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Expression of hepatitis C virus proteins in epithelial intestinal cells in vivo

Short title: HCV protein expression in intestine

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Abbreviations: HCV, hepatitis C virus; LVP, lipo-viro-particles; VLDL, very low density lipoprotein; LDL, low density lipoprotein; apoB, apolipoprotein B; apoE, apolipoprotein E; ER, endoplasmic reticulum.

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Abstract

Previous work on hepatitis C virus (HCV) led to the discovery of a new form of viral particles associating viral and lipoprotein elements. These hybrid particles (LVP for lipo-viro-particles) are enriched in triglycerides and contain at least apolipoprotein B (apoB), HCV RNA and core protein. These findings suggest that LVP synthesis could occur in liver and intestine, the two main organs specialized in the production of apoB containing lipoprotein. To precise the site of LVP production, we studied the genetic diversity and phylogenetic relationship of HCV quasispecies from purified LVP, whole serum and liver biopsies from chronically infected patients. HCV quasispecies from LVP and liver differed significantly suggesting that LVP were not predominantly synthesized in the liver but that they might also originate from the intestine. We thus searched for presence of HCV in the small intestine. Paraffin embedded intestinal biopsies from ten HCV chronically infected patients and from twelve HCV RNA negative controls (10 anti-HCV antibody negative and 2 anti-HCV antibody positive patients) were tested for HCV protein expression. HCV NS3 and NS5A proteins were stained in small intestine epithelial cells in 4 out of 10 chronically infected patients and not in controls. Cells expressing HCV proteins were apoB producing enterocytes but not mucus secreting cells. These data indicate that small intestine can be infected by HCV and identify this organ as a potential reservoir and replication site. This further emphasizes the interaction between lipoprotein metabolism and HCV, and opens new insights in hepatitis C infection and pathophysiology.

Introduction

HCV infects over 170 million people worldwide and is a major cause of chronic liver infection often leading to chronic liver disease, cirrhosis and hepatocellular carcinoma. HCV possesses a single-stranded, positive-sense RNA genome encoding a single polyprotein and the virus has been classified in the *Flaviviridae* family beside the flaviviruses and pestiviruses (Pringle, 1999). While the flaviviruses infect many tissues in a large number of hosts, from insects to primates, and replicate in numerous cell lines *in vitro*, HCV replication is extremely limited and occurs mainly in the human liver (Hoofnagle, 2002, Rice, 1996). Studies on the virus replication and biology have been hampered by the lack of an efficient and reliable cell culture system and because chimpanzee is the only animal model. HCV may be envisioned as a defective flavivirus whose replication relies on specific cellular functions present in specific organs like the liver. A recent report on replication of HCV subgenomic replicons in non hepatic epithelial human and mouse hepatoma cells indicates that the specific cellular factors required for translation and replication of HCV RNA are not restricted to hepatocytes or cells of human origin (Zhu et al., 2003). Therefore, the apparent tropism of HCV for hepatocytes is likely determined at the level of virus entry or assembly.

Several reports revealed the density heterogeneity of ill-defined HCV RNA containing particles. HCV-RNA containing particles were found at density of between 1.03 and 1.25 g/ml in the serum of chronically infected individuals (Miyamoto et al., 1992, Prince et al., 1996, Thomssen et al., 1993). Titration infectivity in chimpanzee established a relationship between density of particles and infectivity, the highest infectivity of plasma being associated with the majority of HCV RNA in low density fraction ($d < 1.06$ g/ml) while HCV RNA found in higher density fractions seemed to be poorly infectious (Bradley et al., 1991, Hijikata et al., 1993). The unusually low density of some HCV RNA containing particles suggested an association of the virus with plasma lipoproteins (Thomssen et al., 1993). Low density lipoproteins ($d < 1.06$ g/ml) are particles which consist of a hydrophobic core of neutral lipid surrounded by a monolayer of amphipathic phospholipids and free cholesterol in which apolipoproteins reside (Fisher & Ginsberg, 2002, Rustaeus et al., 1999). Hepatocytes produce and secrete very low density lipoprotein (VLDL) containing apolipoproteins B and E (apoB and apoE). Transformation of VLDL in the circulation gives rise to particles of smaller size, with intermediate to low density (intermediate-density

lipoprotein [IDL] and low-density lipoprotein [LDL]) (Fisher & Ginsberg, 2002). Chylomicrons are another form of circulating very low density lipoproteins that are secreted by intestinal epithelial cells. They resemble VLDL but contain truncated apoB, are larger and are enriched in triglyceride. Transformation of chylomicrons in the circulation also leads to particles of smaller size, and higher density (Hussain et al., 1996, Yu & Cooper, 2001). In an attempt to better understand the relationship between HCV and lipoprotein metabolism and to unveil specific factors required for viral replication and assembly, we recently conducted a study to precise the nature and the infectivity of HCV particles in the low density plasma fractions (Andre et al., 2002). Low density HCV RNA containing particles (lipo-viro-particles [LVP]) were rich in triglycerides, contained at least apoB, HCV RNA and core protein and appeared as large spherical particles over 100 nm in diameter with internal structures. Delipidation of these particles resulted in capsid-like structures recognized by anti-HCV core protein antibody. These findings suggested that LVP synthesis could occur in organs specialized in production of apoB containing lipoproteins. Because HCV replicates in the liver, this organ is likely an important source of LVP. Alternatively, because of the very high triglyceride content of LVP, intestine may also contribute to the production of these particles. To address this question, we conducted a study to identify the site of LVP production.

Materials and methods

Blood and liver samples.

Volunteers attending the Liver Unit at Necker Hospital, Paris were selected in accordance to the hospital Ethics Committee statements; they were chronically HCV infected patients with chronic active hepatitis and were not given antiviral therapy for more than 6 months. Screening for HBV or HIV infection was negative. EDTA (1mM final concentration) was added to 40 ml of peripheral blood and samples were sent to the laboratory at ambient temperature where plasma and serum were immediately processed for density fraction separation and RNA extraction with the NucleoSpin RNA virus kit (Macherey Nagel, Haerd, France) and eluted in 50 µl RNase free water. Aliquots were stored at -80° C. Liver biopsies performed for regular clinical follow up of three patients were retrospectively included in the study. Frozen liver tissue stored in liquid

nitrogen was disrupted and homogenized with a rotor-stator homogenizer. RNA was extracted with the RNeasy kit (Qiagen) and eluted in 40 μ l RNase-free water.

Small intestine and liver biopsies.

Intestinal and liver biopsies were conserved at the Laboratoire d'Anatomie Pathologique, centre hospitalier Edouard Herriot, Lyon, France. Formalin fixed liver biopsies from one HCV seronegative patient and from four HCV RNA positive patients were selected as negative and positive controls. Formalin fixed and paraffin embedded biopsies of the duodenum from 12 HCV seropositive patients (for one patient, a biopsy of the jejunum was also available) and from 12 HCV sero-negative patients were studied. Intestinal biopsies were taken for diagnostic purposes during endoscopic examinations performed for symptoms not related to HCV infection : dyspepsia in 5 cases, abdominal pain in 5 cases or suspicion of malabsorption in 1 case, melaena (1 case) or evaluation of HCV-related vascularitis (1 case). In all cases but two, histological status of biopsy samples was normal. Aspergillosis was detected in one patient after liver transplantation for HCV-related cirrhosis and chronic non specific duodenitis was observed for the other patient. Ten of the 12 HCV seropositive patients were also HCV RNA positive by the VERSANT® HCV RNA Qualitative Assay (TMA) (Bayer). For RNA extraction, biopsies were sliced in small pieces, deparaffinated and rehydrated as described below for the immuno-staining. 150 μ l of 6 mg/ml Proteinase K (Sigma) complemented with 20 μ g of yeast tRNA were added to the sections and digestion was performed overnight at 45°C. Total RNA was further extracted with Macherey-Nagel NucleoSpin RNA Virus kit and eluted in 50 μ l RNase free water.

Preparation of the low density fractions.

Plasma from infected patients were separated by ultra centrifugation to obtain one low density fraction composed of the apoB containing lipoproteins, including chylomicrons, VLDL, IDL and LDL. Density of the plasma was adjusted to $d = 1.055$ g/ml with NaBr (Sigma). The low density fraction (density $d < 1.055$ g/ml) was obtained by centrifugation of plasma for 4 h at 4°C and 543,000 x g with the TLA100.4 rotor and TL100 ultracentrifuge (Beckman Instruments, S.A., Gagny, France). The top fraction was collected and extensively dialysed at 4°C against 150mM

NaCl, 0.24 mM EDTA pH 7.4 buffer, filtered through 0.22 µm filters (Millipore S.A., Saint Quentin, France) and stored at 4°C in the dark.

Purification of Lipo-viro-particles.

This procedure (Andre et al., 2002) has been extensively described and leads to the purification of HCV lipo-viro-particles (LVP). Briefly, ten µl of protein A coated magnetic beads (Miltenyi Biotec, Paris, France) were incubated at room temperature with 1 ml of the low density fractions in PBS with gentle rocking for 30 min. Beads were then passed through a magnetic column (Miltenyi Biotec), washed and collected in 500 µl PBS or DMEM-0.2% BSA (Gibco/BRL, Life Technologies, Cergy Pontoise, France). Samples with high lipid content were first diluted with two volumes of normal human serum to inactivate PCR inhibitors and RNA was extracted from 10 µl of LVP with the NucleoSpin RNA virus kit (Macherey-Nagel, Haerd, France), eluted in 50 µl and stored at -80°C.

HCV RNA quantitation.

HCV positive strand RNA quantitation was performed by real-time PCR in the 5' HCV non coding region, as previously described with minor modifications (Komurian-Pradel et al., 2001). Briefly, RNA (4 µl) was reverse-transcribed with the Thermoscript™ Reverse transcriptase kit (Gibco/BRL) using the RC21 primer. Real-time PCR were carried out with 2 µl of cDNA and RC1 and RC21 primers using the LC FastStart DNA Master SYBR Green I kit and the LightCycler™ apparatus (Roche Diagnostics, Meylan, France).

An index of HCV RNA association with low density fractions was determined including apolipoprotein B as an internal standard of the lipoprotein compartment (Andre et al., 2002). Apo B concentration in fraction and sera was determined using an immunochemical kit following the manufacturer procedure (Apo B kit, bioMérieux S.A., Marcy l'Etoile, France). The concentration was determined from a calibration curve established with the Apo B kit standard.

Index of HCV RNA association with low density fraction (LDF) was calculated as follow:

$$[(\text{RNA copy number/mg apo B}) \text{ in LDF}] / [(\text{RNA copy number/mg apo B}) \text{ in serum}] \times 100$$

Quantitation of negative strand HCV RNA was performed following the same procedure except that a tag-RC21 primer was used instead of RC21 to initiate the reverse transcription (5' ggc cgt cat ggt ggc gaa taa GTC TAG CCA TGG CGT TAG TA 3') and that the tag was used as primer during the amplification reaction. Details of the negative strand quantitation method was described elsewhere (Komurian-Pradel et al., 2004).

Sequencing and genotyping of the 5'HCV non coding region.

PCR amplification products that were synthesized during the HCV RNA quantitation process with the LightCyclerTM were directly sequenced in both directions using the PRISM Ready Reaction Amplitaq®FS BigDyeTM Terminator Cycle Sequencing Kit (PE/Applied Biosystems, Roissy, France), with the Applied Biosystem 377 and 373A automated DNA Sequencers. The HCV genotype was determined by comparison of nucleotide sequence to the HCVDB hepatitis sequence database (<http://hepatitis.ibcp.fr>).

Cloning of the viral quasispecies.

The HCV core and Envelope 2 (E2) regions were targeted for amplification by nested-RT-PCR. Viral RNA (8 µl) was reverse transcribed in a volume of 20 µl using the anti sense primer (5'-CAT CAT ATC CCA AGCCAT-3') for core and (5'-ACG GTC GAG GTG CGT ART GC-3') for E2 genes and then amplified for 35 cycles (denaturation at 94°C for 45 s, hybridization at 55°C for 30 s and primer extension at 72°C for 60 s) using the sense primer (5'-GCT TGC GAG TGC CCC GGG AGG TCT-3') and (5'-GTA ACA GGT CAC CGC ATG GC -3') for core and E2 genes, respectively. One tenth of the volume of the first PCR products were reamplified for 35 cycles with internal primers (5'-ATG AGC ACG AAT CCT AAA CC-3' and 5'-GGT ATC GAT GAC CTT ACC CA-3') for core gene and (5'-GCA TGG CTT GGG ATA TGA TG-3' and 5'-GCA GTC CTG TTG ATG TGC CA-3') for E2 gene. A 375 bp and a 285 bp fragments were respectively obtained for the core and E2 genes which includes the hyper variable region 1 (HVRI). The amplified PCR products of core and E2 regions were purified by QIAquick PCR purification kit (Qiagen), cloned into TOPO TA Cloning Kit and transformed into *Escherichia coli* strain One Shot Topo 10 competent cells (Invitrogen, San Diego, California). At least 10

clones were selected for each individual and sample. Plasmid DNA was extracted with the Qiagen plasmid Kit (QIAprep Miniprep; Qiagen) and sequenced using the PRISM Ready Reaction AmplitaqFS BigDye Terminator cycle sequencing kit (PE/Applied Biosystem, Rossey, France) and ABI PRISM® 3100 GENETIC ANALYSER (PE/Applied Biosystem).

Quasispecies sequence analysis.

Nucleotide sequences were aligned by using ClustalW 1.74 (Thompson et al., 1994) and refined by visual inspection with Seaview (Galtier et al., 1996). DNA distance matrix and phylogenetic trees were computed with Phylo_win (Galtier et al., 1996). Distances between sequences were computed under the Kimura two-parameters model (Kimura, 1980). Trees were built with the Neighbor-Joining method (Saitou & Nei, 1987) and tree topologies were tested with 1000 bootstrap sampling replicates.

In order to determine whether sequences from a given compartment shared more genetic identity with each other than with sequences from other compartments, we used the Mantel's test (Mantel, 1967). This test was performed using ADE-4 (Thioulouse, 1989). The method consists in comparing a DNA distance matrix to a compartmentalized reference distribution matrix of the same dimensions, where the (i,j) value of the matrix is put to 0 if sequence i is from the same compartment as sequence j and the (i,j) value is put to 1 in the other case. The Pearson correlation coefficient r^2 was computed for all pairs (observed r^2). The null distribution was constructed by permuting the rows and columns of the reference matrix 10,000 times. From this distribution, the number of times where the observed r^2 was exceeded, gave the exact p-value of the correlation observed.

Normalized Shannon entropies were calculated as described (Roque Afonso et al., 1999, Wolinsky et al., 1996). Differences between compartments genetic distances were assessed using the non-parametric Mann-Whitney test (StatView II, Abacus Concept Inc, Berkeley, Calif.).

Immuno-staining of biopsies.

Sections of formaline fixed paraffin embedded biopsies of the liver and of the small intestine were deparaffinated and rehydrated in two baths of methylcyclohexane, two baths of 100%

ethanol, one bath 70% ethanol and one bath of PBS for 10 min each. Sections were placed in 0.01 M citrate buffer pH 6.0 treated for 16 min in microwave oven at 650 W and then allowed to cool slowly to room temperature. Endogenous peroxidase was inhibited by incubation with 1% H₂O₂ in PBS for 10 min and sections were placed in PBS 0.2% BSA for 30 min. Monoclonal antibodies to HCV NS3 (clone 4G10H4), NS5a (clone 4F3H2) and irrelevant Mab (clone 17D1C11) were provided by BioMerieux (France) and were of IgG1 isotype. They were applied for 1 h at final concentration of 0.07 mg/ml at room temperature. After 4 washings in PBS-0.2% BSA, sections were incubated with ENVISIONTM+ System HRP Mouse (DAKO, Ca.) for 45 min. Following 4 washings in PBS-0.2% BSA and 2 washings in PBS, staining was developed for 10 minutes with DAB peroxidase substrate kit (VECTOR, Ca.). Sections were counterstained with Mayer's Hematoxyline (DAKO, Ca.) and mounted in 90% glycerol. Images were recorded with Sony Power HAD digital camera. Monoclonal 4F3H2 antibody anti-NS5A was tested and titrated on cells stably transfected and expressing NS5A provided by D. Moradpour (Polyak et al., 1999) (see supplementary data supplied on line).

Results

LVP quasispecies distribution in blood.

To determine whether LVP quasispecies are evenly distributed within the plasma HCV population or whether they represent a particular subset, we analyzed the genetic diversity of two HCV genome regions in paired LVP-plasma samples from three patients with histologically proven chronic hepatitis C. Patients were infected with HCV genotype 1b and were not given antiviral therapy for at least six months. LVP were present in patient plasma with an index of HCV RNA association to LVP ranging from 12 to 19 % (Table 1). Using RT-PCR, cloning and sequencing, we analyzed 73 capsid and 80 E2 clones [including the E2 hypervariable region (HVR1)] obtained from unfractionated plasma and purified LVP. A mixture of genetically distinct but closely related variants was present in each compartment. The genetic diversity was estimated by calculating the nucleotide and amino acid normalized Shannon entropy which can vary from 0 (no diversity) to 1 (maximum diversity) (Table 2). Entropy of the capsid, E2 and HVR1

sequences were higher in plasma than in LVP for the three patients. The degree of genetic diversity was determined as the average of within-sample genetic distances based on a Kimura two parameters matrix. Mean distances were significantly lower in the LVP fractions than in the plasma for at least one viral genome region for each patient (Table 2). Finally, bootstrapped phylogenetic trees obtained from the viral gene sequences indicated significant grouping according to the origin of the clones for each patient (an example of such grouping is shown in figure 1). Together, these data suggested that LVP quasispecies segregated in a distinct subset within HCV RNA plasma population. LVP could thus originate from a particular site of viral replication.

Comparison of HCV quasispecies from LVP and liver.

We then analyzed the genetic relationship between liver and LVP quasispecies in patient B from which liver and blood have been collected simultaneously. 17 capsid and 13 envelope clones were obtained from the liver and were compared to the corresponding LVP clones. Bootstrapped phylogenetic trees constructed with the capsid and envelope regions indicated that liver and LVP quasispecies segregated in distinct clusters with little or moderate overlapping (figure 2a and 2b). Statistical signification was calculated using Mantel's test to search for a relation between pairwise Kimura two-parameter distances and compartment distribution. Significant genetic differences were observed between liver and LVP quasispecies in the capsid region ($p < 0.0001$) and in the E1/E2 region ($p < 0.04$). Evolutionary constraints acting on E1/E2 quasispecies from plasma, LVP and liver were also determined for this patient by analyzing E1/E2 and HVR1 nucleotide substitution patterns (Table 3). There was a selection for conserved amino acid sequences of E1/E2 (excluding the hypervariable region HVR1) with non-synonymous versus synonymous mutation ratios ranging between 0.59 and 0.79 in the three studied compartments. Peptide variability of the HVR1 region was favored in the plasma and liver compartments with mutation ratios at 2.8 and 3.02. In contrast, the HVR1 region from the LVP fraction does not appear to be subjected to selection pressure since the mutation ratio was close to 1.

HCV detection in small intestine of chronically HCV infected patients.

The above data suggest that LVP may not be predominantly produced by the liver. Due to the biochemical composition of LVP resembling that of chylomicrons, we hypothesized that LVP may originate from the intestine. We therefore search for various signs of HCV infection in the small intestine. This study was conducted with formalin fixed and paraffin embedded small intestinal biopsies performed in 12 HCV seropositive patients and 12 HCV seronegative patients. Ten of the 12 anti-HCV positive patients were also HCV RNA positive while for the two remaining patients, HCV RNA could not be detected in the blood when intestinal biopsies were performed. Immunohistochemistry was used to visualize the presence of NS3 and NS5A HCV proteins in the biopsies. The anti-NS3 antibody detected HCV proteins in three liver samples from four chronically infected patients (Fig. 3 B, C and D). No HCV proteins were detected in HCV RNA negative patients. Intestinal biopsies from 4 of these 10 viremia positive patients were stained with anti-NS3 monoclonal antibodies (Fig. 3 E and G). Three of the four NS3 positive intestinal biopsies were also recognized by anti-NS5A monoclonal antibody on adjacent sections (Fig. 3 H). HCV positive cells were localized within the epithelium, predominantly in the crypts or in the lower part of the villi (Fig. 3 E and G). Interestingly, HCV protein expression was observed in enterocytes which synthesize and secrete chylomicrons (Field & Mathur, 1995) while mucus secreting cells were not stained (Fig. 3 G and H). For one patient, ileal and jejunal biopsies were also stained positive. Two years apart biopsies for another patient were both positive for HCV proteins. Presence of HCV in intestine was further confirmed by HCV RNA detection from positively stained biopsies. Total RNA was extracted from two formalin fixed tissue and HCV RNA was amplified by strand specific RT-PCR. Despite the very low yield of RNA extraction from formalin fixed tissue, positive HCV RNA was detected from one of the two NS3 positive biopsies tested. The intestinal sequence of the HCV 5' non coding region matched the plasma genotype 1b sequence (data not shown). Epithelial intestinal cells could thus be envisioned as an HCV reservoir which expresses viral proteins and replicates HCV RNA.

Discussion

The existence of extrahepatic reservoirs for HCV replication has long been suspected but remains controversial. Several authors have reported the presence of HCV negative strand RNA, the replicative intermediate, in peripheral blood mononuclear cells (PBMC) and in various autopsy

tissues of chronically infected patients (Laskus et al., 1998b, Yan et al., 2000). These data are sometimes not considered as conclusive since the reverse transcription PCR used to detect the negative strand could lack strand specificity and viral protein production is not demonstrated (Lanford et al., 1995, Laskus et al., 1997). However, infection of extrahepatic tissues is supported by the finding that HCV quasispecies composition differs according to the cellular or tissue origin (Cabot et al., 2001, Laskus et al., 1998a, Laskus et al., 2000, Maggi et al., 1997, Roque Afonso et al., 1999). Particularly, genome variants present in plasma and not in liver or in PBMC suggested the existence of other viral compartments. Here, we showed that LVP quasispecies defined a subpopulation of the total plasma quasispecies and that liver and LVP quasispecies were different. These data indicated that the liver is not the only source of LVP and that another organ should contribute to their production. Taking into consideration that LVP are apoB containing particles, enriched in triglyceride, it was assumed that LVP should be synthesized and secreted by cells producing apoB containing lipoproteins. This identified small intestine enterocytes as a potential reservoir and replication site for HCV. Indeed, HCV NS3 and NS5A protein were detected in enterocytes from 4 out of 10 chronically infected HCV RNA positive patients and in none of non infected or non viremic patients. Infected enterocytes were grouped in foci of infected villi leaving numerous villi uninfected. Therefore, 40% of positive intestinal biopsies is a noteworthy percentage suggesting that the small intestine is generally infected in chronically infected patients. A similar percentage of NS3 positive liver biopsies from HCV RNA positive patients has been reported (Wolk et al., 2000). Although we could detect low level of positive strand HCV RNA, we failed to detect HCV negative strand most likely for technical reasons. We found that HCV negative strand RNA in liver is several hundred time less abundant than the positive strand (Komurian-Pradel et al., 2004). Accordingly, negative strand HCV has been found to replicate at a slower rate than the positive strand *in vitro* (Reigadas et al., 2001). Because of the low number of positive strand RNA that could be extracted from formalin fixed tissue, the detection of negative strand RNA was not expected. Altogether, the data show that the small intestine can be infected by HCV and strongly suggest that this organ may be a reservoir for HCV and a source of LVP. To our knowledge, search for HCV in intestine had been performed only once in a study of post-mortem tissues of HCV infected patients (Yan et al., 2000). However in this study, HCV RNA was detected in many tissues, including small intestine, and the nature of infected cells was not determined.

LVP enter hepatoma cell lines through the LDL receptor which recognizes apoB and E on the particle. Binding of purified LVP to HepG2 is very efficient and is competed out by native lipoproteins explaining the poor binding of non purified HCV from infected serum (Andre et al., 2002). The LDL receptor is expressed *in vivo* by intestinal cells (Levy et al., 2000). The entry route in enterocyte is therefore likely to be similar to that of hepatocytes. However, besides the LDL receptor, CD81 and the scavenger receptor B class 1 (SR-B1) have been described as putative receptors for the viral envelope proteins (Pileri et al., 1998, Scarselli et al., 2002). CD81 is not expressed in the intestine (Okochi et al., 1999) but SR-B1 was detected at the apical and basal pole of enterocytes and could be an alternative entry pathway for E1/E2 containing viral particles (Altmann et al., 2002, Cai et al., 2001). However, analysis of LVP quasispecies showed that the binding site of E2 (HVR1) to SR-B1 does not seem to be subjected to any selection. It is thus likely that no constraint is exerted by this receptor on the envelope protein and that LVP do not predominantly enter the intestinal cells via SR-B1.

Thinking of the intestine as a reservoir for HCV producing low density infectious viral particles modifies the current understanding of the pathogenesis of HCV infection and raises several questions. First, HCV infection of small intestine was surprisingly not accompanied by inflammatory response as mild duodenitis was only observed in one case. We recently showed that the lipid composition of apoB containing lipoproteins and of lipid emulsions has important consequences on dendritic cell maturation and function (Coutant et al., 2004, Perrin-Cocon et al., 2001). It is conceivable that the lipid moiety of the low density HCV particles induces a state of immune unresponsiveness. The mechanisms of such inhibition is under investigation. Second, because most chylomicrons and chylomicron remnants are captured by hepatocytes (Hussain et al., 1996, Yu & Cooper, 2001), small intestine might provide a source of infectious particles responsible for continuous liver infection and for infection of liver grafts. Such a mechanism would have important consequences for antiviral therapy. Efficient drugs should target intestinal cells and analysis of drug metabolism in these cells would become an important step in the development of antiviral therapy. Third, infection of intestinal cells *in vitro* may provide opportunities to better understand viral replication and assembly. The Caco-2 cell line is used as a model of human intestinal cells to study apoB metabolism and lipoprotein synthesis (Levy et al., 1995). The possibility to induce the differentiation of Caco-2 *in vitro* and to modulate the lipoprotein secretion, should allow a detailed analysis of the relationship between LVP and

lipoprotein assembly, leading to a cell based HCV replication system. Synthesis of chylomicron and VLDL depends on apoB synthesis and on its translocation into the lumen of the ER. If nascent lipoproteins are not loaded with triglyceride, apo B is retro-translocated into the cytoplasm where it is degraded by the proteasome (Hussain et al., 1996, Luchoomun & Hussain, 1999, Yu & Cooper, 2001). Because triglycerides are synthesized by enterocytes from dietary fatty acids and because chylomicron synthesis and secretion follow lipid digestion, it would be of interest to assess the influence of starving and of lipid rich meal on HCV viremia. Recently supporting this idea, a negative correlation has been reported between plasmatic apoB concentration and HCV viral load, particularly for patients infected with genotype non-1 (Petit et al., 2003).

In conclusion, presence of HCV proteins in enterocytes further emphasizes the interaction between lipoprotein metabolism and HCV, and modifies our current understanding of hepatitis C.

References.

- Altmann, S. W., Davis, H. R., Jr., Yao, X., Lavery, M., Compton, D. S., Zhu, L. J., Crona, J. H., Caplen, M. A., Hoos, L. M., Tetzloff, G., Priestley, T., Burnett, D. A., Strader, C. D. & Graziano, M. P. (2002). The identification of intestinal scavenger receptor class B, type I (SR-BI) by expression cloning and its role in cholesterol absorption. *Biochim Biophys Acta* **1580**, 77-93.
- Andre, P., Komurian-Pradel, F., Deforges, S., Perret, M., Berland, J. L., Sodoyer, M., Pol, S., Brechot, C., Paranhos-Baccala, G. & Lotteau, V. (2002). Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J Virol* **76**, 6919-28.
- Bradley, D., McCaustland, K., Krawczynski, K., Spelbring, J., Humphrey, C. & Cook, E. H. (1991). Hepatitis C virus: buoyant density of the factor VIII-derived isolate in sucrose. *J Med Virol* **34**, 206-8.
- Cabot, B., Martell, M., Esteban, J. I., Piron, M., Otero, T., Esteban, R., Guardia, J. & Gomez, J. (2001). Longitudinal evaluation of the structure of replicating and circulating hepatitis C virus quasispecies in nonprogressive chronic hepatitis C patients. *J Virol* **75**, 12005-13.
- Cai, S. F., Kirby, R. J., Howles, P. N. & Hui, D. Y. (2001). Differentiation-dependent expression and localization of the class B type I scavenger receptor in intestine. *J Lipid Res* **42**, 902-9.
- Coutant, F., Agaugue, S., Perrin-Cocon, L., Andre, P. & Lotteau, V. (2004). Sensing environmental lipids by dendritic cell modulates its function. *J Immunol* **172**, 54-60.
- Field, F. J. & Mathur, S. N. (1995). Intestinal lipoprotein synthesis and secretion. *Prog Lipid Res* **34**, 185-98.
- Fisher, E. A. & Ginsberg, H. N. (2002). Complexity in the secretory pathway: the assembly and secretion of apolipoprotein B-containing lipoproteins. *J Biol Chem* **277**, 17377-80.
- Galtier, N., Gouy, M. & Gautier, C. (1996). SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *Comput Appl Biosci* **12**, 543-8.

- Hijikata, M., Shimizu, Y. K., Kato, H., Iwamoto, A., Shih, J. W., Alter, H. J., Purcell, R. H. & Yoshikura, H. (1993). Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. *J Virol* **67**, 1953-8.
- Hoofnagle, J. H. (2002). Course and outcome of hepatitis C. *Hepatology* **36**, S21-9.
- Hussain, M. M., Kancha, R. K., Zhou, Z., Luchoomun, J., Zu, H. & Bakillah, A. (1996). Chylomicron assembly and catabolism: role of apolipoproteins and receptors. *Biochim Biophys Acta* **1300**, 151-70.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111-20.
- Komurian-Pradel, F., Paranhos-Baccala, G., Sodoyer, M., Chevallier, P., Mandrand, B., Lotteau, V. & Andre, P. (2001). Quantitation of HCV RNA using real-time PCR and fluorimetry. *J Virol Methods* **95**, 111-9.
- Komurian-Pradel, F., Perret, M., Deiman, B., Sodoyer, M., Lotteau, V., Paranhos-Baccala, G. & Andre, P. (2004). Strand specific quantitative real-time PCR to study replication of hepatitis C virus genome. *J Virol Methods* **116**, 103-6.
- Lanford, R. E., Chavez, D., Chisari, F. V. & Sureau, C. (1995). Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. *J Virol* **69**, 8079-83.
- Laskus, T., Radkowski, M., Wang, L. F., Cianciara, J., Vargas, H. & Rakela, J. (1997). Hepatitis C virus negative strand RNA is not detected in peripheral blood mononuclear cells and viral sequences are identical to those in serum: a case against extrahepatic replication. *J Gen Virol* **78 (Pt 11)**, 2747-50.
- Laskus, T., Radkowski, M., Wang, L. F., Jang, S. J., Vargas, H. & Rakela, J. (1998a). Hepatitis C virus quasispecies in patients infected with HIV-1: correlation with extrahepatic viral replication. *Virology* **248**, 164-71.
- Laskus, T., Radkowski, M., Wang, L. F., Nowicki, M. & Rakela, J. (2000). Uneven distribution of hepatitis C virus quasispecies in tissues from subjects with end-stage liver disease: confounding effect of viral adsorption and mounting evidence for the presence of low-level extrahepatic replication. *J Virol* **74**, 1014-7.
- Laskus, T., Radkowski, M., Wang, L. F., Vargas, H. & Rakela, J. (1998b). Search for hepatitis C virus extrahepatic replication sites in patients with acquired immunodeficiency syndrome: specific detection of negative-strand viral RNA in various tissues. *Hepatology* **28**, 1398-401.
- Levy, E., Beaulieu, J. F., Delvin, E., Seidman, E., Yotov, W., Basque, J. R. & Menard, D. (2000). Human crypt intestinal epithelial cells are capable of lipid production, apolipoprotein synthesis, and lipoprotein assembly. *J Lipid Res* **41**, 12-22.
- Levy, E., Mehran, M. & Seidman, E. (1995). Caco-2 cells as a model for intestinal lipoprotein synthesis and secretion. *Faseb J* **9**, 626-35.
- Luchoomun, J. & Hussain, M. M. (1999). Assembly and secretion of chylomicrons by differentiated Caco-2 cells. Nascent triglycerides and preformed phospholipids are preferentially used for lipoprotein assembly. *J Biol Chem* **274**, 19565-72.
- Maggi, F., Fornai, C., Vatteroni, M. L., Giorgi, M., Morrica, A., Pistello, M., Cammarota, G., Marchi, S., Ciccorossi, P., Bionda, A. & Bendinelli, M. (1997). Differences in hepatitis C virus quasispecies composition between liver, peripheral blood mononuclear cells and plasma. *J Gen Virol* **78 (Pt 7)**, 1521-5.
- Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Res* **27**, 209-20.

- Miyamoto, H., Okamoto, H., Sato, K., Tanaka, T. & Mishiro, S. (1992). Extraordinarily low density of hepatitis C virus estimated by sucrose density gradient centrifugation and the polymerase chain reaction. *J Gen Virol* **73** (Pt 3), 715-8.
- Okochi, H., Mine, T., Nashiro, K., Suzuki, J., Fujita, T. & Furue, M. (1999). Expression of tetraspans transmembrane family in the epithelium of the gastrointestinal tract. *J Clin Gastroenterol* **29**, 63-7.
- Perrin-Cocon, L., Coutant, F., Agaogue, S., Deforges, S., Andre, P. & Lotteau, V. (2001). Oxidized low-density lipoprotein promotes mature dendritic cell transition from differentiating monocyte. *J Immunol* **167**, 3785-91.
- Petit, J. M., Benichou, M., Duvillard, L., Jooste, V., Bour, J. B., Minello, A., Verges, B., Brun, J. M., Gambert, P. & Hillon, P. (2003). Hepatitis C virus-associated hypobetalipoproteinemia is correlated with plasma viral load, steatosis, and liver fibrosis. *Am J Gastroenterol* **98**, 1150-4.
- Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D., Grandi, G. & Abrignani, S. (1998). Binding of hepatitis C virus to CD81. *Science* **282**, 938-41.
- Polyak, S. J., Paschal, D. M., McArdle, S., Gale, M. J., Jr., Moradpour, D. & Gretch, D. R. (1999). Characterization of the effects of hepatitis C virus nonstructural 5A protein expression in human cell lines and on interferon-sensitive virus replication. *Hepatology* **29**, 1262-71.
- Prince, A. M., Huima-Byron, T., Parker, T. S. & Levine, D. M. (1996). Visualization of hepatitis C virions and putative defective interfering particles isolated from low-density lipoproteins. *J Viral Hepat* **3**, 11-7.
- Pringle, C. R. (1999). Virus taxonomy--1999. The universal system of virus taxonomy, updated to include the new proposals ratified by the International Committee on Taxonomy of Viruses during 1998. *Arch Virol* **144**, 421-9.
- Reigadas, S., Ventura, M., Sarih-Cottin, L., Castroviejo, M., Litvak, S. & Astier-Gin, T. (2001). HCV RNA-dependent RNA polymerase replicates in vitro the 3' terminal region of the minus-strand viral RNA more efficiently than the 3' terminal region of the plus RNA. *Eur J Biochem* **268**, 5857-67.
- Rice, C. M. (1996). Flaviviridae: the viruses and their replication. In *Fields Virology*, Third edn, pp. 931-959. Edited by B. N. Fields, Knipe, D.M., Howley, P.M. et al. Philadelphia: Lippincott-Raven Publishers.
- Roque Afonso, A. M., Jiang, J., Penin, F., Tareau, C., Samuel, D., Petit, M. A., Bismuth, H., Dussaix, E. & Feray, C. (1999). Nonrandom distribution of hepatitis C virus quasispecies in plasma and peripheral blood mononuclear cell subsets. *J Virol* **73**, 9213-21.
- Rustaeus, S., Lindberg, K., Stillemark, P., Claesson, C., Asp, L., Larsson, T., Boren, J. & Olofsson, S. O. (1999). Assembly of very low density lipoprotein: a two-step process of apolipoprotein B core lipidation. *J Nutr* **129**, 463S-466S.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-25.
- Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R. & Vitelli, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *Embo J* **21**, 5017-25.
- Thioulouse, J. (1989). Statistical analysis and graphical display of multivariate data on the Macintosh. *Comput Appl Biosci* **5**, 287-92.

- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-80.
- Thomssen, R., Bonk, S. & Thiele, A. (1993). Density heterogeneities of hepatitis C virus in human sera due to the binding of beta-lipoproteins and immunoglobulins. *Med Microbiol Immunol (Berl)* **182**, 329-34.
- Wolinsky, S. M., Korber, B. T., Neumann, A. U., Daniels, M., Kunstman, K. J., Whetsell, A. J., Furtado, M. R., Cao, Y., Ho, D. D. & Safrit, J. T. (1996). Adaptive evolution of human immunodeficiency virus-type 1 during the natural course of infection. *Science* **272**, 537-42.
- Wolk, B., Sansonno, D., Krausslich, H. G., Dammacco, F., Rice, C. M., Blum, H. E. & Moradpour, D. (2000). Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J Virol* **74**, 2293-304.
- Yan, F. M., Chen, A. S., Hao, F., Zhao, X. P., Gu, C. H., Zhao, L. B., Yang, D. L. & Hao, L. J. (2000). Hepatitis C virus may infect extrahepatic tissues in patients with hepatitis C. *World J Gastroenterol* **6**, 805-811.
- Yu, K. C. & Cooper, A. D. (2001). Postprandial lipoproteins and atherosclerosis. *Front Biosci* **6**, D332-54.
- Zhu, Q., Guo, J. T. & Seeger, C. (2003). Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. *J Virol* **77**, 9204-10.

Figure 1. Phylogenetic tree of serum and LVP clones from patient C, E1/E2 region. Whole plasma clones in blue and LVP clones in red. The phylogenetic grouping of LVP clones within the plasma clones was confirmed by a significant correlation between genetic proximity and compartment appartenance ($P < 0.0001$ by Mantel's test).

Figure 2. Phylogenetic trees of liver and LVP clones from patient B, capsid (A) and E1/E2 (B) regions. LVP clones are indicated in red and liver clones in green. The phylogenetic grouping of LVP and liver clones was confirmed by a significant correlation between genetic proximity and compartment appartenance ($P < 0.0001$ with the capsid region and $P < 0.04$ with the envelope region by Mantel's test).

Figure 3. Immunohistochemical staining of HCV proteins in small intestine. Anti-NS3 monoclonal antibody stains HCV infected liver biopsies. Sections of paraffin embedded liver biopsies were stained as described in materials and methods for NS3. Biopsy from one HCV negative patient (A) and from 3 HCV RNA positive patients (B, C and D). Detection of NS3 and NS5A HCV proteins in intestinal biopsies of viremic patients. Sections of paraffin embedded biopsies of intestine were stained as described in materials and methods for NS3 (E and G) and NS5A (H) HCV proteins or with an irrelevant isotype matching monoclonal antibody (F and I). Original magnification 20x (E and F) and 100x (G to I).

Table 1. Clinical characteristics of patients with chronic hepatitis C.

Patient (HCV genotype)	Age	Gender	ALT/AST (IU/L)	HCV RNA load		Liver Histology		Index* of RNA association
				Plasma	copies/ml	Metavir	Knodel	
				Liver	copies/ μ g			
B (1b)	47	M	64/58	4.1x10 ⁶	1.6x10 ⁶	A1F1	1111	12%
C (1b)	47	M	201/140	1.7x10 ⁶	0.6x10 ⁶	A3F4	4334	19%
G (1b)	43	M	39/27	4.1x10 ⁶	2.0x10 ⁶	A1F2	3133	14%

* Index of HCV RNA association to lipoproteins (see materials and methods).

Table 2. Genetic distances of capsid and E1/E2 (including HVR1) quasispecies from plasma and LVP in three chronically infected patients.

Patient and compartment	Number of clones*		Normalized nucleotide entropy [†]			Normalized amino acid entropy [†]			Mean genetic distance [‡] (standard deviation)	
	capsid	E1/E2	capsid	E1/E2	HVR1	capsid	E1/E2	HVR1	capsid	E1/E2
Patient B										
Plasma	10	10	0.76	0.88	0.58	0.41	0.88	0.35	0.0072 [§] (0.0049)	0.0359 (0.0313)
LVP	12	14	0.54	0.87	0.35	0.34	0.59	0.31	0.0042 [§] (0.0059)	0.0436 (0.0387)
Patient C										
Plasma	14	18	0.92	0.80	0.50	0.54	0.79	0.40	0.0097 [§] (0.0046)	0.0265 [§] (0.0213)
LVP	15	13	0.63	0.21	0.11	0.39	0.21	0.11	0.0056 [§] (0.0043)	0.0014 [§] (0.0017)
Patient G										
Plasma	10	14	0.88	0.89	0.71	0.53	0.74	0.45	0.0092 [§] (0.0064)	0.0488 [§] (0.0433)
LVP	12	11	0.81	0.87	0.64	0.44	0.61	0.36	0.0083 [§] (0.0043)	0.0286 [§] (0.0359)

* Number of independantly analyzed clones.

[†] Shannon entropy values were compared for the three patients and compartments by the Wilcoxon test ($P < 0.05$).

[‡] Within sample genetic distances were calculated with PHYLO_WIN based on a Kimura two parameter matrix with a transition to transversion ratio of 2. Mean genetic distances between plasma and LVP for each patient were compared by using the Mann and Whitney test ([§] indicates $P < 0.01$).

Table 3. Genetic diversity of E1/E2 quasispecies from plasma, LVP and liver in patient as determined by E1 and HVR1 nucleotide substitution analysis.

Compartments	E1/E2			HVR1		
	d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
Plasma	0.036 (0.036)	0.045 (0.029)	0.79	0.144 (0.177)	0.051 (0.058)	2.80
LVP	0.038 (0.041)	0.066 (0.049)	0.59	0.173 (0.193)	0.135 (0.100)	1.28
Liver	0.017 (0.025)	0.025 (0.032)	0.69	0.082 (0.113)	0.027 (0.069)	3.02

d_N and d_S , mean and standard deviation (in brackets) of proportions of nonsynonymous substitutions per nonsynonymous sites and synonymous substitutions per synonymous sites.

Figure 1

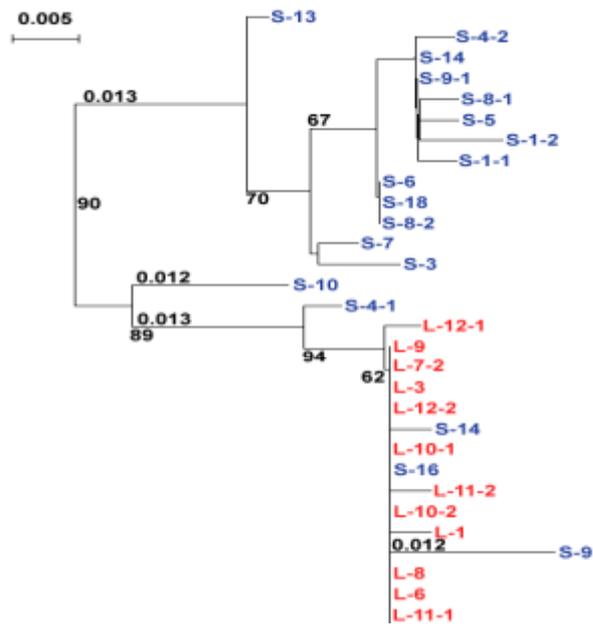


Figure 2

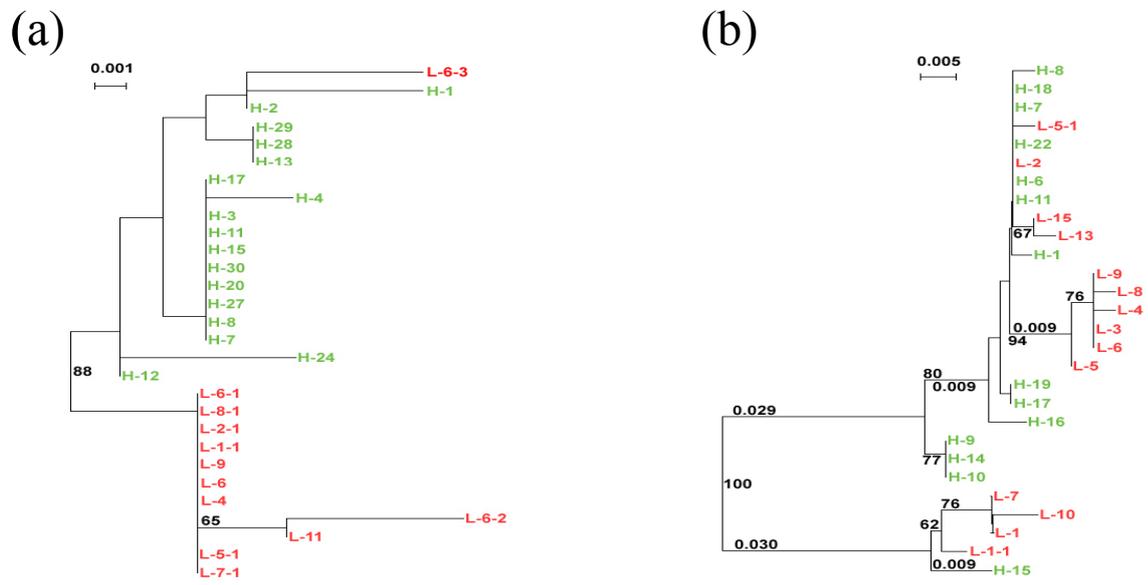


Figure 3

