The particle size of hepatitis C virus estimated by filtration through microporous regenerated cellulose fibre

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To estimate the particle size of hepatitis C virus (HCV), a major causative agent of post-transfusion non-A, non-B hepatitis, we filtered plasma or serum samples through microporous cellulose fibres with different pore sizes. The amount of HCV particles in samples before and after filtration was determined by a quantitative reverse transcriptase polymerase chain reaction (PCR) method. Since there is no quantitative biological assay for HCV, except for that in chimpanzees, the HCV titre obtained from the PCR method was used in an equation constructed previously for application to filtration experiments with a flavivirus which is distantly related to HCV. The particle was estimated to be between 30 and 38 nm in diameter, although the possibility remained that larger HCV particles or HCV aggregates with a diameter of more than 39 nm might exist. Double-step filtration through microporous cellulose fibres with a pore size of 35 nm reduced the HCV content to below levels detectable by our PCR method, indicating that it is possible to eliminate HCV particles by simple filtration techniques.

Hepatitis C virus (HCV) genomic cDNA was cloned from an experimentally infected chimpanzee (Choo et al., 1989). World-wide seroepidemiological studies have revealed that HCV is a major causative agent of post-transfusion and many sporadic cases of non-A, non-B hepatitis (Alter et al., 1989; Esteban et al., 1989; Kuo et al., 1989; Miyamura et al., 1990). HCV contains a positive-stranded RNA molecule of about 9400 nucleotides which contains a single large open reading frame encoding a viral polyprotein precursor of up to 3011 amino acids (aa) (Choo et al., 1989). The nucleotide and deduced aa sequences of the genomes of different isolates have shown that HCV is distantly related to flaviviruses and pestiviruses (Choo et al., 1989; Miller & Purcell, 1990; Takeuchi et al., 1990; Kato et al., 1990; Takeda et al., 1991; Choo et al., 1991; Han et al., 1991). These characteristics, as well as the gene organization of HCV, are consistent with the theory that at least one of the causative agents of post-transfusion non-A, non-B hepatitis is a small enveloped virus, such as a togavirus or flavivirus (Bradley et al., 1983). This assumption was derived mainly from chimpanzee infection experiments showing that the infectious agent has an envelope containing lipid and is less than 80 nm in diameter (Bradley et al., 1983, 1985; Feinstone et al., 1983). It has been shown also that the buoyant density of the infectious particles in sucrose is 1.09 to 1.11 g/ml (D. W. Bradley, personal communication).

Although HCV has yet to be visualized by electron microscopy, it is possible to detect a small amount of HCV in plasma or serum samples using a polymerase chain reaction (PCR) method (Saiki et al., 1988; Kubo et al., 1989; Weiner et al., 1990). Testing of serial 10-fold dilutions of samples by a reverse transcriptase PCR method enabled us to determine the amount of HCV particles semi-quantitatively. In this study, we determined the amount of HCV particles in samples before and after filtration through cellulose fibres with different pore sizes.

HCV was filtered through the newly developed microporous regenerated cellulose fibre, a hollow fibre type referred to as Bemberg Microporous Membrane (BMM; Asahi Chemical Industry), which is designed so that the adsorption of protein is minimal (Manabe et al., 1989; Tsurumi et al., 1990). A detailed method for the preparation of BMM of specific pore size has been
described (Manabe et al., 1986). By comparing the infectious titre before and after filtration through various BMMs of different pore sizes, the diameter of Japanese encephalitis (JE) virus has been determined to be precisely 43 nm (G. Ishikawa, T. Hirasaki, S.-I. Manabe, S.-I. Uematsu & N. Yamamoto, unpublished data) and the equation relating rejection coefficient (RC) values to virus particle size was constructed. On the basis of these data, hepatitis B virus (Dane particles) was removed efficiently from contaminated plasma by filtration through BMM fibres with a pore size of 40 nm (Sekiguchi et al., 1989), and human immunodeficiency virus by filtration through those with a pore size smaller than 105 nm (Hamamoto et al., 1989).

For HCV filtration studies, we first selected plasma samples which were thought to contain a large number of HCV particles, although we did not know the infectivity of these sources in chimpanzees. Donated blood samples, which were unsuitable for transfusion because they were positive for antibody to the non-structural protein of HCV (anti-C100), were collected. During the course of this screening, we established a method to estimate the HCV titre using serial dilutions of samples and PCR. One of these samples (M-8) and pooled serum from a post-transfusion hepatitis C patient (SK) were used. All samples were stored at -80 °C until use.

Cellulose hollow fibres, regenerated using cuprammonium [Cu(NH3)4]2+ with mean pore sizes of 20, 35, 40, 50 and 75 nm were prepared. The mean pore sizes were determined using the water filtration method (Tsurumi et al., 1989, 1990) and the logarithmic RC (RC = log N0 - log Nf, where N0 and Nf are the amounts of particle before and after filtration, respectively), which was determined by filtration of gold colloid particles of known diameter, determined by electron microscopy, through BMM with known pore sizes. For example, RC values for 30 nm gold particles were 2.70, 1.95, 0.85 and 0.0 through BMM 35, BMM 40, BMM 50 and BMM 75, respectively. The pore size of each BMM used was calibrated by determining the RC values of gold colloid particles (Manabe et al., 1986). The cross-sectional area of a single hollow fibre was 0.6 cm² and a single hollow fibre or a bundle of 180 fibres was used for filtration, which was carried out under a constant transmembrane pressure of 26664.4 Pa at 25 °C. The volume of the filtered samples was less than 300 µl per cm². Duplicate filtrations were done with fibres of the same pore size.

After filtration, serial 10-fold dilutions of the sample before filtration and of the filtrate were made with HCV-free plasma. RNA was extracted from each diluted sample by the guanidinium thiocyanate method (Chomczynski & Sacchi, 1987) and PCR was performed in duplicate as described previously (Kubo et al., 1989; Yoneyama et al., 1990) using the Perkin Elmer Cetus kit.

Each RNA sample, obtained from 50 µl of diluted sample, was used for cDNA synthesis with 10 units of reverse transcriptase (Bio-Rad) and an antisense primer (J513A; 5′ CGTATGAGACACTTCCACAT 3′). After the addition of a sense primer (J469S; 5′ GTCACTCAGCGGTCGATT 3′), the cDNA was amplified by 30 cycles of PCR, producing the expected 440 bp fragment from within the NS3 region, as described previously (Kubo et al., 1989). A one-tenth volume of the first-step PCR product was then amplified by an additional 30 cycles of PCR after the addition of a sense primer (J486S; 5′ GTCTATGTGAGTGTTATGA 3′) and an antisense primer (J509A; 5′ GCTGTGTGCTAGCTACAGGTA 3′), producing the expected 230 bp product. Amplified DNA was separated electrophoretically on a 2% agarose gel and stained with ethidium bromide. The amount of HCV particles (N) was expressed as the exponent of the 10-fold dilution where 50% of samples showed positive results (50% endpoint titre); HCV RNA in samples M-8 and SK could be detected up to a dilution of 10⁻³ to 10⁻⁴ per 50 µl by this method.

Fig. 1 shows the relationship between the logarithmic RC values for HCV particles and the average filtration rate, and fibre pore size; RC values decreased and the filtration rate increased with increasing mean pore size. HCV in the filtrate was below the level of detection when the pore size was 20 nm. When fibres with a mean pore size greater than 35 nm were used, HCV RNA was detected in the filtrate with an RC value of 4-0; values were 4-0 ± 0.5, 3-5 ± 0.5, 1-5 ± 0.5 and 1-5 ± 0.5 when the mean pore size was 35 nm, 40 nm, 50 nm and 75 nm, respectively. From these RC values, we estimated the size of HCV particles using the following equations.

![Fig. 1. Relationship between the log RC of HCV (○) and the average filtration rate (O), and the mean pore size (nm). Virus concentrations before and after filtration were determined by PCR.](image-url)
deduced previously from RC values of infectivity assays on JE virus (G. Ishikawa, T. Hirasaki, S.-I. Manabe, S.-I. Uematsu & N. Yamamoto, unpublished data); for BMM 35, RC = 0.158P - 1.11; for BMM 40, RC = 0.071P - 0.78; for BMM 50, RC = 0.071P - 0.78; for BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm). Equations have not been derived for BMM 20 and BMM 30. Applying these equations to BMM 75, RC = 0.067P - 1.10, where P is the mean particle diameter (nm).

### Table 1. Double-step filtration of HCV-positive specimens using BMM

<table>
<thead>
<tr>
<th>BMM mean pore size (nm)</th>
<th>1st filtrate</th>
<th>2nd filtrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCV titre*</td>
<td>RC</td>
</tr>
<tr>
<td>SK (serum)</td>
<td>1.3 ± 0.5</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>35</td>
<td>1.8 ± 0.5</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-8 (plasma)</td>
<td>3.5 ± 0.5</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>35</td>
<td>2.3 ± 0.5</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>40</td>
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</tbody>
</table>

* The HCV titre is expressed as the exponent of the 10-fold dilution giving the 50% endpoint titre. The HCV titres in samples SK and M-8 before filtration were 4.8 ± 0.5 and 5.8 ± 0.5, respectively.

† RNA samples obtained from 1300 μl of the filtrate were used for PCR.

Finally, but most importantly, we have shown that it is possible to remove HCV particles from plasma or serum using BMM fibres of appropriate pore size. As shown in Table 1, double filtration using a fibre with a pore size of 35 nm can reduce HCV to levels undetectable by our PCR method. Since important blood factors such as albumin or globulin can pass through BMM fibres with a pore size of 35 nm, this may be an efficient and safe method of eliminating HCV. At a transmembrane pressure of 26664.4 Pa, 100 ml of serum can be filtered through BMM 35 within 30 min.

We thank Dr D. W. Bradley for providing his experimental results before publication and for his critical review of the manuscript. We thank Dr Y. Watanabe for providing serum from a post-transfusion hepatitis C patient. We also thank Drs H. Yoshikura, K. Komuro, I. Saito and H. Shimojo for helpful discussions. This study was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan for the Comprehensive 10-year Strategy for Cancer Control.

### References


