Association of hepatitis C virus particles with immunoglobulin: a mechanism for persistent infection

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The physical properties of hepatitis C virus (HCV) particles were determined by ultracentrifugation on 20–60% isopycnic sucrose density gradients. We report that (i) two populations of HCV particles were found in the sera of patients with chronic HCV infection [at high density (1.186–1.213 g/ml) and at low density (1.099–1.127 g/ml)], (ii) virus particles with high density values were associated with immunoglobulin, and (iii) virus particles with low density values accumulated base changes within a hypervariable region (HVR) of the E2 envelope domain of the RNA genome. The results indicate that base changes within the HVR of E2 lead to the accumulation of immunoglobulin-free virus particles. Therefore, these findings imply that persistent HCV infection is established as a consequence of sequence variation in the E2 envelope domain.

The genome of hepatitis C virus (HCV), the major aetiological agent of post-transfusion non-A, non-B hepatitis, has been cloned by Choo et al. (1989). Further analysis revealed that (i) it is a positive-stranded RNA virus with a genome size of 10 kb and had one large open reading frame that encodes a polypeptide of 3011 amino acids and (ii) it has some predicted structural homology with flaviviruses and pestiviruses (Miller et al., 1990). However, very little is known about the structure and the physicochemical properties of HCV particles. HCV is an enveloped virus, as indicated by inactivation after chloroform treatment (Feinstone et al., 1983) and the buoyant density of the virus in sucrose has been determined to be between 1.08 and 1.11 g/ml (Bradley et al., 1991; Hijikata et al., 1993; Kanto et al., 1994; Miyamoto et al., 1992). Recently, electron microscopic examination revealed the spherical morphology of HCV particles with a mean diameter of 55–65 nm (Kaito et al., 1994).

We were interested in the characterization of HCV particles derived from the serum of patients with chronic HCV infection. Physical properties of the virus particles were determined by centrifugation on 20–60% isopycnic sucrose density gradients. We report here that (i) two populations of HCV particle were found in the serum of patients with chronic HCV infection, (ii) high density HCV particles were associated with immunoglobulin, and (iii) the immunoglobulin-free HCV particles contained viral genomes with accumulated variation within the hypervariable region (HVR) of the E2 envelope domain.

To quantify a very small copy number of the viral RNA, it was essential to develop a quantitative and competitive polymerase chain reaction (QCPCR) assay as used for titration of human immunodeficiency virus (Kato et al., 1993; Piatak et al., 1993). The strategy of the QCPCR for titration of HCV RNA is described in Fig. 1(a). We used the 5′ untranslated region (UTR) as a template, since the 5′ UTR of the HCV genome is the most conserved region (Han et al., 1991). As a first step, the cDNA encoding the 5′ UTR of HCV that was previously reported (So et al., 1994) was subcloned into a pGEM vector (pGEM5′UTR). Using PCR, the internal control plasmid, pGEM5′UTRDel, was constructed by deletion of nucleotides between nt 87 and 165 of the 5′ UTR of the HCV genome. The internal control RNA was synthesized in vitro by T7 RNA polymerase from a linearized template derived from the pGEM5′UTRDel plasmid. The amount of RNA synthesized in vitro was determined by measurement of the absorbance at 260 nm. A known copy number of the RNA was included as an internal control in order to quantify the viral RNA (Fig. 1a). Since the internal control RNA contains 79 base deletions, as indicated in Fig. 1(a), a PCR product derived from the internal control RNA could be separated from that of HCV RNA by gel electrophoresis. By comparing these two PCR products we were able to quantify HCV RNA. Using this assay, we were able to detect as little as ten copies of specific RNA (data not shown).
To assess the accuracy of the QCPCR assay we titrated H serum that contained $1 \times 10^7$ copies RNA/ml, as reported previously (Hijikata et al., 1993). RNA was extracted from the H serum by the guanidinium isothiocyanate method (Chomczynski & Sacchi, 1987). Aliquots of extracted RNA were mixed with twofold serial dilutions of the internal control RNA. Subsequently, RNAs were mixed with primer KL70 (Fig. 1 a) in a $10 \mu l$ volume of $1 \times$ reverse transcriptase buffer (50 mM-Tris-HCl, pH 8.3, 3 mM-MgCl$_2$, 75 mM-KCl, 10 mM-DTT, 250 nM-dNTPs). The mixture was incubated at 65 °C for 5 min and kept at room temperature for 5 min for annealing. The reverse transcriptase reaction was carried out according to the manufacturer's instructions (BRL). One-tenth of the cDNA was added to the PCR reaction mixture containing 4.5 pmol of KL70 and QC1 primer (Fig. 1 a), in a final volume of 20 μl (10 mM-Tris-HCl, pH 9.0, 50 mM-KCl, 1.5 mM-MgCl$_2$, 0.1% Triton X-100, 0.3 U Tag DNA polymerase, 200 μM-dNTPs). The mixture was covered with mineral oil and amplified in a DNA thermal cycler (Perkin-Emer) by 40 cycles of PCR (95 °C for 1 min, 55 °C for 1 min and then 72 °C for 2 min). The second PCR was carried out as the first PCR except two nested primers were used, QC2 and QC3 (Fig. 1 a). One-tenth of the first PCR product was used for amplification by 25 cycles of PCR. Gel electrophoresis indicated that the band intensity of the PCR product derived from the H serum was equal to that of 250 copies of internal control RNA (Fig. 1 b, lane 4). After normalization of the dilution factor, it was found that the H serum contained $2 \times 10^7$ copies RNA/ml (Fig. 1 b). Thus, the QCPCR assay is not only sensitive but is also an accurate method for quantifying HCV RNA in serum. In addition, it was noted that the 79 base deletion introduced in the internal control RNA did not affect the efficiency of PCR amplification (data not shown).

To determine the density of virus particles, serum obtained from a patient with chronic HCV infection was subjected to isopycnic ultracentrifugation through sucrose density gradients (20-60%) (Fig. 2a). Serum samples were obtained from the Daejeon blood bank in Korea. HCV infection was determined by ELISA (HCD kit, LG Chem, Korea). The ELISA absorbance values for the individual HCV antigens were as follows: 2.41 (core), 1.76 (E1), 1.31 (E2), 2.15 (NS3) and 1.06 (NS5). The alanine aminotransferase (ALT) level was 160 U/ml, i.e. far above the normal level (Sigma). Five-hundred microlitre portions of 100-fold dilution of plasma were layered onto a 10ml 20-60% (w/v) linear sucrose density gradient in STE buffer (0.1 M-NaCl, 10 mM-Tris-HCl, pH 8.0, 1 mM-EDTA). The gradient was centrifuged in an SW 40 rotor at 30000 r.p.m. for 16 h at 4 °C. Twenty fractions were collected from the top by Auto Densi-Flow IIC (Buchler). The viral RNAs in each fraction were extracted and quantified by QCPCR. Interestingly, the majority of viral RNAs were detected in two distinct fractions with densities of 1.099-1.127 g/ml (fraction nos 5-9) and 1.186-1.213 g/ml (fraction nos 11-16). This result indicated that there are two populations of virus particles with different densities in the serum of patients with chronic HCV infection (Fig.
Fig. 2. Equilibrium density gradient ultracentrifugation of serum from patients with chronic HCV. (a) Sucrose density gradient analysis before removal of the immune complex. Sucrose density gradient analysis was performed as described in the text. Fractions were collected from the top of the gradient (starting from lane 1 and finishing at lane 20). The amount of HCV RNA in each fraction was determined by QCPCR. The density of each fraction was determined by refractometer (Fisher). Density values for each fraction are indicated in parentheses: 1 (1.042), 2 (1.053), 3 (1.064), 4 (1.076), 5 (1.085), 6 (1.096), 7 (1.105), 8 (1.115), 9 (1.127), 10 (1.139), 11 (1.152), 12 (1.166), 13 (1.176), 14 (1.187), 15 (1.194), 16 (1.206), 17 (1.214), 18 (1.224), 19 (1.229), 20 (1.230). (b) Sucrose density gradient analysis after removal of the immune complex. Sucrose density gradient analysis was done as described in (a). Density values for each fraction are indicated in parentheses: 1 (1.050), 2 (1.059), 3 (1.069), 4 (1.078), 5 (1.087), 6 (1.099), 7 (1.117), 8 (1.127), 9 (1.142), 10 (1.151), 11 (1.161), 12 (1.172), 13 (1.186), 14 (1.190), 15 (1.199), 16 (1.213), 17 (1.226), 18 (1.229), 19 (1.229), 20 (1.230).

2a). These data are consistent with the findings of others (Hijikata et al., 1993; Kanto et al., 1994).

We then asked what the differences were between the two populations of HCV particles with different densities. It has been suggested that the high density HCV particles derived from the serum of patients with chronic infection are associated with immunoglobulin (Hijikata et al., 1993). To determine directly whether the high density HCV particles are associated with immunoglobulin, the serum was treated with Protein A-bearing cells (Pansorbin; Calbiochem) prior to sucrose density gradient analysis. Briefly, a 20 μl volume of the serum of patients was resuspended in 500 μl of PBS and incubated with 100 μl of Pansorbin for 1 h. The mixture was centrifuged for 5 min and the supernatant was taken and treated with Pansorbin twice more as before. After centrifugation, the relative RNA copy number in each fraction was determined by QCPCR with 400 copies of the internal control RNA. The viral RNAs in the high density fractions were barely detectable (or not detectable), whereas there was no significant change in the amount of viral RNAs in the low density fractions (Fig. 2b). It is conceivable that the association of viral particles with immunoglobulin increases the density of the particles. Since the high density population of the virus was removed after Pansorbin treatment, we concluded that the high density population of the virus was associated with immunoglobulin.

Then, the question arises as to what determines whether a subset of the virus population in chronic serum is associated with immunoglobulin. It was reported that the N terminus of the putative E2 envelope glycoprotein encodes an HVR of 28 amino acids (Weiner et al., 1991). Further, it was suggested that hyper-variation of amino acids in the HVR of the E2 envelope protein was involved in virus escape from immuno-
surveillance (Kato et al., 1994). Therefore, we examined whether immunoglobulin-free HCV particles contained sequence changes within the HVR of the E2 domain.

Briefly, RNA was extracted from the low and high density fractions as described above. The cDNA was made with primer 1864A [5' CAACAGGCTTGGG-GTGAACA 3', corresponding to positions 1885–1864 of HCV-L2 (So et al., 1994)] using reverse transcriptase. Nested PCR was performed with external and internal primer pairs: external primer pair 1311S (5' CACCGC-ATGGCTTGGGAG 3', corresponding to positions 1288–1311 of HCV-L2) and 1846A; internal primer pair KR89 (5' GTATCATCCCGATGGCC-TGG GATATGATGATGAA 3', corresponding to positions 1864–1885 of HCV-L2). The amplified DNAs were subcloned into M13 phage as described in the text. Amino acid positions 384 and 411 are indicated. Amino acids are denoted by the single letter code. Base substitutions that result in amino acid changes are indicated by closed circles. (b) Summary of amino acid substitutions within the HVR between high and low density HCV particles. Amino acid positions 398 and 407 are indicated.

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**Fig. 3.** Sequence analysis of the HVR of the HCV genome. (a) Nucleotide and deduced amino acid sequences from HCV particles isolated from high and low density fractions of the sucrose density gradient. The HVRs of HCV RNA in fractions 7 and 17 were amplified by RT-PCR and subcloned into M13 phage as described in the text. Amino acid positions 384 and 411 are indicated. Amino acids are denoted by the single letter code. Base substitutions that result in amino acid changes are indicated by closed circles. (b) Summary of amino acid substitutions within the HVR between high and low density HCV particles. Amino acid positions 398 and 407 are indicated.

We sequenced ten cDNA clones of the HVR of virus derived from both the high and low density populations (Fig. 3). Sequence analysis revealed that (i) several quasispecies of virus co-existed simultaneously in a single individual (Higashi et al., 1993), (ii) the low density particles contained additional multiple base changes within the HVR that were clustered between amino acids 401 and 407. Furthermore, these base changes were accumulative (e.g. L-7-2 vs L-7-1, L-7-15, L-7-22 and L-7-28). The amino acid difference between high and low density HCV particles are indicated in Fig. 3(b). Interestingly enough, this area of the HVR domain was also recognized by others as a B cell epitope (Kato et al., 1994; Taniguchi et al., 1993; Weiner et al., 1992). The results further support the hypothesis that this area of the HVR domain is under strong immune selection. It is possible that the virus assembled by the E2 antigen with a modified HVR domain could escape immune surveillance. Therefore, the hypervariability as a consequence of immune selection could provide a mechanism for persistent infection (Kato et al., 1994; Weiner et al., 1992).

One of the hallmarks of HCV infection is a high incidence of chronic infection (Alter et al., 1992). More than 60% of infection resulted in chronic infection. One important question related to HCV infection is the mechanism by which persistent infection is established. Several mechanisms for persistent infection have been proposed, including (i) escape from immunosurveillance, (ii) suppression of the host immune system and (iii) generation of variants (Oldstone, 1989). Our results indicated that hypervariation of the E2 HVR accounts for the establishment of persistent HCV infection. In combination, hypervariation of the envelope protein as well as the existence of multiple genotypes (Simmonds et al., 1993) provide the major obstacles for vaccine development (Weiner et al., 1992).

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