Extraordinarily low density of hepatitis C virus estimated by sucrose density gradient centrifugation and the polymerase chain reaction

Hideaki Miyamoto,1 Hiroaki Okamoto,2 Koei Sato,1 Takeshi Tanaka1 and Shunji Mishiro3*

1Japanese Red Cross Blood Center, Saitama 388, 2Immunology Division, Jichi Medical School, Tochigi 329-04 and 3Institute of Immunology, Koraku 1-1-10, Bunkyo-ku, Tokyo 112, Japan

The genomic RNA of hepatitis C virus (HCV) in the plasma of volunteer blood donors was detected by using the polymerase chain reaction in a fraction of density 1.08 g/ml from sucrose density gradient equilibrium centrifugation. When the fraction was treated with the detergent NP40 and recentrifuged in sucrose, the HCV RNA banded at 1.25 g/ml. Assuming that NP40 removed a lipid-rich surface coat from HCV, the 1.08 g/ml and 1.25 g/ml HCV RNA may correspond to intact HCV virions and nucleocapsids, respectively. The extraordinarily low density of the virion is unusual in comparison to the density of classified viruses.

Hepatitis C virus (HCV) has been identified as the major causative agent of blood-borne non-A, non-B hepatitis (Choo et al., 1989). Despite the recent accumulation of data suggesting the importance of this virus in many seroepidemiological settings, HCV has not yet been cultured or visualized, and we know only a little about its physicochemical properties: the infectivity of plasma for chimpanzees is destroyed by lipid solvents (Bradley et al., 1983; Feinstone et al., 1983); the particle size is estimated to be between 30 and 80 nm by filtration studies (Bradley et al., 1985; He et al., 1987); the buoyant density of the factor VIII-derived isolate in sucrose has been shown by infectivity studies to be significantly lower than those of conventional viruses (Bradley et al., 1991). On the other hand, the RNA of several HCV isolates has been fully sequenced. It is a positive-stranded RNA of about 9500 nucleotides in length, containing a single open reading frame which can encode a presumed viral precursor polyprotein of 3000 or more amino acid residues (Kato et al., 1990; Choo et al., 1991; Takamizawa et al., 1991; Okamoto et al., 1991). A comparative study of the hydropathy profile of the presumed polyprotein of HCV with those of proteins of known viruses has suggested a possible evolutionary relationship between HCV and flaviviruses, pestiviruses and some plant viruses (Miller & Purcell, 1990). Additional information on the physicochemical properties of HCV is needed for its relationship with other viruses to be assessed.

With the advent of gene amplification techniques using the polymerase chain reaction (PCR), we are now able to detect as few as two or three copies of HCV RNA (Weiner et al., 1990; Okamoto et al., 1990a). In this study, we utilized this technique to estimate the buoyant density of HCV, not yet detectable by electron microscopy or other means. Sucrose was selected for the density gradients after several candidate media had been examined to assess the stability of HCV in them. These values may help in the development of procedures to purify HCV from cell culture or other virus-rich sources, and may also contribute to further understanding of the properties of HCV in comparison to those of known viruses.

Fresh plasma or serum from voluntary blood donors apparently hepatitis-free but seropositive for anti-HCV c100 antibodies (Kuo et al., 1989) was used as the source of HCV. As internal controls, plasma from donors persistently infected with hepatitis B virus (HBV) and seropositive for hepatitis B e antigen were also included in the experiments.

Five sources of density media were examined for their influence upon the stability of HCV. Sucrose, potassium tartrate, potassium bromide, metrizamide and caesium chloride were dissolved in TEN buffer (50 mm-Tris–HCl pH 8.0, 1 mm-EDTA, 150 mm-NaCl) to a final density of 1.28, 1.30, 1.30, 1.30 and 1.55 g/ml, respectively, and each mixed with a one-tenth volume of human plasma containing HCV and HBV. After incubation at 4°C for 30 h, each mixture was examined for HCV RNA by the PCR method described below.

HCV-containing plasma was mixed with an equal volume of TEN buffer and a 1/2000 volume of HBV-containing plasma and clarified by centrifuging at 3000 r.p.m. for 10 min. The supernatant (0.8 ml) was layered
on top of 6.8 ml of a stepwise density gradient of sucrose prepared from 2.8 ml of 60% and 0.8 ml each of 50%, 40%, 30%, 20% and 10% (w/w) sucrose in TEN buffer. The mixture was overlaid with 25 ml of TEN buffer and centrifuged at 27800 r.p.m. for 44 h at 10 °C in a Beckman SW28 ultracentrifuge rotor. Alternatively, to allow for the presence of less starting material, 0.2 ml of clarified plasma was layered onto a 2 ml stepwise gradient and centrifuged at 40000 r.p.m. for 44 h at 10 °C in a Beckman SW65Ti rotor. Fractions (800 µl each from the SW28 or 200 µl from the SW65Ti rotor) were collected from the bottom of the tube. The density of each fraction was determined by refractometry; HCV RNA and HBV DNA in each fraction were detected by the PCR procedures described below.

The HCV RNA-positive fraction obtained from the sucrose equilibrium centrifugation was mixed with a 1/2000 volume of HBV-containing plasma, and then incubated with an equal volume of 0.32% NP40/TEN buffer on ice for 5 min, a condition used previously for the uncoating of Japanese encephalitis virus (Shapiro et al., 1971). Another aliquot was mixed with an equal volume of 0.2% NP40/0.2% 2-mercaptoethanol/0.2% pronase E/TEN buffer at 37 °C for 2 h, a method reported to liberate core particles from HBV virions (Takahashi et al., 1979). The reaction product in each case was recentrifuged in a sucrose density gradient as above.

Plasma or serum samples, mixtures with various density media or processed fractions obtained from the sucrose density gradient equilibrium ultracentrifugation were diluted with 10 volumes of TEN buffer and centrifuged at 90000 r.p.m. for 15 min using a Beckman TLA100.2 rotor and a TL100 table-top ultracentrifuge. The precipitates were resuspended in 200 µl of buffer containing 50 mM-Tris–HCl pH 8.0, 200 mM-NaCl, 10 mM-EDTA, 2% (w/w) SDS and 1 mg/ml proteinase K, and incubated at 60 °C for 1 h. The nucleic acids were then extracted from the reaction mixture with phenol/chloroform, precipitated by adding ethanol and dissolved in distilled water. An aliquot of the nucleic acid solution was heated at 70 °C for 1 min, and subjected to first strand cDNA synthesis utilizing an antisense primer (no. 36; see below) and cloned reverse transcriptase from Moloney murine leukaemia virus (BRL). HCV-derived cDNA was then amplified by 35 cycles of PCR using the HCV-specific primer pair no. 32 (5' CTGTGAGGGAAC-TACTGTCTT 3'; nucleotides 28 to 47)/no. 36 (5' AACACTACTCGGCTAGGCTACTGTCTT 3'; nucleotides 248 to 229) deduced from the 5' non-coding region of the HCV genome (Okamoto et al., 1990a) and the GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus). The HBV sequence was simultaneously amplified by adding the HBV-specific primer pair PC1 (5' CATAA-

GAGGACTCTTGGACT 3'; nucleotides 1653 to 1672 of the plus strand)/PC2 (5' AAAGAATTCGAGG-GCAAAAGA 3'; nucleotides 1972 to 1949 of the minus strand), deduced from the sequence of the pre-C region of HBV DNA (Okamoto et al., 1990b), into the same reaction. Each reaction cycle involved denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1.5 min and primer extension at 72 °C for 3 min. Reaction products were electrophoresed in an agarose gel (2% composite gel of NuSieve and SeaKem, 1:1; FMC) and stained with ethidium bromide. Expected molecular sizes for amplified HCV and HBV sequences were 221 bp and 320 bp, respectively.

The amount of HCV RNA originally in the starting materials was reduced significantly when metrizamide, potassium bromide or caesium chloride was added to the HCV source material. In contrast, sucrose and potassium tartrate did not produce any decrease in the amount of HCV RNA detected compared to that detected using TEN buffer alone (Fig. 1). Potassium tartrate, although it did not affect the stability of HCV, induced visible aggregation of serum proteins when mixed with plasma or serum. Therefore sucrose was selected for the estimation of the density of HCV. The amount of HBV DNA present was similar in all the density media.

In sucrose density gradient equilibrium ultracentrifugation, native HCV virions in plasma banded at a buoyant density of 1.08 g/ml; the HCV RNA was detected by PCR in two neighbouring fractions having a density of 1.09 and 1.07 g/ml (fractions 9 and 10, respectively, Fig. 2a). The HBV DNA-associated particles (Dane particles) used as an internal control banded at 1.21 g/ml, and were detected in fraction 5 (Fig. 2a). HCV was obviously far less dense than HBV.

The peak HCV RNA fraction obtained from the first centrifugation (fraction 10, Fig. 2a) was treated with NP40 (final concentration 0.16%) in the presence of HBV and re-centrifuged. The density of the HCV RNA-associated particles then shifted to 1.25 g/ml (fraction 3, Fig. 2b), whereas that of the Dane particles remained unchanged (fraction 5, Fig. 2b). When the same HCV fraction (fraction 10, Fig. 2a) and HBV were re-centrifuged after treatment under much stronger conditions, i.e. with NP40/2-mercaptoethanol/3% pronase E, the HCV RNA was no longer detectable in any fraction, but HBV DNA was detected at a density of 1.26 g/ml (fraction 2, Fig. 2c), corresponding to that of HBV core particles.

In combination, the elucidation of the nucleotide sequence of the HCV genome and the advent of PCR techniques have enabled us to identify HCV RNA associated with a low density particulate structure. We believe this represents intact virions. Only a small amount of plasma from HCV-infected humans was
Fig. 1. Influence of density media on the stability of HCV. Human plasma containing HCV and HBV was incubated with TEN buffer (lane 1), sucrose (lane 2), potassium tartrate (lane 3), potassium bromide (lane 4), metrizamide (lane 5) or caesium chloride (lane 6), and subjected to PCR amplification of viral sequences, products of which were electrophoresed in an agarose gel and stained with ethidium bromide. Expected lengths of PCR products (arrows; 221 bp for HCV and 320 bp for HBV) are shown. Molecular size markers (123 bp ladder; BRL) were run in lane M.

Fig. 2. Isopycnic banding in sucrose of HCV RNA-associated particles. (a) Human plasma containing HCV and HBV was subjected to sucrose density gradient equilibrium ultracentrifugation. Each fraction was examined for the presence of HCV RNA and HBV DNA by PCR, and for density by measuring refractive indices. PCR products were electrophoresed and stained as in Fig. 1. Lanes 1 to 11 correspond to fractions 1 to 11 (the densities of which were 1.27, 1.26, 1.25, 1.23, 1.21, 1.18, 1.15, 1.12, 1.09, 1.07 and 1.05 g/ml, fractions 1 to 11; similarly in b and c below) collected from the bottom of the centrifugation tube. Arrows indicate the expected sizes of PCR-amplified HCV and HBV sequences (221 bp for HCV and 320 bp for HBV). (b) Fraction 10 mixed with HBV was treated with NP40, and re-centrifuged. (c) Fraction 10 mixed with HBV was treated with NP40, 2-mercaptoethanol and pronase E, and re-centrifuged.

needed to estimate the buoyant density of the presumed HCV virions and nucleocapsid particles. The values obtained for the density of HBV virions and core particles (Fig. 2a and 2c), included in the experiment as internal controls, were comparable to those estimated by classical methods utilizing relatively large quantities of virus particles and immunological and morphological detection tools (Takahashi et al., 1976).

The buoyant density of HCV in sucrose, estimated in the current study, was 1.08 g/ml, much lower than that of HBV which was used in the experiments as an internal control (Fig. 2a). That HCV has a low density had already been predicted from chimpanzee infectivity studies, showing the chloroform sensitivity of the blood-borne non-A, non-B hepatitis agent (Bradley et al., 1983; Feinstone et al., 1983). In addition, the buoyant density of the factor VIII-derived HCV isolate in sucrose was estimated by a bioassay to be significantly lower than that of conventional viruses (Bradley et al., 1991). Our data are consistent with these observations. The value of 1.08 g/ml for the density of HCV is relatively close to that of bovine viral diarrhoea virus (1.09 to 1.15 g/ml) and hog cholera virus (1.12 to 1.16 g/ml) of the pestivirus family (Horzinek, 1981), and considerably different to that of flaviviruses (1.19 to 1.20 g/ml; Westaway et al., 1985). This might suggest that the structural region of the presumed precursor polyprotein of HCV is more comparable with that of pestiviruses, whereas the non-structural region is more akin to that of flaviviruses. Some common properties shared by the structural proteins of HCV and pestiviruses could account for the relative richness of surface lipids on both viruses.

The nucleocapsid of HCV, isolated by different detergent treatments, was estimated to have a buoyant density of 1.25 g/ml in sucrose, slightly lower than that of HBV core particles (1.26 g/ml). The presumed nucleocapsid of HCV would incorporate a viral RNA genome of nearly 10000 nucleotides, whereas the HBV core particle contains partially double-stranded circular DNA of less than 6000 nucleotides. Therefore, the HCV core is predicted to have a higher density than the HBV core particle. A possible explanation for the observed lower density of the HCV nucleocapsid may originate with the presumed core protein of HCV. The presumptive core protein of HCV, consisting of 191 amino acids, has a transmembrane segment-like sequence at its C terminus, and this portion could integrate lipids into the architecture of the HCV nucleocapsid. Therefore the core particles of HCV might contain a lipid component, although it is yet to be determined whether the C-terminal hydrophobic domain of the putative core protein is really a component of the HCV capsid.

A low density component of HCV was easily removed by NP40 treatment alone (Fig. 2b), as is the case for the
flavivirus Japanese encephalitis virus (Shapiro et al., 1971), whereas that of HBV required additional treatments (Fig. 2b and c). This suggests a structural relationship between HCV and flaviviruses. In addition, HCV RNA was not detectable by PCR in several density media, such as caesium chloride (Fig. 1). Thus HCV is suggested to be relatively fragile. Also noteworthy is the possible influence of density media on the estimated value of the buoyant density of HCV; it was 1.11 g/ml in potassium bromide (Takahashi et al., 1992).

Preliminary observations indicated that the density of HCV virions varied among the samples tested, ranging from 1.06 to 1.10 g/ml, depending upon the individual specimen. Possible explanations include an anisomorphism of HCV in different sera, specific antibodies or other proteins attached to the surface, or partial loss of low density lipid or glycoproteins.

We owe a great deal to Dr Lacy R. Overby for his careful reading and revision of the manuscript for both scientific content and language.

References


(Received 13 August 1991; Accepted 19 November 1991)