exploits similar properties of SR-BI
during cell entry in vitro. As expected, some polyclonal and monoclonal antibodies that
strongly neutralised HCVpp could also neutralise HCVcc (Fig. 6C), though seemingly less
efficiently than the HCVpp. Overall, 10-fold higher concentrations of AP33 (IC50: 1.35 vs.
13.33 µg/ml, Fig. 7A vs. Fig. 12C) or E2mAb-1 (IC80: 3.35 vs. 50 µg/ml, Fig. 11A vs.
Fig. 12C) antibodies were required to neutralise HCVcc vs. HCVpp. Likewise, the
polyclonal antibody purified from HCV chronic patients was unable to neutralise HCVcc
by more than 50%, even at a concentrations higher than 1 mg/ml (Fig. 12C) although it
neutralised over 90% of HCVpp at concentrations higher than 100 µg/ml [Bartosch, 2005
#1371]. Of note, HCVcc were produced from Huh-7 cells that express HDL [Yamamoto,
1987 #1389], which could thus impair neutralisation through interaction with SR-BI.
Indeed, treatment of target cells with BLT-4 considerably enhanced the potency of the
E2mAb-1 and patient polyclonal antibodies. This allowed neutralisation efficiencies higher
than 99% and 95%, respectively, in contrast to the 80% and 50% neutralization efficiencies achieved at the same antibody concentrations in the absence of the SR-BI inhibitor (Fig. 12C). In contrast to these latter antibodies, the E1 polyclonal antibody efficiently neutralised HCVcc (Fig. 12C). Identical IC95, of ca. 20 μg/ml, were obtained for either HCVpp or HCVcc, whether or not human serum was present in the infection assay. These results suggest that E1 is a promising target for efficient neutralization of infectious HCV particles.

Discussion

Facilitation of infection in vivo has previously been reported for several viruses. Virus-specific antibodies can enhance viral infectivity, both in vitro and in vivo, through the binding of virus-antibody complexes to cellular Fc receptors (expressed in e.g., monocytes/macrophages) via the Fc portion of the antibodies (35). Fixation of the C3 or C1q complement proteins, activated by virus-antibody complexes, can also facilitate virus entry, as shown for HIV (12), Dengue virus (15) and Ebola virus (44). Finally, antibody-independent enhancement of HIV infection in vitro, via a mechanism that involves receptors of the classical and alternative complement pathways has also been reported (14). However, none of these previously described mechanisms appear to be involved in the facilitation of HCVpp infection observed in the present study. Indeed, sera from healthy donors did not loose their ability to facilitate HCVpp infection after de-complementation by heat-treatment, and incubation with normal purified human immunoglobulin or monoclonal antibodies did not augment infectivity. The inventors’ results demonstrate that HCVpp infection is significantly increased by factors within HS, of which HDL appears to be a major component. Furthermore, the HDL-mediated infection enhancement requires the HCV receptor candidate scavenger receptor BI (SR-BI), involves the hyper-variable region 1 (HVR1) of the E2 glycoprotein and leads to protection from neutralizing antibodies.

Cell entry of HCV is thought to involve several cell surface molecules, including the LDL receptor (1), the type C lectins (DC-SIGN and L-SIGN), which act as virion capture receptors (34), the tetraspanin CD81 (33) and the SR-BI receptor (41) that act as cell entry co-receptors. The involvement of some of these molecules in cell entry has been confirmed by using HCVpp (3, 4, 17, 47). SR-BI is a 509-amino acid glycoprotein with two C- and N-terminal cytoplasmic domains separated by a large extracellular domain. Although a direct interaction between soluble E2 glycoproteins and SR-BI could be
demonstrated (41), how this receptor mediates HCV entry requires further investigation. It has become clear that the HVR1 domain located at the amino-terminal end of the E2 glycoprotein is a critical region required for the functional interaction between E2 and SR-BI. Indeed, deletion of this region decreases E2 binding to SR-BI (41) and SR-BI-mediated cell entry (4), and lowers infectivity of HVR1-deleted HCVpp by over 10 fold.

SR-BI is a lipoprotein receptor responsible for selective uptake of cholesteryl ester from HDL, via a two-step mechanism involving the binding of lipoproteins to its extracellular domain followed by lipid uptake (8). It remains possible that HDL interacts with HCVpp, via lipid/protein or protein/protein interactions, and hence 'stimulates' SR-BI to augment HCVpp entry. However, binding of HDL to SR-BI is of a transient nature due to loss of affinity to SR-BI after cholesteryl uptake (24). While E2 can bind to SR-BI in the absence of HDL (4, 41), HDL-virus interactions may stimulate the binding of the viral glycoprotein to SR-BI. So far, the inventors have not been able to detect a stable physical association of HDL with HCVpp nor an enhancement of HCVpp binding to SR-BI. These results could mean that interactions between HDL and HCVpp are transient and/or that enhancement of infection occurs at a post-binding step.

Alternatively, the inventors proposed another mechanism of action. Accordingly, the desensitisation of neutralisation induced by HDL operates via an original mechanism involving the biological activity of SR-BI, a cell surface molecule which binds both E2 (4) and HDL (19). Interestingly, HCV appears to have adopted SR-BI not only as a receptor for E2-binding (4) but also for exploiting its physiological activity, i.e., the capacity to mediate uptake of cholesteryl ester from HDL. Indeed, the inventors found that the interplay between HCV E2, SR-BI, and HDL promotes enhancement of infection. It is thus noteworthy that protection against neutralizing antibodies is also intimately linked to such a mechanism of cell entry enhancement. Remarkably, BLT-4, a drug that inhibits SR-BI-mediated cholesterol ester uptake from HDL fully restored the potency of neutralising antibodies in infection assays conducted in the presence of human serum or HDL. Similarly, activation of cholesterol uptake by SR-BI also enhances cell entry of HCVpp and HCVcc. The mechanism by which HDL enhances HCVpp or HCVcc infectivity is unclear at the moment, although it has been proposed that the enhancing component is ApoC1, an apolipoprotein located on the surface of HDL particles. HCV particles may interact with SR-BI and HDL to specifically target cholesterol-enriched microdomains and/or to stimulate local cholesterol enrichment, which would thus enhance its entry, perhaps by facilitating membrane fusion events. Alternatively, interaction(s) of HDL with
HCV particles and SR-BI may induce or accelerate conformational changes within the HCV glycoproteins or SR-BI, which are required for the fusion process. While further investigations are required to decipher the role of cholesterol uptake in HCV entry, it is possible that increasing the cell entry rate by SR-BI activation could reduce the time required by antibodies to neutralise the virions and would have strong impact for antibodies with slow neutralisation kinetics. However, this hypothesis may not explain how HDL/SR-BI interaction can desensitise the immune complexes formed by pre-incubating the viral particles with neutralising antibodies before adding HDL and proceeding to infection (Table 5). Furthermore, it is worth noting that desensitisation differentially affects neutralising antibodies, depending on the targeted epitope. Finally, it is important to highlight that HDL/SR-BI interaction with HCVpp also induces desensitisation of compounds that inhibit binding to CD81, such as CD81-LEL. This highlights a complex interrelation between SR-BI and CD81 during HCV cell entry for which HDL and/or cholesterol seems to play a critical role.

The data described here have important implications for active and passive antibody-based immunotherapy strategies against hepatitis C. The present data show that while the neutralising activity of most mAbs retrieved from patients (e.g., CBH-2, CBH-5, CBH-7 and E2mAb-1) or from mice immunised with E2-based antigens (e.g., AP33, 3/11, H35, H48, H54, H57) is reduced by HDL/SR-BI interaction, some antibodies that resist to the desensitisation can be retrieved from patients (e.g., H111) or from immunised rodents (e.g., 9/27, H60). The repartition of the epitopes, or the E2 functions blocked by these antibodies clearly indicate that the antibodies that are not desensitised are those which do not block E2/CD81 interaction. This provides a mean to screen neutralising mAbs or to develop vaccine formulations that induce such antibodies.

The involvement of HVR1 in infection enhancement via HDL and SR-BI is highly significant, given that its genetic variability, which is thought to be the result of a continuous selection process by the host humoral immune response, may allow the virus to adapt to its host. Indeed, the early development of HCV quasispecies and particularly the variation of HVR1, have been suggested to correlate with persistent infection (9) whereas reduction of genetic diversity, leading to increasingly homogenous virus populations, was shown to be a consistent feature associated to viral clearance in sustained responders (11). Consistently, the HVR1 has been shown to contain at least one neutralization epitope (2, 4, 10, 17, 42). Furthermore, emergence of antibodies against HVR1 in inoculated
chimpanzees was associated to variations in HVR1 whereas no variation was detected in the absence of detectable HVR1-antibodies (46). These and other evidence in support of HVR1 selection by the humoral response (5, 39) has led to the notion that HVR1 may function as a ‘immunological decoy’, stimulating a strong immune response causing variant selection but ineffective for viral clearance (29).

The present results shed new light on the functions of HVR1 as an essential component of sustained HCV infection. Indeed, the inventors found that via interplay with HDL, HVR1 not only promotes infection enhancement but also protects from neutralization by over 10-fold (Fig. 5). These data are consistent with the present findings that HCVpp are protected from neutralizing antibodies in the presence of HDL but not LDL (Fig. 5). The implications of these results are significant for the understanding of viral propagation and persistence *in vivo*. Indeed, HDL strongly stimulates HCVpp infectivity not only by enhancing infection but also because neutralization in the presence of HDL requires at least 10-fold more antibody. Importantly, a polyclonal antibody purified from a pool of chronically infected patients could not neutralize more than 80% of the virions treated with HDL or normal HS, even when it was used at high concentrations. Interestingly, at saturation, a set of E2 monoclonal antibodies targeted to epitopes outside HVR1 neutralized HCVpp significantly less efficiently in the presence of normal HS or HDL, while a monoclonal antibody against the HVR1 could almost totally inhibit infection, most likely as a result of inactivation of the protection mechanism itself.

Furthermore, the inventors found that HVR1 impaired the detection of neutralizing and cross-neutralizing antibodies, in both acutely and chronically infected patients. A recent study of the humoral response in a cohort of acute phase patients infected by a single source HCV revealed that some patients underwent partial control of HCV RNA loads which correlated with the progressive emergence of neutralizing antibodies of narrow specificity, while in other patients high and stable HCV RNA levels correlated with a lack of neutralizing antibodies despite sero-conversion (21). Strikingly, the use of HVR1-deleted HCVpp in neutralization assays with sera of this latter group of patients revealed the existence of a relatively strong neutralization response against both homologous and heterologous HCV sequences. Moreover, the use of HVR1-deleted HCVpp, in contrast to wild-type HCVpp, lead to the detection of a stronger and more broadly-specific neutralizing response in sera from the first group of patients, indicating that HS/HDL may somehow mask HCVpp from the cross-neutralizing antibodies present within these sera. Altogether, results from the inventors and others assign to HVR1 three different roles,
which are complementary in their aim to help the virus to survive within its host: enhancement of cell entry, masking of virions from (cross)-neutralizing antibodies by HDL and escape from a selective humoral immune response by mutation. Preserving the three functions of HVR1 may be essential for viral persistence and is consistent with the notion that, despite its high degree of genetic variability, highly conserved amino acid positions are found throughout HVR1 and that, even at variable positions, the physico-chemical properties of amino acids are maintained (32). Indeed, the inventors found that non-conservative substitution of conserved amino acids had a dramatic effect on infection enhancement (Fig. 4), suggesting that genetic diversification of HVR1 may compromise immune escape and enhancement of cell entry. While the conserved amino acids may be responsible for maintaining HVR1 in a conformation that allows interaction with HDL and/or SR-BI, the truly variable positions are likely involved in HVR1 antigenicity; a type of organization similar to those of immunoglobulin and T-cell receptor variable domains that exhibit variable sequences but conserved conformations (13). Deciphering the molecular aspects of HVR1 in immune escape and infection enhancement will provide valuable information on HCV biology and the development of antiviral therapies.
Table 1. Enhancement of HCVpp infection by human serum or HDL

<table>
<thead>
<tr>
<th>Treatment of Huh-7 target cells</th>
<th>% infection$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HS</td>
</tr>
<tr>
<td>1) Pre-mix no FF/HCVpp (1 min)$^b$</td>
<td>100</td>
</tr>
<tr>
<td>1) Pre-mix FF/HCVpp (1 min)$^b$</td>
<td>411±23</td>
</tr>
<tr>
<td>1) Pre-mix FF/HCVpp (1 hr)$^b$</td>
<td>422±53</td>
</tr>
<tr>
<td>No FF (1 min)$^c$</td>
<td>-</td>
</tr>
<tr>
<td>FF (1 min)$^c$</td>
<td>-</td>
</tr>
<tr>
<td>FF (1 hr)$^c$</td>
<td>-</td>
</tr>
<tr>
<td>FF (1 min)$^c$</td>
<td>wash</td>
</tr>
<tr>
<td>FF (1 hr)$^c$</td>
<td>wash</td>
</tr>
<tr>
<td>Pre-bind HCVpp (1 hr)$^d$</td>
<td>-</td>
</tr>
<tr>
<td>Pre-bind HCVpp (1 hr)$^d$</td>
<td>-</td>
</tr>
<tr>
<td>Pre-bind HCVpp (1 hr)$^d$</td>
<td>wash</td>
</tr>
<tr>
<td>Pre-bind HCVpp (1 hr)$^d$</td>
<td>wash</td>
</tr>
</tbody>
</table>

$^a$: results of infection expressed as percentages of the average infectious titers ± standard deviations (n=3) relative to titers determined in the absence of the indicated facilitating factor (FF): HS; human serum (2.5%), HDL (6 
µg/ml) or LDL (39 
µg/ml).

$^b$: Huh7 cells were treated with a mixture of virus and facilitating factor, which had been preincubated for the indicated times, at room temperature.

$^c$: Huh7 target cells were incubated with the facilitating factor for the indicated time at room temperature and then directly infected with HCVpp or washed twice to remove unbound facilitating factors before infection with HCVpp.

$^d$: HCVpp were pre-bound to Huh7 cells for 1 hr at 4°C. Then facilitating factor were either directly added to the infection, or unbound virus was removed by washing the Huh7 cells twice before addition of facilitating factor. Removing unbound virus from the infection reaction resulted in a 15 fold reduction in titre.
Table 2. Neutralizing responses in sera from acute-phase HCV-infected patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>HCVpp-1b&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HCVpp-del1b&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HCVpp-1a&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HCVpp-del1a&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt-1</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Pt-2</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Pt-3</td>
<td>++</td>
<td>+++</td>
<td>+/-</td>
<td>+++</td>
</tr>
<tr>
<td>Pt-4</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pt-5</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Pt-6</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Pt-7</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Pt-8</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Pt-9</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pt-10</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pt-11</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pt-12</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pt-13</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

<sup>a</sup>: the cohort consisted of hemodialysis patients (mean age 63.7 years, range 37-77) with HCV RNA-positive acute HCV infection who had all been infected by a single HCV source, genotype 1b, during a nosocomial outbreak that occurred in an hemodialysis ward in mid-2002, as described in (21). They were all followed for 6 months without treatment before a therapeutic decision was made. Acute HCV infection was confirmed in patients Pt-1 to Pt-13 by characterizing seroconversion profiles with a line immunoassay (INNO-LIA HCV IV, Innogenetics, Gent, Belgium). One group of patients (Pt-1 to Pt-7) was characterized by a strong decrease of HCV replication over time correlated with the emergence of neutralizing responses, whereas a second group (Pt-8 to Pt-13) did not clear or control the virus (21).

<sup>b</sup>: the neutralizing response was analyzed by incubating patient sera with viral particles harboring the E1E2 glycoproteins of 1b genotype (HCVpp-1b), 1a genotype (HCVpp-1a) and the HVR1-deleted versions of these glycoproteins (HCVpp-del1b and HCVpp-del1a, respectively). The results display the maximal neutralizing activity detected during the 6-month follow up and were expressed as the mean percentages of inhibition of the average infectious titers relative to incubation with medium devoid of human serum: -, no detectable neutralization; +/-, <25% neutralization; +, 25-80% neutralization; ++, 80-90% neutralization; ++++, >90% neutralization. The results were derived from at least three independent experiments and the standard deviations (see Fig. 5A) did not exceed 15% of the mean values.
Table 3. Modulation of HCVpp antibody-mediated neutralization by human serum or HE

<table>
<thead>
<tr>
<th>Antibody:</th>
<th>Neutralization in the presence of</th>
<th>Antibody concentrations (µg/ml) at&lt;sup&gt;d&lt;/sup&gt;:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ID60</td>
</tr>
<tr>
<td>E2mab-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>no serum</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>HDL</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>6.2</td>
</tr>
<tr>
<td>9/27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>no serum</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>HDL</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>1.54</td>
</tr>
<tr>
<td>AP33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>no serum</td>
<td>4.53</td>
</tr>
<tr>
<td></td>
<td>HDL</td>
<td>18.86</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>21.46</td>
</tr>
<tr>
<td>HCV Ig&lt;sup&gt;b&lt;/sup&gt;</td>
<td>no serum</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>LDL</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>HDL</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>90</td>
</tr>
<tr>
<td>Anti-RD114&lt;sup&gt;c&lt;/sup&gt;</td>
<td>no serum</td>
<td>0.431</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>0.459</td>
</tr>
</tbody>
</table>

a: HS (2.5%) or HDL (6 µg/ml) or LDL (39 µg/ml) were added to target cells concomitantly to the indicated antibodies, before infection with HCVpp or RD114pp.
b: neutralization of HCVpp with the indicated antibodies.
c: neutralization of RD114pp with a polyclonal antibody against RD114 glycoproteins.
d: concentration of antibodies in infection medium required to neutralize the HCVpp or RD114pp at the indicated ID values. "-", not applicable as neutralization could not reach the indicated ID value at saturating antibody concentrations.
Table 4: Neutralising activity of selected antibodies

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Neutralisation in the presence of: b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nothing</td>
</tr>
<tr>
<td>E1 (192-202)</td>
<td>+++</td>
</tr>
<tr>
<td>5 E1 (polyclonal)</td>
<td>+++</td>
</tr>
<tr>
<td>HVR1 (396-407)</td>
<td>+++</td>
</tr>
<tr>
<td>E2 (412-423)</td>
<td>+++</td>
</tr>
<tr>
<td>E2 (432-443)</td>
<td>+++</td>
</tr>
<tr>
<td>10 E2 (436-447)</td>
<td>+++</td>
</tr>
<tr>
<td>E2 (conformational)</td>
<td>+++</td>
</tr>
<tr>
<td>E1E2 (polyclonal)</td>
<td>+++</td>
</tr>
</tbody>
</table>

a: epitope location as numbered in the HCV polyprotein

b: maximal neutralising % of HCVpp in infection assays in the presence of saturating concentrations of antibodies: ++++, over 95%; ++, 80%; +, 60%

Table 5. Desensitisation of HCVpp neutralisation by human serum

Sequential treatment of viral particles and Huh-7 target cells

<table>
<thead>
<tr>
<th>Step A: Treatment of HCVpp a</th>
<th>Step B: Infection of Huh-7 cells c</th>
<th>% neutralisation d</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>HCVpp + mAb</td>
<td>72.23%±10.1</td>
</tr>
<tr>
<td>NA</td>
<td>HCVpp + mAb + HS</td>
<td>19.73%±10.7</td>
</tr>
<tr>
<td>Preincubation HS + HCVpp</td>
<td>Mix Step A + mAb</td>
<td>21.46%±9.4</td>
</tr>
<tr>
<td>Preincubation mAb + HCVpp</td>
<td>Mix Step A</td>
<td>76.48%±3.8</td>
</tr>
<tr>
<td>Preincubation mAb + HCVpp</td>
<td>Mix Step A + HS</td>
<td>38.36%±7.5</td>
</tr>
<tr>
<td>Preincubation mAb + HCVpp</td>
<td>Purified Mix Step A</td>
<td>56.92%±10.2</td>
</tr>
<tr>
<td>then purification b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preincubation mAb + HS+</td>
<td>Purified Mix Step A</td>
<td>64.31%±5.5</td>
</tr>
<tr>
<td>HCVpp then purification b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preincubation mAb + HCVpp</td>
<td>Purified Mix Step A + HS</td>
<td>44.90%±0.8</td>
</tr>
<tr>
<td>then purification b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preincubation mAb + HS+</td>
<td>Purified Mix Step A + HS</td>
<td>29.01%±0.8</td>
</tr>
<tr>
<td>HCVpp then purification b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
a: HCVpp and 2.5% human serum (HS) or 5 µg/ml of the AP33 monoclonal antibody (mAb) were preincubated for 45 min at room temperature, before addition of the purified or non-purified mixtures to Huh-7 cells. NA, not applicable.

b: HCVpp preincubated or non preincubated with HS and/or antibody were purified by ultracentrifugation through sucrose cushion.

c: Huh-7 target cells were simultaneously incubated with the indicated reagents for 4 hrs at 37°C.

d: Results are expressed as the mean percentages (mean ± SD, n=4) of inhibition of the infectious titres relative to incubation with medium devoid of antibodies.

Table 6. Determination of CD81-NOB activity of antibodies.

<table>
<thead>
<tr>
<th></th>
<th>HS</th>
<th>HDL</th>
<th>LDL</th>
<th>NOB</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBH2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CBH5</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CBH7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>E2mAb-1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3/11</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>H35</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>H48</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>H54</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>H57</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AP33</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>9/27</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H60</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H111</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-80Ab</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a: Determination of desensitised (+) or non-desensitised (-) neutralising property by human serum as described in Fig.5.

b: Determination of desensitised (+) or non-desensitised (-) neutralising property by HDL and low density lipoproteins (LDL).

c: Neutralisation of binding to CD81.
REFERENCES


C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. Proc Natl Acad Sci U S A 93:15394-15399.


glycoprotein that blocks virus attachment and viral infectivity. J Virol, 78, 7257-7263


CLAIMS

1. A method for selecting an HCV epitope which is not sensitive to HDL-mediated decrease of the neutralizing capacity of anti-HCV antibodies, said method comprising:
   - testing an antibody against an HCV epitope for its capacity to neutralise HCV or HCV pseudoparticles in the presence of HDL and/or human serum and in the absence of HDL and/or human serum;
   - comparing the capacity to neutralise HCV or HCV pseudoparticles in the presence of HDL and/or human serum and in the absence of HDL and/or human serum; and,
   - selecting the HCV epitope if the capacity of an antibody to neutralise HCV or HCV pseudoparticles is not substantially decreased in the presence of HDL and/or human serum.

2. The method of claim 1, wherein the tested antibody is directed against the HVR1 region of E2.

3. The method of claim 1, wherein the tested antibody (i) is directed against E2 and (ii) does not neutralise HCV by inhibiting E2/CD81 interaction.

4. The method of claim 1, wherein the tested antibody is directed against E1.

5. A method for treating HCV infection in a patient, the method comprising administering to the patient a therapeutically effective amount of a compound inhibiting HCV/HDL/SR-BI interplay.

6. The method of claim 5, wherein said compound is an inhibitor of HDL/SR-BI interaction.

7. The method of claim 6, wherein said compound is a selected from the group consisting of BLT-1, BLT-2, BLT-3, BLT-4, and BLT-5.

8. The method of claim 6, wherein said compound is an anti-SR-BI antibody.
9- The method according to claim 5, further comprising the administration of at least one HCV-neutralizing antibody to said patient.

10- The method according to claim 5, further comprising the administration of an HCV antigen for eliciting HCV-neutralizing antibodies in said patient.

11- The method according to claim 5, wherein the compound inhibiting HCV-HDL complexes is a compound which decreases the plasma loads of HDL in said patient.

12- A pharmaceutical composition comprising a compound inhibiting HCV/HDL/SR-BI interplay and at least one HCV-neutralizing antibody, for simultaneous, separate or sequential administration.

13- A pharmaceutical composition comprising a compound inhibiting HCV/HDL/SR-BI interplay and at least one HCV antigen for eliciting HCV-neutralizing antibodies, for simultaneous, separate or sequential administration.

14- A method for identifying a drug useful for treating or preventing HCV infection, comprising incubating HDL and HCV or a HCV pseudo-particle with a candidate drug and identifying a candidate drug which inhibits the HCV/HDL/SR-BI interplay, said drug being useful for treating or preventing HCV infection.

15- The method of claim 14, wherein said candidate drug is an inhibitor of HDL/SR-BI interaction.

16- A method for increasing the therapeutic activity of an HCV-neutralizing antibody in a subject, the method comprising administering to the subject, prior to, together with or after said HCV-neutralizing antibody, a compound that inhibits HCV/HDL/SR-BI interplay.
17- A method for treating HCV infection in a patient, the method comprising reducing the plasma loads of HDL in said patient.

18- The method according to claim 17, further comprising the administration of at least one HCV-neutralizing antibody to said patient.

19- The method according to claim 17, further comprising the administration of an HCV antigen for eliciting HCV-neutralizing antibodies in said patient.

20- The method according to claim 17, wherein reducing the plasma loads of HDL in said patient is performed by depletion of HDL by plasmapheresis.

21- The method according to claim 17, wherein reducing the plasma loads of HDL in said patient is performed by administering to the patient a compound which decreases the plasma loads of HDL in a direct or indirect way.
Figure 2C

![Graph showing % infection across different conditions.](image-url)
Figure 3A

Figure 3B

Figure 3C

Figure 3D

No HS/HDL

HS

HDL

% infection

% infection

% infection

% infection

BLT concentration (µM)

BLT concentration (µM)

BLT concentration (µM)
Figure 4A

6/16 Viral pellets

E1

E2

MLV CA

Figure 4B

Infectious titer (IU/ml)

10^3
10^4
10^5
10^6

HCvpp
HCvpp-del
G389L HCvpp
L399R HCvpp
G405R HCvpp
G406L HCvpp
Y276F HCvpp

Figure 4C

% Infection

no HS/HDL
HS
HDL

HCvpp
HCvpp-del
G389L HCvpp
L399R HCvpp
G405R HCvpp
G406L HCvpp
Y276F HCvpp
RD114pp
Figure 5A

- □ - HCVpp-1b  ■ - HCVpp-del1b
- ○ - HCVpp-1a  ● - HCVpp-del1a

---

Figure 5B

---

% neutralization

Antibody concentration (µg/ml)
Figure 5C

![Graphs showing antibody neutralization for different HCVpp variants](image)

- **HCVpp**
  - No HS vs. HS neutralization profiles for antibody concentration (μg/ml)

- **L399R HCVpp**
  - No HS vs. HS neutralization profiles for antibody concentration (μg/ml)

- **G406R HCVpp**
  - No HS vs. HS neutralization profiles for antibody concentration (μg/ml)

- **G406L HCVpp**
  - No HS vs. HS neutralization profiles for antibody concentration (μg/ml)
Residual infection (%) upon treatment with:

- Control serum
- HCVpp-E1 serum
- Chronic HCV serum

FIGURE 6
FIGURE 7
FIGURE 8A
12/16

HCVpp capture

FIGURE 8B
**FIGURE 9**

**A**

HDL: $R^2 = 0.8202$

HS: $R^2 = 0.9294$

**B**

[Graphical data showing the effect of BLT4 on HCVpp/HS and HCVpp/HDL interactions, with fold increase of infection and BLT4 concentration]
FIGURE 10
FIGURE 11
FIGURE 12
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. GOIN33/576

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

GOIN

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, CHEM ABS Data, BIOSIS, EMBASE, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>WO 2004/032716 A (MASSACHUSETTS INSTITUTE OF TECHNOLOGY; CENTER FOR BLOOD RESEARCH, INC) 22 April 2004 (2004-04-22) cited in the application page 2, line 26 - page 3, line 11; claims 1-5; tables I-III</td>
<td>12, 13</td>
</tr>
<tr>
<td>Y</td>
<td>WO 03/040726 A (ISTITUTO DI RICERCHE DI BIOLOGIA MOLECOLARE P. ANGELETTI SPA; CORTESE,) 15 May 2003 (2003-05-15) page 14, line 12 - line 19; claim 16; example 5 page 5, line 21 - line 31</td>
<td>1-4</td>
</tr>
<tr>
<td>A</td>
<td>See patent family annex.</td>
<td>5-21</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C.

Date of the actual completion of the international search 26 April 2006

Date of mailing of the international search report 24/05/2006

Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentissen 2 NL - 2280 HJ Rijswijk Tel. (431-70) 340-2040, Tx. 31 651 epo nl Fax: (431-70) 340-3016

Authorized officer Van Bohemen, C
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>VOISSET CÉCILE ET AL: &quot;High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I.&quot; THE JOURNAL OF BIOLOGICAL CHEMISTRY. 4 MAR 2005, vol. 280, no. 9, 4 March 2005 (2005-03-04), pages 7793-7799, XP002377477 Rockville MD USA ISSN: 0021-9258 page 7797, column 2, paragraph 3; figure 5</td>
<td>14, 15</td>
</tr>
<tr>
<td>Y</td>
<td></td>
<td>1-4</td>
</tr>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2501685 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1562605 A2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1444523 A2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2005509160 T</td>
</tr>
</tbody>
</table>