Preferential association of *Hepatitis C virus* with apolipoprotein B48-containing lipoproteins

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Hepatitis C virus (HCV) in cell culture has a density comparable to that of other members of the family *Flaviviridae*, whereas *in vivo* infectious particles are found partially in low-density fractions, associated with triacylglycerol (TG)-rich lipoproteins (TRLs). In the blood of infected patients, HCV circulates as heterogeneous particles, among which are lipo-viroparticles (LVPs), globular particles rich in TG and containing viral capsid and RNA. The dual viral and lipoprotein nature of LVPs was addressed further with respect to apolipoprotein composition and post-prandial dynamic lipid changes. The TRLs exchangeable apoE, -CII and -CIII, but not the high-density lipoprotein apoA-II, were present on LVPs, as well as the viral envelope proteins. apoB100 and -B48, the two isoforms of the non-exchangeable apoB, were represented equally on LVPs, despite the fact that apoB48 was barely detectable in the plasma of these fasting patients. This indicates that a significant fraction of plasma HCV was associated with apoB48-containing LVPs. Furthermore, LVPs were enriched dramatically and rapidly in triglycerides after a fat meal. As apoB48 is synthesized exclusively by the intestine, these data highlight the preferential association of HCV with chylomicrons, the intestine-derived TRLs. These data raise the question of the contribution of the intestine to the viral load and suggest that the virus could take advantage of TRL assembly and secretion for its own production and of TRL fate to be delivered to the liver.

Received 15 March 2006
Accepted 6 June 2006

INTRODUCTION

Hepatitis C virus (HCV) has been classified within the family *Flaviviridae* according to the structure of its genome (Pringle, 1999). However, in contrast to flaviviruses and closely related viruses, cell culture of HCV remained problematic for 15 years and this lack of an appropriate *in vitro* replication system and of a small-animal model impeded the understanding of HCV structure and replication cycle. Therefore, most of our knowledge of the virus cell receptors and of HCV RNA replication relied on pseudotyped viruses and on biscistronic and subgenomic replicons, which do not allow the study of HCV assembly or secretion, or the identification of the elusive nature of the virion. Recently, complete replication and production of infectious HCV particles in tissue culture were performed with HCV genotype 2a full-length replicons derived from a patient with fulminant hepatitis (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). This major breakthrough identified a viral structure with size, morphology and density (1·15 g ml^{-1}) appropriate for a member of the family *Flaviviridae*. The structure of these virions probably matches that of virions found in the plasma of chronically infected patients, with a density of 1·15 g ml^{-1} and recognized by anti-HCV envelope antibodies (Kaito *et al.*, 1994; Petit *et al.*, 2005; Takahashi *et al.*, 1992).

Several forms of HCV particles coexist in the plasma of infected patients (Carrick *et al.*, 1992; Kanto *et al.*, 1994; Miyamoto *et al.*, 1992) with a wide range of density (from 1·30 g ml^{-1} to an unusual low density of $<$1·06 g ml^{-1}). Low-density viral particles are of particular interest, as they correlate with plasma infectivity in chimpanzees (Bradley *et al.*, 1991; Hijikata *et al.*, 1993). Interestingly, chimpanzee infection with *in vitro*-produced HCV with a density of 1·14 g ml^{-1} led to plasma HCV particles whose specific infectivity was recovered in fractions of lower density, indicating that a shift to lower buoyant density was correlated with an increased specific infectivity of HCV grown *in vitro* (Lindenbach *et al.*, 2006). The low density of some HCV particles was attributed to an association of the virus with triacylglycerol (TG)-rich lipoproteins (TRLs)

(Prince *et al.*, 1996; Thomssen *et al.*, 1992). Proportions of plasma HCV RNA found associated with TRLs vary from patient to patient, with a mean value close to 40 %, but can reach almost 100 % for some patients (André *et al.*, 2002; Nielsen *et al.*, 2004, 2006; Thomssen *et al.*, 1992, 1993). Some of these TRL-like structures have been described as lipo-viroparticles (LVPs), whose structure and origin remain to be better defined (André *et al.*, 2002; Nielsen *et al.*, 2006).

TRLs are very-low-density particles ($d \leqslant 1{\cdot}006$ g ml^{-1}) made of a hydrophobic core of neutral lipids, TG and cholesterol esters, surrounded by a monolayer of phospholipids (PL) and free cholesterol, associated with apoB and other apolipoproteins (Fisher & Ginsberg, 2002). TRLs are formed by the assembly of one molecule of apoB with TG within the endoplasmic reticulum lumen. apoB is a non-exchangeable apolipoprotein that remains associated with the particle until its capture and internalization by lipoprotein receptors. In humans, hepatocytes secrete very-low-density lipoproteins (VLDLs), which comprise one apoB100 molecule per particle, whereas enterocytes secrete another class of TRL, chylomicrons, which contain one molecule of apoB48, the truncated form of apoB resulting from the enterocyte-specific editing of apoB mRNA (Patterson *et al.*, 2003). In the circulation, TRLs are subjected to TG hydrolysis by lipoprotein lipase, releasing free fatty acids, the remodelling of surface lipids and of exchangeable apolipoproteins A, C and E. These modifications give rise to particles of smaller size and higher density, i.e. remnants from chylomicrons and intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL) from VLDLs.

LVPs are low-density, globular, HCV RNA-containing particles covered with natural antibodies allowing their purification from plasma low-density fractions ($d < 1{\cdot}055$ g ml^{-1}) (André *et al.*, 2002). They are rich in TG and contain internal structures that appear as capsids recognized by anti-core protein antibodies after delipidation. Binding and entry of purified LVPs into cells was competed by native VLDLs and by anti-apoB and anti-apoE antibodies, and increased by upregulation of the LDL receptor (Agnello *et al.*, 1999; André *et al.*, 2002). Therefore, LVPs appear to display some features of TRL-like structures. To further characterize the TRL-like nature of LVPs, the apolipoprotein composition of LVPs was analysed, as well as their lipid composition during the dynamic transition from the pre-prandial to the post-prandial period.

METHODS

Materials. Unless indicated, all chemicals were from Sigma. Silica gel thin-layer chromatography plates were from Whatman. Anti-E1 (A4) or E2 (H52 and H47) mAbs and 293T cells expressing E1 and E2 were obtained from Dr J. Dubuisson (Institut de Biologie de Lille-Institut Pasteur de Lille, France). Anti-apoB (clone 1D1) mAb was from the Heart Institute (University of Ottawa, Ontario, Canada) and peroxidase-conjugated goat anti-apoB antibodies were from Biodesign. Anti-apoCII polyclonal antibody was purchased from Merck Calbiochem. Anti-apoAII and anti-apoCIII polyclonal antibodies and anti-apoE mAb were obtained from Chemicon.

Blood samples and patients. Plasma samples from HCV-negative and HCV-positive blood donors were obtained from the Etablissement de Transfusion Sanguine, Lyon, France. Eight volunteers attending the Service d'Hépato-Gastro-Entérologie at the Hôtel Dieu Hospital, Lyon, France, were selected in accordance with hospital ethics committee statements and enrolled in the study of the transition from the pre- to post-prandial states and of the lipidomic analysis of their plasma viral population (Table 1). These patients were chronically HCV-infected and had not been given antiviral therapy for at least 6 months. HCV genotypes were determined by sequencing of the 5′-untranslated region, and presence of cryoglobulinaemia was checked by routine laboratory examination. Patients were given a breakfast of a 900 kcal meal containing 30 % fat after an overnight fasting. Peripheral blood was drawn just before breakfast and 90 min after the first phlebotomy. EDTA was added to 0·1 mM final concentration and samples were processed immediately.

Preparation of low-density fractions. Plasma samples were adjusted to 1·055 g ml^{-1} with NaBr and centrifuged for 4 h at 4 °C

Table 1. Clinical characteristics of patients with chronic hepatitis C

Patient (HCV genotype)	HCV RNA load			(1) Index of HCV RNA association (%)*	(2) (apoB48/total apoB) × 100 in LVPs	Plasma HCV RNA associated with apoB48 (%)†
	Plasma [copies (mg apoB)$^{-1}$]	Low-density fraction [copies (mg apoB)$^{-1}$]	Purified LVPs [copies (mg apoB)$^{-1}$]			
A (1a)	$3{\cdot}76 \times 10^7$	$5{\cdot}63 \times 10^6$	$9{\cdot}98 \times 10^8$	15·0	41·9	6·3
B (1a)	$9{\cdot}97 \times 10^6$	$7{\cdot}95 \times 10^6$	$1{\cdot}45 \times 10^8$	79·8	47·2	37·7
C (1a)	$6{\cdot}02 \times 10^5$	$1{\cdot}13 \times 10^6$	$7{\cdot}32 \times 10^7$	95·0	40·0	40·0
D (1a)	$5{\cdot}62 \times 10^7$	$1{\cdot}28 \times 10^7$	$4{\cdot}39 \times 10^9$	13·7	56·4	7·7
E (1b)	$8{\cdot}02 \times 10^6$	$8{\cdot}13 \times 10^5$	$1{\cdot}56 \times 10^8$	10·1	50·3	5·1
F (1b)	$1{\cdot}98 \times 10^7$	$6{\cdot}83 \times 10^6$	$4{\cdot}52 \times 10^8$	34·5	51·3	17·7
G (1b)	$1{\cdot}15 \times 10^7$	$2{\cdot}65 \times 10^6$	$3{\cdot}19 \times 10^9$	23·1	53·2	12·3
H (1b)	$3{\cdot}93 \times 10^6$	$2{\cdot}08 \times 10^6$	$9{\cdot}60 \times 10^7$	52·9	51·8	27·4

*Index of HCV RNA association with low-density fraction: see Methods.
†Calculated as value of (column 1 × column 2)/100.

and 543 000 ***g*** with a TL100 (Beckman Instruments). Upper low-density fraction was dialysed extensively at 4 °C against 150 mM NaCl/0·24 mM EDTA (pH 7·4) buffer, filtered through 0·22 μm pore-size filters and stored at 4 °C in the dark in the presence of 2 % inhibitor cocktail.

LVP purification. LVP purification was performed as described previously (André *et al.*, 2002). Briefly, protein A-coated magnetic beads (Miltenyi Biotec) were incubated at room temperature with 2 ml low-density fractions in PBS with gentle rocking for 30 min. Samples were then passed through a magnetic column (Miltenyi Biotec), washed with PBS and collected in 500 μl Dulbecco's modified Eagle's medium/0·2 % BSA. Immunocaptured particles (purified LVPs) were stored at 4 °C in the dark in the presence of 2 % inhibitor cocktail.

Protein, apoB and lipid quantification. Protein concentration was determined according to the Lowry method and calculated from a calibration curve by using BSA as a standard. apoB concentration in low-density fractions and sera was determined by using immunochemical kits (ApoB kit, bioMérieux, or ApoB kit, SFRI Diagnostics). Total cholesterol, PL and TG concentrations in sera were calculated with Cholesterol RTU, Phospholipides Enzymatique PAP 150 and Triacylglycerols Enzymic PAP 150 kits (bioMérieux), with the inclusion of standard curves to calculate the concentrations.

apoB concentrations in purified LVPs were determined by ELISA. Ninety-six-well flat-bottomed ELISA plates (Maxisorb; Nunc) were coated overnight at 4 °C with 100 μl monoclonal anti-human apoB antibody (5 μg ml^{-1}; clone 1609) in PBS and then saturated with 2 % BSA for 1 h. Samples were first incubated for 30 min at room temperature in PBS/0·2 % BSA supplemented with 10 μg human IgG ml^{-1} before being distributed at 100 μl per well. After 2 h incubation at 37 °C and washing with PBS/0·05 % Tween 20, peroxidase-conjugated goat anti-human apoB antibody (1·6 μg ml^{-1}) 100 μl per well in PBS/0·2 % BSA was added for 90 min at 37 °C. The plates were washed and *o*-phenylenediamine substrate was added (150 μl per well). The reaction was revealed for 10 min and A_{490} was read. Standard curves were established with LDL dilutions ranging from 2 to 100 ng apoB ml^{-1}. Controls included human IgG-saturated protein A-coated magnetic beads prepared under the same conditions.

PL and TG compositions of LVP and low-density fractions were determined by gas chromatography quantification of their fatty acid content. Diheptadecanoyl phosphatidylcholine and triheptadecanoyl glycerol were added to LVPs and low-density fractions before lipid extraction as internal standards. Lipid extracts obtained from 200 μl LVPs or 100 μl low-density fraction were separated on Silica Gel G60 plates (Merck) with the solvent system hexane/diethyl ether/acetic acid (60 : 40 : 1, by vol.). The silica-gel areas corresponding to PL and TG were scraped off and transmethylated. Briefly, 1 vol. 5 % H_2SO_4 in methanol was added to the scraped silica gel and transmethylation was carried out at 100 °C for 90 min in screw-capped tubes. The reaction was terminated by the addition of 1·5 vols ice-cold 5 % (w/v) K_2CO_3 and the fatty acid methyl esters were extracted with isooctane and analysed by using a PerkinElmer Life Sciences chromatograph model 5830, equipped with a capillary column (30 m × 0·32 mm; Supelco) and flame-ionization detection. The column was two-step-programmed from 135 to 160 °C at 2 °C min^{-1} and from 160 to 205 °C at 1·5 °C min^{-1}; the detection temperature was maintained at 250 °C. The vector gas was helium at a pressure of 0·8 p.s.i. (5520 Pa). Peaks were identified by using standard fatty acid methyl esters and the absolute amounts of fatty acid methyl esters present in PL and TG were determined relative to the known amount of added 17 : 0.

HCV RNA quantification. RNA was extracted from 150 μl serum, 10 μl low-density fraction or purified LVPs with a QIAamp viral RNA kit (Qiagen); RNA was eluted in 50 μl water and stored at −80 °C. HCV RNA quantification was performed by real-time PCR of the 5′ HCV non-coding region as described previously, but with minor modifications (Komurian-Pradel *et al.*, 2001). Briefly, RNA (4 μl) was reverse-transcribed with a Thermoscript reverse transcriptase kit (Gibco-BRL) with the RC21 primer (Besnard & Andre, 1994). Real-time PCR was carried out with 2 μl cDNA and the RC1 and RC21 primers by using an LC FastStart DNA Master SYBR Green I kit and a LightCycler apparatus (Roche Diagnostics). The proportion of HCV RNA in low-density fractions was defined as described previously (André *et al.*, 2002).

Western blotting. Fifteen microlitres of purified LVPs and 15 μl 100-fold-diluted low-density fraction were denaturated in Laemmli buffer and separated on a 5 % (apoB) or 10 % (E1 and E2) acrylamide gel. apoB100 and apoB48, used as controls of migration, were obtained respectively from LDLs and chylomicrons isolated from healthy plasma donors. Briefly, for apoB100, plasma density was adjusted to 1·055 g ml^{-1} with NaBr and ultracentrifuged as described above. Fifteen microlitres of 100-fold-diluted low-density fraction was denaturated in Laemmli buffer and loaded on the gel. For apoB48, a post-prandial plasma sample from one healthy volunteer was ultracentrifuged immediately for 4 h at 4 °C and 543 000 ***g*** with a TL100 (Beckman Instruments). Twenty microlitres of 20-fold-diluted apoB48-rich very-low-density chylomicron fraction was denaturated in Laemmli buffer and loaded on the gel. After migration, proteins were electrotransferred onto an Immobilon P membrane (Millipore). Membranes were incubated in blocking solution [20 mM Tris/HCl (pH 7·4), 150 mM NaCl, 0·05 % Tween 20, 5 % skimmed milk] overnight at 4 °C. All following steps were performed in TBS/Tween 0·05 % at room temperature. After washing, blots were incubated for 90 min with 1D1 anti-human apoB mAb (1/10 000) or with anti-E1 (A4) or E2 (H52) (1/1000). After washing, membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse antibody (1/5000; Perbio Science). Immunoreactive proteins were visualized by using the ECL detection system (Amersham Biosciences) or SuperSignal FEMTO system (Perbio Science) and Biomax MR-film (Kodak). Bands were quantified with a videodensitometric software analyser (Imagemaster; Amersham Biosciences).

HCV envelope ELISA. Ninety-six-well ELISA plates (Nunc MaxiSorp) were coated with 10 ng protein A per well (Sigma) in 100 μl PBS overnight at 4 °C. Plates were washed three times with washing buffer [PBS containing 0·02 % (v/v) Tween 20] and non-specific binding was blocked by addition of 200 μl blocking buffer [PBS containing 2 % (w/v) BSA] per well for 2 h at 37 °C. Low-density fractions ($d < 1·055$ g ml^{-1}), prepared as described in the low-density fraction preparation section above from infected (patient) or non-infected (control) plasma, were diluted from 10 to 0 μg protein ml^{-1} in PBS (10, 5, 3·33, 2·5, 1·67, 1·25, 0·63 and 0 μg). One hundred microlitres of each dilution was transferred to the protein A-coated plate and incubated for 60 min at 37 °C. After washing, free protein A binding sites were blocked by 150 μl human IgG (2·5 μg ml^{-1}) for 2 h at 37 °C. An additional wash was performed and 100 μl monoclonal anti-E1 A4 (upper panel), anti-E2 H47 (lower panel) or anti-measles virus H protein (clone 55) (negative control) antiserum (diluted 1/1000 in PBS) was added to each well and incubated at 37 °C for 1 h. A further wash step was performed and 100 μl alkaline phosphatase-conjugated anti-mouse IgG (A2429; Sigma) diluted 1/1500 in PBS was incubated in each well for 1 h at 37 °C. After a final wash step, 100 μl of a 2 mg PNPP ml^{-1} (N2765; Sigma) substrate solution was added to each well and developed for 45 min. A_{405} was read.

RESULTS

Apolipoprotein distribution in purified LVP and in the whole $d<1{\cdot}055$ g l^{-1} plasma fraction

apoB are non-exchangeable apolipoproteins and apoB100 and -48 are specific for hepatic VLDLs or intestinal chylomicrons, respectively, in humans. We first determined whether HCV was associated randomly with TRLs by looking at the composition of LVPs in apoB isoforms. As expected, in the whole $d<1{\cdot}055$ g l^{-1} plasma fraction, apoB100 was the major form of apoB, whilst apoB48 was barely detectable, reflecting the massive predominance of liver-derived lipoproteins in the plasma of fasting patients (Fig. 1a, b). In contrast, apoB100 and -48 were represented equally in LVPs purified from the same whole fraction. Considering that apoB is a non-exchangeable apolipoprotein, these data indicated that viral low-density particles are associated preferentially with intestine-derived lipoproteins. For the eight patients studied (Table 1), the proportion of plasma HCV RNA found in the $<1{\cdot}055$ g ml^{-1} fraction varied from patient to patient from 10 to 95 % (mean 39 %), accordingly with a previously report (André *et al.*, 2002). Therefore, we could estimate that 5–45 % (mean 18 %) of the total viral load in the plasma of these patients was in the form of chylomicron-like particles, considering that apoB48 was found in half of purified LVPs. The presence of apoE, -CII and -CIII, but not of apoAII, in purified LVPs further supported the TRL-like nature of these particles (Fig. 2), whereas their viral nature was confirmed by Western blot experiments with anti-envelope antibodies showing the presence of both E1 and E2 glycoproteins in purified particles (Fig. 3a). In addition, anti-envelope antibodies recognized native LVPs captured by protein A in an ELISA assay, indicating that E1 and E2 viral glycoproteins are localized at the surface of the particle (Fig. 3b).

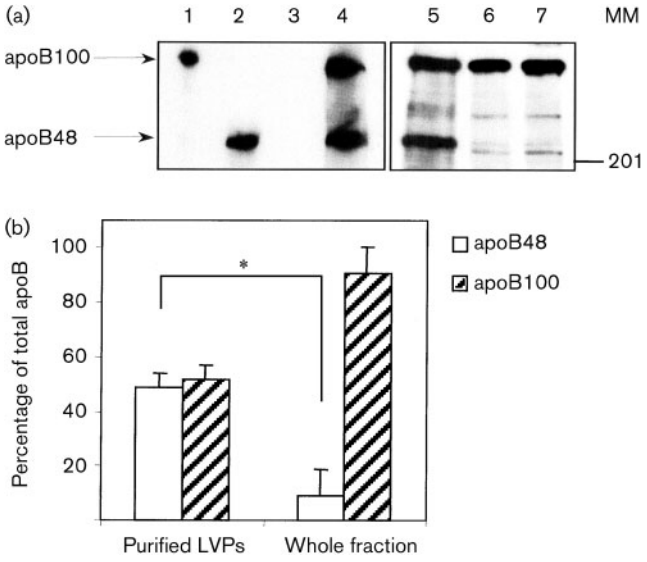

Fig. 1. apoB100 and apoB48 are present in purified LVPs. (a) Nature of apoB in purified LVPs. Plasma samples from HCV-infected or healthy donors were adjusted to a density of $1{\cdot}055$ g ml^{-1} and centrifuged for 4 h at 4 °C and 543 000 ***g***. LVPs were immunopurified from the low-density fraction as described in Methods. Samples of LVPs and of the whole fraction were analysed by SDS-PAGE (5 % gel) under reducing conditions and immunoblotted with 1D1 anti-apoB mAb. Lanes 1 and 2, the $d<1{\cdot}055$ g ml^{-1} fraction and chylomicrons isolated from a healthy blood donor; respectively; lane 3, mock-prepared LVPs from a healthy blood donor; lanes 4 and 5, purified LVPs from patient H; lanes 6 and 7, the $d<1{\cdot}055$ g ml^{-1} fraction from which LVPs were purified from the same patient and a healthy subject, respectively. MM, Molecular mass (kDa). (b) Relative proportions of apoB48 and apoB100 in purified LVPs and in the $d<1{\cdot}055$ g ml^{-1} fraction prepared from eight infected patients. apoB48 and apoB100 spots were quantified by videodensitometry and expressed as a percentage of total apoB. The proportion of apoB48 was significantly higher in purified LVPs than in the whole $d<1{\cdot}055$ g ml^{-1} fraction (Student's *t*-test, $P<0{\cdot}01$).

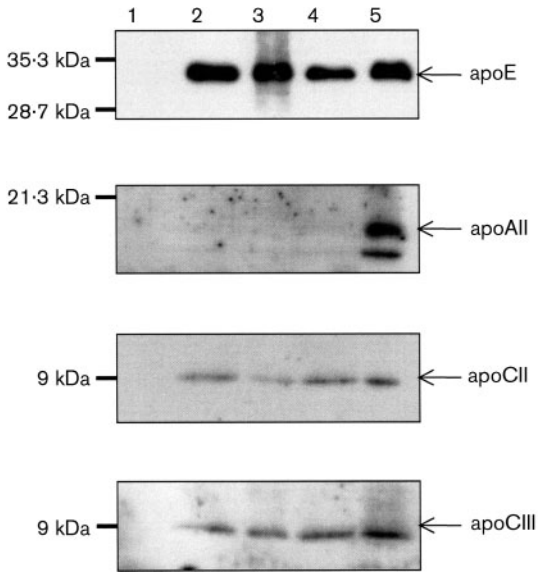

Fig. 2. Presence of apoE, -CII and -CIII in LVPs. LVPs were immunopurified from the $d<1{\cdot}055$ g ml^{-1} fraction as described in Methods, analysed by SDS-PAGE [12 % (apoE) or 15 % (apoAII, apoCII and apoCIII) gel] under reducing conditions and immunoblotted with anti-apoE, anti-apoAII or anti-apoCIII mAbs (Chemicon International) or anti-apoCII polyclonal antibody (Merck Calbiochem). Lanes 1 and 2, mock-prepared LVPs and the $d<1{\cdot}055$ g ml^{-1} fraction from a non-infected blood donor, respectively; lanes 3 and 4, LVPs and the $d<1{\cdot}055$ g ml^{-1} fraction from a chronically infected patient, respectively; lane 5, control plasma from a blood donor. apoE, -CII and -CIII were present in the $d<1{\cdot}055$ g ml^{-1} fraction where apoB-containing lipoproteins reside and in purified LVPs. apoAII, a component of HDLs, was detected neither in the $d<1{\cdot}055$ g ml^{-1} fraction nor in LVPs.

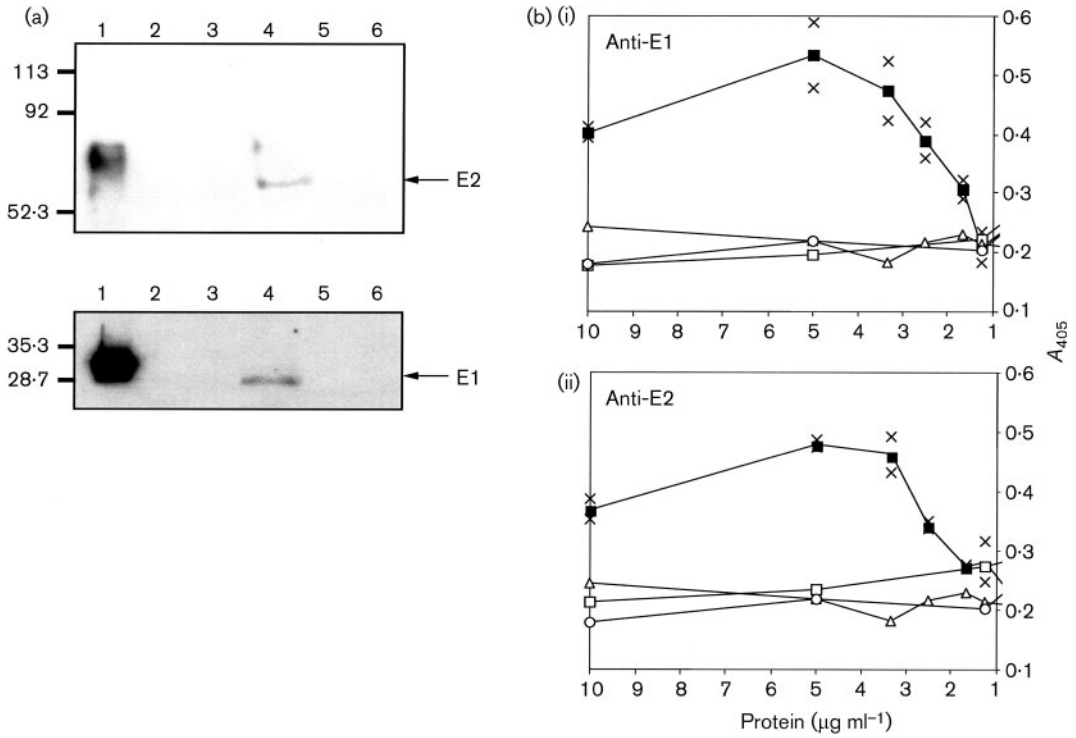

Fig. 3. Presence of envelope glycoproteins in LVPs. (a) LVPs were immunopurified from the $d<1{\cdot}055$ g ml^{-1} fraction as described in Methods, analysed by SDS-PAGE (10 % gel) under reducing conditions and immunoblotted with the A4 anti-E1 (lower panel) or the H52 anti-E2 (upper panel) mAbs. Lanes 1 and 2, lysates of 293T cells expressing or not E1 and E2 glycoproteins, respectively; lane 3, mock-prepared LVPs from plasma of a healthy blood donor; lane 4, purified LVPs from a chronically infected patient; lanes 5 and 6, the $d<1{\cdot}055$ g ml^{-1} fraction from which LVPs were purified from an HCV patient or a healthy subject, respectively. Western blots are representative of experiments performed with plasma from three infected individuals. Note that both viral glycoproteins were detected in purified LVPs. (b) Detection of protein A-captured LVPs in an ELISA with anti-E1 and anti-E2 antibodies. The $d<1{\cdot}055$ g ml^{-1} fractions were prepared as described in Methods from chronically infected patients and from non-infected blood donors. The $d<1{\cdot}055$ g ml^{-1} fraction from infected (■) or non-infected (□) patients was revealed by anti-E1 A4 (i) or anti-E2 H47 (ii). As a control, the $d<1{\cdot}055$ g ml^{-1} fraction from infected (△) or non-infected (○) patients, stained by anti-H measles clone 55, is presented. Results are means of duplicates (×). Note that protein A-captured LVPs from infected patients were only recognized by anti-HCV E1 and E2 envelope antibodies and not by anti-measles virus H envelope antibodies. No material from the non-infected control was recognized by any antibody.

Post-prandial modifications of LVPs

In order to further support the hypothesis of a preferential association of HCV with chylomicrons that contributes to LVP production, we studied the dynamic transition between the pre-prandial and the post-prandial periods. To that end, eight HCV-infected volunteers were given a 900 kcal breakfast with 30 % fat after an overnight fast (one of these patients was excluded from the comparison between the pre- and post-prandial states because of lipid characterization technical failure). Peripheral blood was drawn before breakfast and 90 min after the first phlebotomy. The increase observed in plasma TG in all post-prandial samples indicated that fat absorption and chylomicron secretion had occurred during this time period for all patients (Table 2). The TG/apoB and PL/apoB ratios of purified LVPs increased significantly in 90 min (Table 2). By contrast, the TG/PL ratio did not significantly differ in the inter- and post-prandial periods, indicating that the TG and PL contents of the particle increased in the same proportion. Moreover, the TG/apoB mass ratios in both the pre-prandial and post-prandial periods were largely higher in LVPs than in the $d<1{\cdot}055$ g ml^{-1} fraction from which LVPs were purified (Fig. 4a), indicating that LVPs are TG-enriched circulating particles in plasma. The fatty acid composition of TG and PL in purified LVPs and in the $d<1{\cdot}055$ g ml^{-1} fraction was similar in the pre-prandial and in the post-prandial periods (Table 3), and very similar between the two periods (Fig. 5). These results confirmed the lipoprotein nature of LVPs. The rapid and dramatic post-prandial changes observed in the composition of LVPs while the composition of the corresponding whole $d<1{\cdot}055$ g l^{-1} fraction remained steady further suggested an active contribution of the intestine to LVP production.

DISCUSSION

In the plasma of chronically HCV-infected patients, infectious particles are found partially in low-density fractions, associated with TRLs forming LVPs. Some of these LVPs are naturally coated with antibody and can be protein A-precipitated. Previous analysis of these captured LVPs showed that they are globular particles that are rich in TG and contain HCV core protein and RNA. In the present study, we show that, in addition to HCV RNA, similarly purified LVPs contain at their surface HCV envelope glycoproteins and TRL apolipoproteins apoB, apoE, apoCII and apoCIII, but not apoAII, which is a component of HDLs. A major finding is that, among TRL apolipoproteins, the two isoforms of apoB, apoB100 and -48, are represented equally, whereas apoB48 is barely detectable in the fasting-patient plasma. A direct indication of the association of HCV RNA with apoB48 would require an immunoprecipitation with an anti-apoB48 antibody and the detection of HCV RNA in the captured material. However, apoB48 results from the edition in enterocytes of a stop codon within the open reading frame of the apoB mRNA (Patterson *et al.*, 2003), leading to a protein lacking the C-terminal end of the complete apoB100 molecule. As a result, there is no direct way to capture apoB48-containing lipoproteins and therefore to demonstrate directly the association of HCV RNA with apoB48-containing lipoproteins. Despite this limitation, the strong apoB48 enrichment in protein A-captured, HCV RNA-positive LVPs compared with the plasma lipoproteins strongly suggests a direct association of HCV RNA with apoB48-containing lipoprotein. The rapid and dramatic increase in TG of these purified LVPs after lipid ingestion further strengthens the HCV–apoB48 association. These results raise the question of the nature, origin and functions of such particles.

The strong relationship between apoB-containing lipoproteins and viral particles is a specificity of HCV and related viruses (Sato *et al.*, 1996). Although ultrastructural analysis of LVPs is necessary, these data suggest that LVPs are TRL-like particles in which the two hydrophobic domains of the core protein could be embedded in the neutral lipids of the lipoprotein core (Hope & McLauchlan, 2000; McLauchlan *et al.*, 2002). Glycoproteins E1 and E2 may display an amphipathic helix conformation (Charloteaux *et al.*, 2002) as apolipoproteins and insert into the surface layer of the particle. With respect to apoE, which is borne by LVPs, it has recently been shown that the *E2*,*E3* and *E2*,*E4* genotypes were associated respectively with a significant three- and fivefold reduction in the risk of chronic HCV infection compared with E3E4 or E3 and E4 homozygotes (Price *et al.*, 2006). In addition, the *E2*,*E2* genotype was never found in HCV-positive patients. The E2 isoform of apoE binds poorly to the LDL receptor (Mahley & Rall, 2000). As LVP binding to cells can be blocked by anti-apoE antibody (Agnello *et al.*, 1999; André *et al.*, 2002), it is likely that the defective binding of the apoE2 isoform could result in a poor uptake of LVPs. Moreover, these data support a biological role for LVPs,

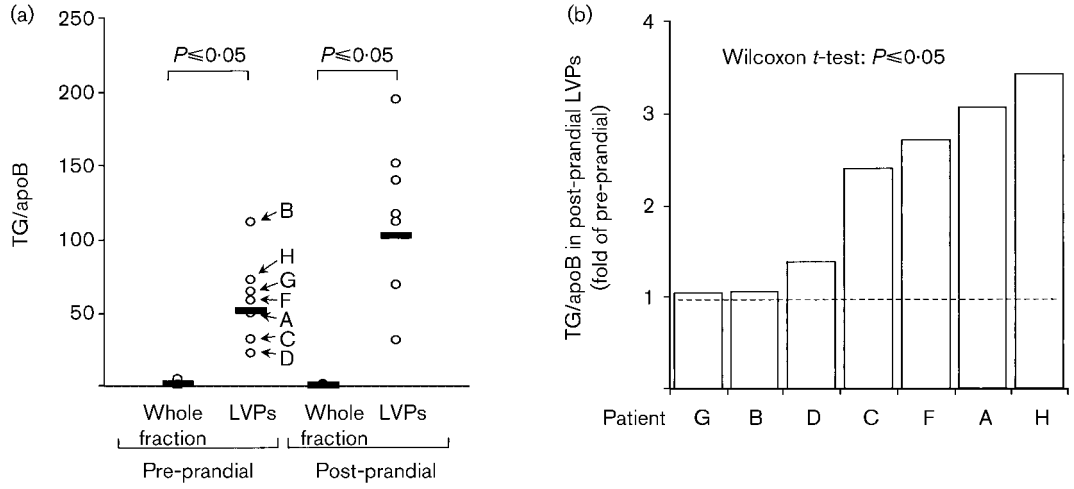

Fig. 4. Evolution of the TG/apoB mass ratio in LVPs between the pre-prandial and post-prandial periods. (a) Mean (dash) and individual (spots) TG/apoB mass ratios in both fractions from seven patients. Lipids from purified LVPs and the whole $d < 1{\cdot}055$ g ml^{-1} fraction were extracted and separated by thin-layer chromatography. TG spots were scraped off and the fatty acids were transmethylated and quantified by gas chromatography and the apoB content of purified LVPs was determinated by ELISA, as described in Methods. Note that the TG/apoB ratio was significantly higher in LVPs than in their respective whole fraction (Wilcoxon *t*-test, $P \leqslant 0{\cdot}05$). (b) TG/apoB mass ratio in LVPs increases between the pre-prandial and post-prandial periods. Results are expressed as the ratio between the TG/apoB mass ratio in LVP in the post-prandial period vs that in the pre-prandial period for each patient. Patients are identified as A–G, indicated by arrows in (a). Note that the TG/apoB mass ratio was increased significantly in LVPs during the post-prandial period (distribution-free Wilcoxon *t*-test, $P \leqslant 0{\cdot}05$).

Table 2. Effect of lipid intake on LVP characteristics: lipid and protein mass ratios

Lipid and protein mass ratios in the whole $d<1{\cdot}055$ g ml^{-1} fractions and purified LVPs in the pre-prandial and post-prandial periods. Results are means from seven patients. TG, Triglycerides; PL, phospholipids; apoB, apolipoprotein B.

	Plasma fraction	Mass ratio of indicated lipids and protein in purified LVPs		
	TG (g/L)	TG/apoB	PL/apoB	TG/PL
Pre-prandial state	0·64 ± 0·16*	58·99 ± 29·21†	25·76 ± 19·99‡	3·81 ± 3·03
Post-prandial state	0·81 ± 0·17*	116·69 ± 54·02†	46·34 ± 17·94‡	2·99 ± 2·12

*P value for a comparison of TG between the pre-prandial and post-prandial states in the plasma fraction was $<0{\cdot}0005$.
†P value for a comparison of the TG/apoB ratio between the pre-prandial and post-prandial periods was 0·012.
‡P value for a comparison of the PL/apoB ratio between the pre-prandial and post-prandial periods was 0·045.

which, like TRLs, may have their fate and their site of clearance directed by their apolipoprotein composition (Field & Mathur, 1995).

Several mechanisms could be involved in the production of LVPs. First, LVPs could be formed within the blood circulation by the association of mature HCV virions with circulating TRLs. However, a recent study reported that HCV RNA quasispecies found in LVPs corresponded to a subgroup of the whole plasma viral population (Deforges *et al.*, 2004). This indicates, at least, that LVPs are not issued from a random fusion of circulating HCV virions with plasma lipoproteins. Although natural antibodies against LVPs may introduce some bias in selecting a particular LVP subpopulation, the most likely hypothesis is that LVPs are formed within the endoplasmic reticulum of lipoprotein-secreting cells, in which apoB and TG are assembled to form TRLs. Indeed, immunoprecipitation of TRLs with an anti-apoB antibody precipitated 50 % of HCV RNA from HCV-infected liver macerate, indicating that a substantial amount of HCV RNA was already associated with apoB in hepatocytes (Nielsen *et al.*, 2004). Altogether, these studies suggest that HCV association with apoB-containing lipoproteins probably occurs within lipoprotein-secreting cells, rather than resulting from binding of HCV to TRLs in the circulation.

Therefore, one should consider the hypothesis of an intestinal production of LVPs, based on the association of HCV RNA and envelope glycoproteins with apoB48-containing TRLs. Indeed, the expression of Apobec1, the editing enzyme of the apoB mRNA leading to apoB48 synthesis, is strictly restricted to enterocytes (Patterson *et al.*, 2003) and HCV infection has not been reported to induce Apobec1 expression in hepatocytes (Jacobs *et al.*, 2005; Smith *et al.*, 2003; Su *et al.*, 2002). This hypothesis is further

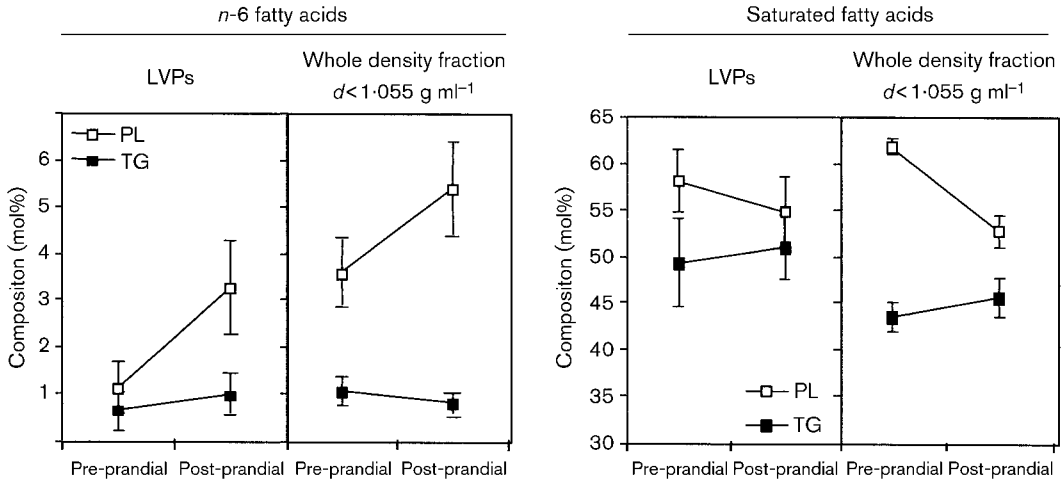

Fig. 5. Effect of lipid intake on LVP characteristics. Parallel modifications of fatty acid composition of LVPs and of the $d<1{\cdot}055$ g ml^{-1} fraction between the pre-prandial and post-prandial periods. *n*-6 and saturated fatty acids were quantified by gas chromatography as described in Methods and expressed as mol% in triacylglycerol (TG) and PL of purified LVPs and of the whole $d<1{\cdot}055$ g ml^{-1} fraction. Results are means from seven patients. Note that the fatty acid compositions of TG and PL vary in the same proportion in LVPs and the whole $d<1{\cdot}055$ g ml^{-1} density fraction between the pre-prandial and post-prandial periods.

Table 3. Fatty acid composition of phospholipids and triacylglycerols from purified LVPs and whole low-density fraction before (pre-prandial) and after (post-prandial) the meal

Fatty acid compositions are given by weight (mol%); values represent means ± SEM. PL, Phospholipids; TG, triacylglycerol; ND, not detected.

Fatty acid	Purified LVPs ($n=7$)				Whole fraction ($n=7$)			
	PL		TG		PL		TG	
	Pre-prandial	Post-prandial	Pre-prandial	Post-prandial	Pre-prandial	Post-prandial	Pre-prandial	Post-prandial
14:0	5·91 ± 2·26	5·75 ± 1·78	3·56 ± 0·90	5·16 ± 1·47	0·61 ± 0·11	0·55 ± 0·11	2·91 ± 0·68	4·84 ± 0·37
16:0	38·82 ± 2·30	35·61 ± 2·57	36·36 ± 2·14	36·63 ± 2·74	38·32 ± 1·27	35·85 ± 2·63	34·90 ± 1·44	35·84 ± 2·13
16:1*n*-7	0·49 ± 0·17	1·19 ± 0·25	6·46 ± 1·92	5·30 ± 1·28	0·65 ± 0·20	0·87 ± 0·19	3·16 ± 0·61	3·35 ± 0·63
18:0	15·51 ± 2·36	14·98 ± 2·42	10·18 ± 3·50	9·09 ± 3·77	12·95 ± 1·39	16·32 ± 2·29	6·20 ± 1·31	4·89 ± 0·31
18:1*n*-9	16·90 ± 4·07	13·64 ± 1·47	32·42 ± 3·48	32·35 ± 2·78	11·04 ± 1·04	10·25 ± 1·25	35·39 ± 3·68	37·14 ± 2·14
18:2*n*-6	16·24 ± 1·74	16·78 ± 1·61	11·85 ± 1·77	9·43 ± 1·71	20·05 ± 1·18	19·27 ± 1·45	14·70 ± 1·61	10·67 ± 1·13
18:3*n*-6	ND	ND	ND	ND	0·18 ± 0·10	0·66 ± 0·37	0·14 ± 0·05	0·13 ± 0·05
20:3*n*-6	3·38 ± 1·34	1·89 ± 0·41	0·97 ± 0·45	0·14 ± 0·07	2·35 ± 0·27	2·38 ± 0·32	0·54 ± 0·29	0·22 ± 0·07
20:4*n*-6	6·56 ± 0·95	6·64 ± 0·64	1·82 ± 0·47	1·54 ± 0·44	6·72 ± 0·87	6·58 ± 0·63	2·24 ± 0·64	1·12 ± 0·23
20:5*n*-3	0·40 ± 0·11	1·27 ± 0·48	ND	0·64 ± 0·36	1·62 ± 0·90	2·04 ± 1·37	ND	ND
22:4*n*-6	9·87 ± 2·19	7·48 ± 2·67	2·46 ± 1·06	1·13 ± 0·29	5·53 ± 1·11	1·70 ± 0·78	0·33 ± 0·13	0·24 ± 0·08
22:5*n*-3	0·44 ± 0·11	2·92 ± 0·78	1·56 ± 0·69	0·88 ± 0·24	0·64 ± 0·10	0·66 ± 0·09	0·34 ± 0·07	0·23 ± 0·06
22:5*n*-6	1·01 ± 0·07	7·06 ± 1·40	1·11 ± 0·27	1·75 ± 0·85	1·07 ± 0·48	3·45 ± 1·81	0·67 ± 0·39	0·12 ± 0·03
22:6*n*-3	2·19 ± 0·49	2·82 ± 0·62	0·68 ± 0·26	0·56 ± 0·24	2·33 ± 0·41	3·23 ± 0·70	0·92 ± 0·26	0·50 ± 0·14
ΣsatFA	58·10 ± 3·34	54·78 ± 3·81	49·17 ± 4·73	50·87 ± 3·42	61·82 ± 0·89	52·72 ± 1·76	43·45 ± 1·50	45·57 ± 2·11
Σ*n*-3	23·98 ± 3·15	27·68 ± 4·21	12·93 ± 2·54	11·34 ± 2·02	32·99 ± 1·68	30·60 ± 2·28	16·78 ± 2·94	12·48 ± 1·35
Σ*n*-6	1·10 ± 0·53	3·21 ± 0·98	0·61 ± 0·39	0·95 ± 0·43	3·56 ± 0·71	5·37 ± 1·00	1·04 ± 0·32	0·78 ± 0·21

supported by the variation in the lipid enrichment of circulating LVPs between the pre- and the post-prandial period of the patient, as expected for intestinal TRLs after food intake (Field & Mathur, 1995). Such a hypothesis is consistent with a previous study reporting that the quasispecies populations of LVPs and liver HCV RNA did not match completely, suggesting a second reservoir beside the liver and with the presence of HCV proteins in enterocytes of chronically infected patients (Deforges *et al.*, 2004). Further investigations of chronically infected patients for detection of HCV RNA in intestinal biopsies and comparative quasispecies analysis between gut, LVPs and plasma are necessary to quantify precisely the contribution of enterocytes to the circulating viral load.

Besides the fundamental challenge to decipher the mechanisms leading to the production of LVPs, considering the intestine as a reservoir and replication site of HCV in the form of LVPs has important pathophysiological consequences. The proportion of intestinal LVPs might be substantial (mean calculated value, 18 % of the plasma viral load). As the final destination of intestinal lipoprotein remnants is the liver (Field & Mathur, 1995), an intriguing possibility could be a permanent inoculation of the liver with LVPs from the intestine. Binding and internalization of naturally antibody-coated LVPs was shown to be mediated by lipoprotein receptors that recognize apolipoproteins on the viral particles (André *et al.*, 2002). Neutralizing antibodies directed to the envelope glycoproteins may therefore not be sufficient to control infection of the liver by LVPs. Therefore, classical virions, like those produced *in vitro*, and LVPs could deliver the virus with the possibility to infect the host both acutely and chronically, a feature not achieved by other flaviviruses.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministère de la Jeunesse, de l'Éducation Nationale et de la Recherche, INSERM and the ANRS (Agence Nationale de Recherche sur le SIDA et les Hépatites Virales), by the 'Programme de Recherche Clinique des Hospices Civils de Lyon'. Lipid analysis was performed at the 'Lipidomics Platform' set up at UMR 585 INSERM/INSA-Lyon (IMBL).

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