Demonstration of a hepatitis C virus-specific antigen predicted from the putative core gene in the circulation of infected hosts

Kazuaki Takahashi,1 Hiroaki Okamoto,2 Shinya Kishimoto,1 Eisuke Munekata,3 Katsumi Tachibana,4 Yoshihiro Akahane,5 Hiroshi Yoshizawa,6 and Shunji Mishiro7*

1Department of Public Health, Hamamatsu University School of Medicine, Shizuoka 431-31, 2Immunology Division, Jichi Medical School, Tochigi 329-04, 3Institute of Applied Biochemistry, University of Tsukuba, Ibaraki 305, 4Japanese Red Cross Blood Center, Saitama 388, 5First Department of Internal Medicine, Yamanashi Medical College, Yamanashi 429-38, 6Department of Hygiene, Hiroshima University School of Medicine, Hiroshima 734 and 7Institute of Immunology, Koraku 1-1-10, Bunkyo-ku, Tokyo 112, Japan

An ELISA was used to detect a protein derived from the core gene of the hepatitis C virus (HCV) in human plasma. The solid phase antibody in the assay was a murine monoclonal antibody against a synthetic peptide deduced from the putative core gene of HCV (residues 39 to 74). An enzyme-labelled affinity-purified human antibody directed at another region within the HCV core (residues 5 to 23) was the second antibody tracer. The ELISA had a sensitivity capable of detecting a few ng/ml of the HCV core polypeptide expressed in Escherichia coli. Core antigen activity in plasma of infected hosts was detected after treatment of HCV RNA-rich fractions from buoyant density centrifugation with the detergent Tween 80. There was a direct correlation between core antigen ELISA values of a plasma fraction and intensities of polymerase chain reaction signals for HCV RNA. These observations are consistent with the proposal that the N-terminal sequence of the predicted polyprotein of HCV is a nucleocapsid protein, and that improved core antigen assays may correlate with viraemia.

Introduction

Hepatitis C virus (HCV) has been identified as a causative agent for the majority of parenterally transmitted non-A, non-B hepatitis (Choo et al., 1989). Several complete sequences of HCV genomic RNA have been reported (Kato et al., 1990; Choo et al., 1991; Takamizawa et al., 1991). Despite the growing importance of this virus in many seroepidemiological settings, very little is known about the biological properties of HCV because it has not been grown in culture, and the only sources of HCV available (i.e. plasmas or livers of infected humans and/or chimpanzees) do not contain sufficient virus to allow it to be easily visualized or analysed by classical methods. In contrast, hepatitis B virus (HBV) was well characterized biophysically before molecular cloning of the viral genome; HBV virions (Dane particles; Dane et al., 1970) and core particles (Almeida et al., 1971) were directly recovered and visualized from plasmas of infected humans.

The nucleotide sequences of HCV RNA indicate that it is a positive (sense) strand of about 10000 bases with a single open reading frame (ORF). The presumed polyprotein coded for by the ORF is comparable with those of flaviviruses and pestiviruses at the level of amino acid sequence similarity (Miller & Purcell, 1990) and hydrophopy profiles (Choo et al., 1991). The protease cleavage points and possible function of each part of the precursor polyprotein have been predicted by analogy with flaviviruses and pestiviruses. The first 191 amino acids of the polyprotein, for example, have been proposed to represent the core protein of HCV. No direct evidence is available to identify this as the nucleocapsid protein of HCV, whereas antibodies directed to this region can be detected in some human infections concurrently with viraemia (Okamoto et al., 1990c) as in the case of anti-HBc antibodies in HBV infection (Hoofnagle et al., 1973).

In this study, we have demonstrated core antigen activity in the circulation of infected hosts by using a newly developed ELISA the specificity of which is based on the amino acid sequence of the predicted core protein of HCV. The antigenic activity was specifically found in the HCV RNA-rich fractions of antibody-positive plasmas separated by buoyant density ultracentrifugation. The antigen was detected only after treating the...
fractions with a detergent. This suggests that it was an internal antigen of HCV and was released by removal of a lipid-rich envelope.

**Methods**

**Human plasmas.** Plasmas were obtained from Japanese Red Cross Blood Center voluntary blood donors who were asymptomatic but seropositive for anti-HCV-c100 antibody (Kuo et al., 1989). High antibody titre plasmas were selected on the basis of having absorbance values higher than 3:000 in a commercially available ELISA (Anti-HCV Ab ELISA; Ortho). As internal controls, plasmas from donors persistently infected with HBV and which were seronegative for anti-HCV-c100 antibody were included in the experiments.

**Synthetic peptides and recombinant polypeptide deduced from the putative core gene of HCV.** Two oligopeptides, RRGPRLGVVRATKTSERQPRGRQRPVPKVRPPGRCV (CP9; residues 39 to 74) and PKPQRTKRNTRPQDVK (CP10; residues 5 to 23) were synthesized from the putative core protein encoded by the HCV-1 isolate of HCV (Okamoto et al., 1990a,c). A recombinant polypeptide (P20) having residues 1 to 180 of the presumed core protein of the HC-J4 isolate of HCV (Okamoto et al., 1990a) was expressed in Escherichia coli.

**Antibodies.** A murine monoclonal antibody was prepared for use in the ELISA as a solid phase capture reagent. Female BALB/c mice (7 weeks of age) were injected intraperitoneally with 100 µg of CP9 peptide conjugated with ovalbumin as an emulsion with Freund’s complete adjuvant (Difco). After 6 weeks, they were boosted by intravenous administration of 20 µg of CP9. Three days later, spleen cells from the mice were hybridized with NS-1 myeloma cells by a conventional method (Oi & Herzenberg, 1980). Clones were selected by an ELISA with CP9 as the solid phase antigen.

An antibody from human plasma was affinity-purified and used as an enzyme-labelled second antibody in the ELISA. Human plasmas were tested for reactivity with synthetic peptide CP10 by ELISA. The antibody in the plasma was then purified by affinity chromatography using Sepharose 4B (Pharmacia) coupled to oligopeptide CP10. The bound antibody was eluted with 5 M-MgCl2, and coupled to horseradish peroxidase by standard procedures (Nakane & Kawaoi, 1974).

**ELISA for HCV core antigen.** A murine monoclonal antibody (9380-B) raised against CP9 was immobilized on polyvinyl microtiter plate wells (Costar) for use as the capture antibody. Each well of the plate received 50 µl of 5 µg/ml of 9380-B dissolived in 10 mM-Tris-HCl pH 7.5. After remaining at 4°C overnight, the plate was washed five times with 0.05% Tween 20 in 150 mM-NaCl, and 50 µl of horseradish peroxidase-labelled anti-CP10 antibody was then added. After incubation with the tracer antibody at room temperature for 30 min with constant agitation, the plate was washed as above and subjected to a colour-developing reaction with o-phenylene diamine according to conventional methods. Absorbance of the reaction products at 492 nm was measured, and compared with standard curves produced with the recombinant polypeptide, P20.

**Specificity of the ELISA.** To confirm the specificity of the ELISA, the CP9 and CP10 peptides were used separately in the ELISA system for neutralization. CP9 was co-incubated with the test samples at a concentration of 100 µg/ml during the first stage reaction of the ELISA to compete with binding of ELISA antibody. In the second step, CP10 was mixed with the enzyme-labelled antibody at 100 µg/ml and incubated to compete with the analyte for binding antibody.

**Detection of HCV-RNA by polymerase chain reaction (PCR).** Plasmas or their processed fractions obtained by ultracentrifugation were centrifuged at 90000 r.p.m. for 15 min using a Beckman TL100.2 rotor with a TL100 tabletop ultracentrifuge. Prior to centrifugation, the processed fractions were diluted with 40 volumes of 50 mM-Tris-HCl pH 8.0, 1 mM-EDTA, 150 mM-NaCl. The pellets from the ultracentrifugations were resuspended in 200 µl of buffer containing 50 mM-Tris-HCl pH 8.0, 200 mM-NaCl, 10 mM-EDTA, 2% (w/v) 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 cDNA strand synthesis utilizing an antisense primer (no. 36; see below) and cloned reverse transcriptase of Moloney murine leukemia virus (BRL). HCV-derived cDNA was then amplified by PCR using HCV-specific primer pair no. 35 (5'CTCTAGAAGTCGGTCTGTCTT 3'; nucleotides 28 to 47) and no. 36 (5'AACAC-TACTCGGCTAGCAGT 3'; nucleotides 248 to 229) deduced from the 5'-non-coding region of the HCV genome (Okamoto et al., 1990b), and the GeneAmp DNA amplification reagent kit (PerkinElmer Cetus). The PCR was cycled 30 times for samples from centrifugation fractions and 35 times for native plasmas. Negative specimens were amplified another 30 times using a nested primer pair with the sequences described by Okamoto et al. (1990b). Reaction products were subjected to electrophoresis in agarose gel (2% composite gel of NuSieve and SeaKem, 1:1; FMC) and stained with ethidium bromide. The expected molecular size for the amplified HCV sequence was 221 bp.

**Buoyant density ultracentrifugation.** A 1600 ml pool of plasma was prepared from 12 donors seropositive for both anti-HCV-c100 antibody (ELISA absorbance higher than 3:000) and anti-CP10 antibody (ELISA absorbance higher than 2:000). The plasma pool was clarified by centrifuging at 2000 r.p.m. for 10 min, and the supernatant was centrifuged at 30000 r.p.m. for 3 h using a Beckman Type 35 rotor. The pellet was collected and suspended in 10 ml of 20 mM-Tris-HCl pH 7.5 (Tris buffer). Five grams of potassium bromide (KBr) was added to this suspension to adjust the density to 1.30 g/ml. The suspension was transferred to the bottom of a 35 ml ultracentrifuge tube and overlaid with 17 ml of KBr in Tris buffer (density 1.28 g/ml) followed by 6 ml of Tris buffer (density 1.00 g/ml). The mixture was centrifuged in a Beckman SW27 rotor at 25000 r.p.m. for 14 h. Fractions, each of 1 ml, were collected from the top of the tube and tested for HCV RNA by PCR and for HCV core antigen activity by ELISA, before and after treatment with Tween 80.

In addition to the large pool of plasma from 12 donors, 18 plasmas from individual anti-HCV-c100 antibody-positive donors and six plasmas from negative controls were also tested. In these cases 30 ml of each plasma was pre-mixed with 30 ml of Tris buffer, and clarified and pelleted by ultracentrifugation, as above. The pellets were resuspended in 1 ml of Tris buffer and mixed with 9.5 g of KBr to adjust the density to 1.30 g/ml. The mixture was transferred to the bottom of a 5 ml centrifuge tube and overlaid with 3.5 ml of KBr in Tris buffer with a density of 1.28 g/ml, followed by 0.6 ml of Tris buffer. After centrifuging at 30000 r.p.m. at 10°C in a Hitachi RPS50 rotor for 14 h, successive 200 µl fractions were collected from the top of the tube. The top five fractions had densities ranging from 1.00 to 1.20 g/ml. Only these fractions were tested for core antigen, and the total ELISA results
in these fractions were added and interpreted as the core antigen activity in each plasma.

Results

Sensitivity and specificity of the ELISA for HCV core antigen

The recombinant polypeptide possessing the first 180 amino acids of the predicted HCV core protein, P20, was tested at various dilutions in ELISA for HCV core antigen activity. A linear correlation was found between the ELISA values \( A_{492} \) and the concentration of P20 from 0-5 to 100 ng/ml (Fig. 1). The minimum antigen detected by this assay system was estimated to be about 1 ng of P20 per ml. The ELISA values showed neutralization by CP9 and CP10 peptides derived from the putative core gene Table 1), indicating that the two-site immunoassay was specific for the recombinant core polypeptide.

Demonstration of the HCV core antigen activity in the circulation of seropositive hosts

A 1600 ml human plasma pool obtained from anti-HCV-c100 antibody-seropositive blood donors was fractionated by an equilibrium buoyant density centrifugation using KBr. The fractions were tested for HCV core antigen activities by ELISA and for HCV RNA by PCR. The ELISA readings for HCV core antigen were significantly increased after treatment of each fraction with Tween 80. A peak was observed at the fraction with a density of 1.115 g/ml (Table 2). HCV RNA was also concentrated at the peak intensity of the ELISA signal (Table 2 and Fig. 2).

HCV core antigen activities in individual plasmas

Most (15 of 18) plasmas from anti-HCV-c100 antibody-positive subjects showed HCV core antigen ELISA activities with an absorbance higher than 0.05, and all of six negative controls showed ELISA values lower than 0.02 (Fig. 3). Of the three c100 antibody-positive subjects who lacked HCV core antigen activity, two were negative for circulating HCV RNA. Another was positive for HCV RNA only at the minimal detectable concentration (+ in Fig. 3). Plasmas containing the highest concentrations of HCV RNA (+ + + in Fig. 3) showed higher activity of the HCV core antigen than two plasmas with an intermediate amount of HCV RNA (+ + in Fig. 3). These results are consistent with HCV core antigen and RNA association in the nucleocapsid of an intact virion.
K. Takahashi and others

- HCV RNA complexes in plasmas of infected humans have been determined recently (Miyamoto et al., 1992). These studies identified two fractions of HCV RNA at different densities in sucrose gradients. A lighter fraction (a presumed enveloped virus) was transformed into a higher density fraction (a presumed nucleocapsid) after detergent treatment. However, these PCR techniques did not provide information about the proteins associated with HCV. The viral proteins have been deduced from the genomic RNA nucleotide sequences by comparison with known flavivirus and pestivirus precursor polyproteins. The N-terminal 191 amino acids of the HCV precursor polyprotein sequence has been proposed to form the HCV core protein, but there is no direct evidence.

In this study we found HCV RNA and the predicted core protein cosedimenting in gradient centrifugations. The ELISA system used for the core antigen analysis was entirely dependent upon the deduced amino acid sequences of the HCV precursor polyprotein. The capture antibody was a murine monoclonal antibody raised against a synthetic peptide from the presumptive core protein. The enzyme-conjugated tracer antibody was prepared from human antiserum and bound specifically to a similar but different peptide within the core protein.
protein sequence. Prior to analysis for HCV core antigen activity the human plasmas were subjected to buoyant density centrifugation to separate particulate structures from immunoglobulins and other plasma proteins. The core antigen was detected by ELISA only after treating the gradient fractions with Tween 80 (Table 2). Core antigen ELISA activity was found in the same density gradient fractions as PCR signals for HCV RNA (Fig. 2). These data suggested that the antigenic activity detected by ELISA was internally associated with HCV RNA, representing a nucleocapsid.

The purified recombinant HCV core protein (residues 1 to 180), P20, expressed in E. coli, served as a standard for quantifying the ELISA. In the standard assay an absorbance of 0.000 equated to about 100 ng of P20 per ml (Fig. 1). For the 18 plasmas that were tested individually we were able to calculate an approximate number of nucleocapsids, assuming that all the core antigen activity was involved in particulate structures. For these analyses, the ultracentrifugation pellet from 30 ml of plasma was banded in the equilibrium density gradient. The core antigen was found only in the upper five fractions (total 10 ml) of the gradient. The plasmas with the highest PCR RNA values had an average ELISA absorbance of about 0.20 for these 1-0 ml fractions (Fig. 3), equivalent to about 20 ng of the P20 standard per 30 ml of original plasma (0.67 ng/ml). Assuming that 500 P20 molecules of 20K are associated with each RNA molecule, we can estimate a concentration of about 4 x 10^7 nucleocapsid particles per ml of plasma. In comparison, it has been estimated that the number of Dane particles in HBV carriers seropositive for hepatitis B e antigen is more than 10^10 per ml of plasma with chimpanzee infectious titres of 10^8 to 10^9 per ml (Shikata et al., 1977). The above calculations for HCV are consistent with chimpanzee infectivity studies of human plasma, where infectivity titres of about 10^3/ml were usually observed (Bradley et al., 1983).

We found that two of 18 plasmas were negative for HCV RNA despite seropositivity for anti-HCV-c100 antibody (Fig. 3). HCV core antigen was detected in 15 of 16 HCV RNA-positive plasmas. The one ELISA-negative plasma had the lowest amount of RNA detectable by PCR. Plasmas containing HCV RNA at relatively higher concentrations (+ + or ++ +, Fig. 3) were clearly positive by ELISA. However, the 30 ml samples of plasma used in the analyses involved time-consuming sample preparation and centrifugations. A minimum 10-fold increase in sensitivity of the ELISA and a more convenient pretreatment method will be required for practical HCV antigen serology studies. A scaling-down to 7-5 ml of starting plasma has been achieved so far using the methods described in this paper. A major problem is the presence of anti-CP9 and anti-CP10 antibodies in plasma which would interfere with detection of core antigen by the current ELISA if not separated by ultracentrifugation or other methods.

The present study involved buoyant density centrifugation of HCV in KBr solutions. The presumed enveloped virus was found at a density of 1.11 g/ml, slightly higher than 1.08 g/ml that has been estimated by sucrose equilibrium centrifugation (Miyamoto et al., 1992). The differences in composition and the ionic strength of the separation media probably accounts for these small changes in apparent density of HCV.

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References


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