Hepatitis C virus particle detected by immunoelectron microscopic study

Masahiko Kaito,1* Shozo Watanabe,1 Kyoko Tsukiyama-Kohara,2 Kenjiro Yamaguchi,3 Yoshinao Kobayashi,1 Masayoshi Konishi,1 Masato Yokoi,1 Satoshi Ishida,1 Shiro Suzuki1 and Michinori Kohara2

1 The Third Department of Internal Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514, Department of Microbiology, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113 and 3 Fundamental Research Laboratory, Corporate Research and Development Laboratory, The Tonen Company, Nishi-Tsurugaoka, Ohi-machi, Iruma-gun, Saitama 354, Japan

To clarify the morphology of hepatitis C virus (HCV), an indirect immunogold electron microscopic study was carried out on two plasma samples with high HCV RNA titres using polyclonal and monoclonal antibodies specific to the putative HCV envelope protein. Spherical virus-like particles, 55 to 65 nm in diameter with spike-like projections, were found in 1.14 to 1.16 g/ml fractions after sucrose density gradient centrifugation. These particles were found only in HCV-infected blood donors and had morphological features similar to those of flaviviruses. Moreover, these particles specifically reacted with the polyclonal and monoclonal antibodies to the putative HCV envelope protein. This is the first known report in which the morphology of the HCV particle is clearly shown.

Hepatitis C virus (HCV) is the main causative agent of non-A non-B hepatitis. Initially cDNA clones of the HCV genome were isolated from experimentally infected chimpanzee plasma (Choo et al., 1989). To date, almost the entire nucleotide sequence of the HCV genome has been reported, and is known to be RNA, composed of more than 9400 nucleotides, which encode a single polypeptide consisting of a putative core and E1, E2/NS1, NS2, NS3, NS4 and NS5 protein regions (Kato et al., 1990; Takamizawa et al., 1991). Based on structural similarities, HCV is now classified into the family Flaviviridae. A detection system that is highly sensitive for HCV antibodies has already been established using the putative core, NS3 and NS4 protein regions as antigens (Aach et al., 1991). However, there have been very few reports on the morphology of HCV in plasma (Abe et al., 1989; Takahashi et al., 1992) and no confirmation that specific antibodies react with the HCV envelope protein on the surface of virus-like particles. Therefore, the morphology of the HCV particle has remained unknown. In this paper, we visualized virus-like particles with fine spike-like projections existing in human plasma positive for both anti-HCV antibodies and HCV RNA and carried out an immunoelectron microscopic examination using specific antibodies to the putative HCV envelope protein.

Plasma samples were obtained from blood donors with elevated alanine aminotransferase (ALT) who tested negative for hepatitis B virus surface antigen as well as antibodies to human T cell leukaemia virus type I and human immunodeficiency virus. Thirteen samples were positive for antibodies to HCV (anti-HCV; EIA-2, Ortho Diagnostic Systems) and two were negative for anti-HCV. Quantification of HCV RNA was accomplished by competitive PCR of a sequence within the 5' non-coding region (Kaneko et al., 1992; Yoshioka et al., 1992) and two plasma samples (samples A and B) with more than 4 × 10^7 copies/ml of HCV RNA were chosen to examine the existence of HCV particles. The genotype of these two HCV-positive samples was determined by PCR of the core region (Okamoto et al., 1992) and they were found to be type II (type 1b, Simmonds et al., 1993; Table 1).

The two HCV RNA-rich samples A and B (100 ml), and two HCV-negative samples C and D (100 ml), were centrifuged at 75000 g for 6 h at 4 °C. The pellets were suspended in TEN buffer (100 mm-Tris–HCl pH 8.0, 100 mm-NaCl, 1 mm-EDTA) and centrifuged again at 150000 g for 2.5 h at 4 °C. An approximately 1000-fold-concentrated suspension of the sample was layered onto a 20 to 60 % continuous sucrose gradient in TEN buffer (100 mm-Tris–HCl pH 8.0, 1 mm-EDTA) and centrifuged at 100000 g for 16 h at 4 °C in a Hitachi RPS 40T rotor. Sucrose fractions (500 µl) were collected from the tube bottom and the density of each was measured with an Abbé refractometer. HCV RNA titres in each
Table 1. ALT levels and HCV data of four plasma samples from blood donors

<table>
<thead>
<tr>
<th>Sample</th>
<th>ALT* (IU/l)</th>
<th>Anti-HCV (C.O.I.) †</th>
<th>HCV RNA titre (copies/ml) ‡</th>
<th>HCV genotype §</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>103</td>
<td>&gt; 5.0</td>
<td>+</td>
<td>&gt; 4 x 10⁷</td>
</tr>
<tr>
<td>B</td>
<td>109</td>
<td>&gt; 5.0</td>
<td>+</td>
<td>5 x 10⁷</td>
</tr>
<tr>
<td>C</td>
<td>121</td>
<td>0.22</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>87</td>
<td>0.26</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Normal plasma ALT levels are less than 35 IU/L.
† C.O.I., cut-off indices by second generation ELISA (Aach et al., 1991). C.O.I.s greater than 1.0 were defined as positive.
‡ Based on the methods of Kaneko et al. (1992) and Yoshioka et al. (1992).
§ As defined by Okamoto et al. (1992).

For the study of virus particles, fractions from sucrose density gradient centrifugation were diluted in PBS (pH 7.4) and spun down at 150,000 g for 2.5 h at 4 °C. The pellets were suspended in 100 µl of PBS and 3 µl of each suspension was applied to formvar–carbon grids and then negatively stained with 2% phosphotungstic acid pH 6.5, before being investigated under a Hitachi H-800 electron microscope. Virus-like spherical particles with fine spike-like projections, 55 to 65 nm in diameter, were visualized in samples A and B only (Fig. 2). The morphological features of these particles were quite similar to those of flaviviruses (Murphy & Kingsbury, 1990) and their size was consistent with the results of previous filtration studies (Bradley et al., 1985; He et al., 1987). The buoyant density of the fractions containing the flavivirus-like particles was 1.14 to 1.16 g/ml. Bradley et al. (1991) estimated that the density of infectious HCV (using a bioassay in the chimpanzee) was within the 1.09 to 1.21 g/ml range in sucrose, with peak infectivity in the fractions ranging from 1.09 to 1.11 g/ml. This buoyant density was significantly lower than that of conventional viruses. However, it was suggested that the low buoyant density of HCV could be related to its non-specific interaction with plasma components, such as low-density lipoproteins (Bradley et al., 1991). We observed many lipoprotein particles in the lower density fractions (1.05 to 1.11 g/ml) and it was therefore difficult to visualize the flavivirus-like particles. A few naked particles, 30 to
Fig. 3. Immunoelectron micrographs of flavivirus-like particles about 55 to 65 nm in diameter from plasma samples with a high HCV RNA titre, visualized using polyclonal antibodies: virus-like particles from sample A are shown in (a) and from sample B in (b), (c) and (d). In (a), (b) and (d), virus-like particles were reacted with rabbit polyclonal antibody to the HCV envelope protein (RR2) at a dilution of 1:100, and their antibody haloes were identified by binding to goat anti-rabbit IgG-conjugated colloidal gold particles (5 nm) at a dilution of 1:20. (c) Control reaction using rabbit polyclonal antibody to vaccinia virus Lister strain at a dilution of 1:100, following reaction with goat anti-rabbit IgG colloidal gold particles (5 nm) at a dilution of 1:20. (d) Gold particles clustering on the surface of a virus-like particle with a visible inner core (indicated by an arrow). The scale bar represents 100 nm.

35 nm in diameter, were observed in fractions with higher densities (1.23 to 1.27 g/ml).

In order to determine whether the flavivirus-like particles were HCV particles, an immune-aggregating experiment was performed on the fractions with a density of 1.12 to 1.16 g/ml, using an antibody specific to the putative HCV envelope protein. We observed specific, large aggregations of virus-like particles in this fraction. However, this fraction also contained naturally occurring clusters of virus-like particles. This finding
illustrates the difficulties in identifying individual HCV particles by the immune-aggregation method. Therefore we utilized a specific antigen-antibody reaction using indirect immunogold electron microscopy (Murti & Webster, 1986).

Rabbit polyclonal antibody (RR2) to the putative HCV envelope protein was prepared as follows. The putative envelope gene of HCV type II (nucleotide positions 676 to 1607; Kohara et al., 1992; Takamizawa et al., 1991) was cloned under the control of the ATI-P7.5 hybrid promoter of vaccinia virus vector pSFB4 (Funahashi et al., 1991), and allowed to recombine with the Lister strain of vaccinia virus to give vector RVV. Rabbits were infected intradermally with 10^6 p.f.u. of RVV and 2 months later were boosted twice with the purified putative envelope protein. Putative HCV envelope protein was expressed by RVV and purified by lentil lectin column chromatography and affinity chromatography using an anti-envelope monoclonal antibody (Kohara et al., 1992). Mouse monoclonal antibodies (159, 260, 305 and 1905) against the putative HCV envelope protein were prepared by the immunization of mice with purified recombinant envelope protein expressed by RVV, as described above (Kajita et al., unpublished results). The monoclonal antibodies were screened by ELISA and an indirect immunofluorescence assay (IFA) using the putative envelope protein expressed in baculovirus (Matsuura et al., 1992). Rabbit polyclonal antibody RR2 and the four mouse monoclonal antibodies to the HCV envelope protein, 159, 260, 305 and 1905, were used as primary antibody. Each antibody reacted specifically with the HCV putative envelope protein and did not react with the putative core, E2/NS1 or NS2 proteins when expressed by RVV and baculovirus (Matsuura et al., 1992).

Specificity was determined by using primary antibodies from pre-immune normal rabbit serum, serum from a rabbit infected with the Lister strain of vaccinia virus and mouse monoclonal antibody specific to the human blood type A antigen as negative controls, or by omitting the use of the primary antibody. Goat anti-rabbit IgG or staphylococcal Protein A-coupled colloidal gold particles (5 nm; BioCell Research Laboratories) were used as the secondary antibody.

Fig. 4. Immunoelectron micrographs of virus-like particles from plasma samples, revealed using monoclonal antibodies. Shown are virus-like particles from sample A in (a) and (c), and sample B in (b). In (a) and (b), virus-like particles were reacted with a mixture of four monoclonal antibodies to the HCV envelope protein, at a dilution of 1:10, and staphylococcal Protein A-conjugated colloidal gold particles (5 nm), dilution 1:20. (c) Control reaction using mouse monoclonal antibody to human blood type A antigen. The scale bar represents 100 nm.
The immunoelectron microscopic procedure followed the method of Murti & Webster (1986) with a minor modification. Briefly, 3 μl of each of the 1.14 g/ml (sample B) and 1.16 g/ml (samples A, C and D) fractions was adsorbed on a formvar–carbon grid and then floated for 5 min on a drop of BSA–TBS (150 mm-NaCl, 100 mm-Tris–HCl pH 7.6 and 2% BSA) placed on a Parafilm strip in a moist glass Petri dish. The grid was then floated for 1 h on a drop of TBS containing 3% gelatin and then washed on a drop of BSA–TBS. The grid was incubated for 1 h on a drop of primary antibody solution (diluted 1:10 to 1:100 in BSA-TBS) at room temperature and then washed three times on a drop of BSA–TBS. After floating the grid on a drop of secondary antibody solution (diluted 1:20 in BSA–TBS) for 1 h, it was rinsed three times on a drop of BSA–TBS and then stained with 2% phosphotungstic acid for observation by electron microscopy.

Virus-like particles, 55 to 65 nm in diameter, which exhibited specific gold labelling when reacted with the rabbit polyclonal antibody (RR2) to the HCV envelope protein are shown in Fig. 3. Obvious antibody haloes and their specific binding to goat anti-rabbit IgG colloidal gold particles are seen in samples A and B (Fig. 3a) and B (Fig. 3b) but not in samples C or D. Antibody haloes and specific gold labelling were not observed when the normal rabbit serum and the anti-vaccinia virus Lister strain rabbit serum were used as the primary antibody in samples A and B (Fig. 3c). A virus-like particle with a visible inner core structure was also labelled with gold particles specific to the HCV envelope protein (Fig. 3d). The mixture of four clones of monoclonal antibodies to the HCV envelope protein (159, 260, 305 and 1905) also produced specific gold labelling of a virus-like particle (Fig. 4). This kind of specific reaction was confirmed in samples A (Fig. 4a) and B (Fig. 4b) but not in samples C or D. No specific gold labelling occurred in the presence of mouse monoclonal antibody to human blood type A antigen (Fig. 4c). In the reaction with monoclonal antibody (Fig. 4), the clustering of gold particles was specific, although they were fewer in number and the antibody haloes were narrower than those found with polyclonal antibody to the HCV envelope protein. This might indicate that the quantities of antibodies to the HCV envelope protein were higher in the polyclonal samples than in the monoclonal samples. These particles possessed shorter and more slender spike-like projections than the virus-like particles reported by Abe et al. (1989).

In conclusion, this indirect immunogold electron microscopic study showed that HCV particles are 55 to 65 nm diameter spherical particles with fine surface spike-like projections, 6 nm in length, and have a 30 to 35 nm inner core. HCV particles were observed to be similar to flavivirus virions, which consist of an envelope 7 nm in width with fine surface projections surrounding a spherical nucleocapsid.

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### Table 2. Characteristics of polyclonal and monoclonal antibodies to the putative HCV envelope protein

| Serum       | ELISA* titre | IFA† titre | WB‡ titre | Epitope§ (amino acid position) ||
|-------------|--------------|------------|-----------|-----------------------------|
| Polyclonal  |              |            |           |                             |
| RR2         | 10⁶          | > 10³      | 10⁵       |                             |
| Monoclonal  |              |            |           |                             |
| 159         | < 0·1 μg/ml  | < 10 μg/ml | < 1 μg/ml | 192-211                     |
| 260         | < 0·1 μg/ml  | < 10 μg/ml | < 1 μg/ml | 207-226                     |
| 305         | < 0·1 μg/ml  | < 10 μg/ml | < 1 μg/ml | 207-226                     |
| 1905        | < 0·1 μg/ml  | < 10 μg/ml | < 10 μg/ml| 192-211                     |

* Carried out using synthetic peptides and purified recombinant proteins.
† Western blot analysis, using purified recombinant proteins from the putative envelope protein region of HCV type II as antigen (Kohara et al., 1992).
§ Mapped using 20-amino acid oligopeptides, each overlapping the adjacent oligopeptide by 10 amino acids.

### References


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