Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection

I Castillo, E Rodríguez-Iniáigo, J Bartolomé, S de Lucas, N Ortíz-Movilla, J M López-Alcorocho, M Pardo, V Carreño

Background: Occult hepatitis C virus (HCV) infection is characterised by the presence of HCV-RNA in the liver in the absence of anti-HCV and serum viral RNA. Up to 70% of these patients also have HCV-RNA in peripheral blood mononuclear cells (PBMC) but it is not known if HCV is replicating in these cells.

Aim: We studied possible HCV replication in PBMC of 18 patients with an occult HCV infection who were selected on the basis of HCV-RNA positivity in PBMC.

Methods: Detection of HCV-RNA positive and negative strands in PBMC was done by strand specific reverse transcriptase-polymerase chain reaction (RT-PCR) and by in situ hybridisation.

Results: The presence of HCV-RNA positive strand in PBMC was confirmed in all patients by strand specific RT-PCR and by in situ hybridisation. Mean percentage of PBMC which had the HCV-RNA positive strand was 3.3% (95% confidence interval (CI) 2.1–4.4). The HCV-RNA negative strand was found in the PBMC of 11/18 (61%) patients by strand specific RT-PCR and confirmed by in situ hybridisation, and the percentage of PBMC harbouring the HCV-RNA negative strand was 3.1% (95% CI 0.8–5.5). There was a significant correlation (ρ = 0.001, r = 0.84) between the percentage of PBMC with the HCV-RNA positive strand and that of PBMC with the HCV-RNA negative strand.

Conclusion: HCV replicates in the PBMC of patients with occult HCV infection and thus, although these patients do not have serum HCV-RNA, they could be potentially infectious.
Detection of HCV-RNA positive and negative strands in PBMC by fluorescent in situ hybridisation

The HCV-RNA positive strand was detected using a cRNA probe of negative polarity obtained by in vitro transcription of the pC5’NCR in the presence of digoxigenin-11-UTP (Roche Molecular Biochemicals, Indianapolis, Indiana, USA).

Detection of HCV-RNA negative strand was performed with a complementary digoxigenin labelled cRNA probe spanning 390 nucleotides of the HCV core coding region, obtained by in vitro transcription of the pCore plasmid.

For fluorescent in situ hybridisation (FISH), PBMC were resuspended in KCl (0.075 M) for 10 minutes at 37°C, pelleted at 500 g for five minutes, and resuspended in 15 μl of phosphate buffered saline. Resuspended cells were mixed with 35 μl of low melting point agarose maintained at 37°C, deposited onto a glass slide (that was previously precoated with 0.65% standard agarose and dried at 80°C), covered with a cover slip, and allowed to solidify at 4°C. Finally, coverslips were gently removed by immersing the slides in phosphate buffered saline at room temperature. Hybridisation with both HCV-RNA riboprobes was carried out at 42°C for 16 hours in a humid chamber. After hybridisation, samples were washed at 42°C in 2×SSC, 0.5×SSC, and 0.1×SSC (15 minutes each) (20×SSC: 3 M NaCl, 3 mM trisodium citrate). Digoxigenin labelled hybrids were detected with fluorescein isothiocyanate conjugate (Roche Molecular Biochemicals). Signal intensity was amplified using the Fluorescent Antibody Enhancer set for DIG detection kit (Roche Molecular Biochemicals). Cells were counterstained with 4-6 diamidino-2-phenylindole. Fluorescent signals were observed using a Nikon Eclipse E-400 light-microscope (Nikon Co., Tokyo, Japan) and the images were captured with a high resolution monochrome CCD camera (DIC-N; Wared Precision Instruments, Cambridge, UK). At least 2000 cells per sample were counted to obtain the percentage of positive cells.

Table 1 Characteristics of the patients with occult hepatitis C virus (HCV) infection included in the study

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>13/5</td>
</tr>
<tr>
<td>Age (y)</td>
<td>45.7 (41.0–50.3)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.3 (24.2–26.5)</td>
</tr>
<tr>
<td>Estimated duration of abnormal liver function tests (months)</td>
<td>35.5 (14.3–56.7)</td>
</tr>
<tr>
<td>Values are mean (95% confidence interval of the mean) or number.</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis

Data analysis was performed using SPSS version 9.0 for windows (Chicago, Illinois, USA). Normality and homocedasticity of continuous variables were tested by the Kolmogorov-Smirnov and Levene's tests, respectively. The mean was compared with the Student's *t* (variables with normal distribution) or with the Mann-Whitney U test (variables with non-normal distribution). Categorical variables were compared using the χ² or Fisher's exact tests. Pearson (variables with Gaussian distribution) or Spearman (non-normally distributed variables) correlation coefficients were determined to study the existence of correlation between the variables. All tests performed were two sided and statistical significance was considered at a *p* value <0.05.

RESULTS

To assess the sensitivity and specificity of our strand specific RT-PCR assay, serial dilutions of synthetic HCV-RNA positive and negative strands were used as RNA templates. The analysis showed that the positive strand assay detected the HCV-RNA positive strand at 0.1 fg and the HCV-RNA negative strand at 10 pg (fig 1A). Thus, discrimination was 100 000-fold. Similarly, the negative strand assay detected the HCV-RNA negative strand at 1 fg while the RNA positive strand was detected at 10 pg (fig 1B), resulting in a 10 000-fold discrimination.

The results obtained by strand specific RT-PCR assay as well as by the FISH technique confirmed that all 18 patients with occult HCV infection had the HCV-RNA positive strand in their PBMC. In contrast, PBMC from six healthy donors were negative. Mean percentage of PBMC showing fluorescent signals for the HCV-RNA positive strand was 3.3% (95% confidence interval (CI) 2.1–4.4).

When studying the presence of the RNA negative strand in PBMC, we found that 11/18 (61%) patients were positive by the strand specific RT-PCR. The existence of the HCV-RNA negative strand was confirmed by FISH in these 11 cases while no hybridisation signals were seen in the PBMC of the
remaining seven patients. PBMC samples from the healthy donors were negative for the RNA negative strand by both techniques. In 11 patients with occult HCV infection, mean percentage of PBMC with a positive hybridisation signal for the HCV-RNA negative strand was 3.1% (95% CI 0.8–5.5).

When patients were divided according to the status of HCV replication in their PBMC, we found that the mean percentage of PBMC cells harbouring the HCV-RNA positive strand was significantly higher ($p = 0.009$) in those patients with HCV replication in PBMC (mean 4.2% (95% CI 2.5–5.9)) than in those without HCV replication (mean 1.8% (95% CI 1.3–2.2)). No other differences were found between the groups of patients (table 2).

In contrast, there was a significant correlation ($p = 0.001$, $r = 0.84$) between the percentage of PBMC with the HCV-RNA positive strand and that of PBMC with the HCV-RNA negative strand was significantly higher ($p = 0.009$) in those patients with HCV replication in PBMC (mean 4.2% (95% CI 2.5–5.9)) than in those without HCV replication (mean 1.8% (95% CI 1.3–2.2)). No other differences were found between the groups of patients (table 2).

![Figure 1](https://www.gutjnl.com)

**Figure 1** Specificity and sensitivity analysis of the strand specific reverse transcriptase-polymerase chain reaction using synthetic hepatitis C virus (HCV)-RNA. (A) Positive strand RNA assay of HCV-RNA positive strand (0.1–100 fg) and HCV-RNA negative strand (1–100 pg). Lane N, negative control; lane M, 100 bp DNA ladder. (B) Negative strand RNA assay of HCV-RNA negative strand (0.1–100 fg) and HCV-RNA positive strand (1–100 pg). Lane N, negative control; lane M, 100 bp DNA ladder.

### Table 2

Comparison between patients with and without hepatitis C virus (HCV)-RNA negative strand in their peripheral blood mononuclear cells (PBMC)

<table>
<thead>
<tr>
<th></th>
<th>HCV replication in PBMC</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>No of patients</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>8/3</td>
<td>5/2</td>
</tr>
<tr>
<td>Age (y)</td>
<td>46.5 (40.1–52.8)</td>
<td>44.4 (35.4–53.5)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.0 (24.2–27.7)</td>
<td>24.3 (24.3–25.3)</td>
</tr>
<tr>
<td>Estimated duration of abnormal liver function tests (months)</td>
<td>41.4 (6.9–75.8)</td>
<td>26.3 (3.5–49.0)</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>43.7 (21.4–66.0)</td>
<td>25.7 (18.6–32.8)</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>61.3 (22.8–99.8)</td>
<td>43.1 (20.3–66.0)</td>
</tr>
<tr>
<td>GGTP (IU/l)</td>
<td>147.4 (69.6–225.2)</td>
<td>68.0 (39.5–96.5)</td>
</tr>
<tr>
<td>Liver</td>
<td>3.6 (1.0–6.2)</td>
<td>3.9 (0.9–7.0)</td>
</tr>
<tr>
<td>HCV-RNA positive strand (%)</td>
<td>2.2 (0.3–4.0)</td>
<td>2.3 (1.4–3.3)</td>
</tr>
<tr>
<td>PBMC HCV-RNA positive strand (%)</td>
<td>4.2 (2.5–5.9)</td>
<td>1.8 (1.3–2.2)</td>
</tr>
</tbody>
</table>

Values are mean (95% confidence interval of the mean) or number. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGTP, gamma-glutamyl transpeptidase.
Whether this lack of association reflects infection with HCV-RNA positive and negative strands. The specificity of HCV-RNA negative strand detection in PBMC and incorrect strands of RNA. These differentials confirm the existence of HCV-RNA negative strand detection in PBMC as in vivo the HCV-RNA positive-negative ratio is 10–100-fold. Detection of the HCV-RNA positive strand by RT-PCR and FISH confirmed the existence of HCV infection in the PBMC of the 18 patients with occult HCV infection included in the study. Analysis of the HCV-RNA negative strand showed that HCV was replicating in the PBMC of the majority (61%) of patients with occult HCV infection, with concordant results between the two different techniques. This percentage of HCV replication in PBMC of patients with occult HCV infection is similar to that reported in PBMC of patients with chronic hepatitis C.

The existence of HCV infection and replication in PBMC in the absence of detectable serum HCV-RNA has also been described in anti-HCV positive patients years after spontaneous or antiviral therapy induced clearance of serum viral RNA and normalisation of transaminases. As there is no evidence of persistent HCV-RNA negative strand replication in PBMC of patients with occult HCV infection, it could be assumed that HCV infection and replication in PBMC may be related to the status of intrahepatic HCV-RNA. However, in these patients with occult HCV infection, we did not find any correlation between the percentage of hepatocyte showing the HCV-RNA positive or negative strand and these percentages in PBMC. Moreover, the existence of HCV replication in PBMC was not related to the presence of HCV replication in the liver. Whether this lack of association reflects infection with HCV variants of different tropisms or with different replication capacities is currently under investigation.

In contrast, the percentage of PBMC harbouring the HCV-RNA positive and negative strands were highly correlated with each other \( r = 0.84 \); \( p = 0.001 \) and the mean percentage of infected PBMC was significantly higher \( p = 0.009 \) in patients with HCV replication in PBMC than in those without HCV replication. Taking into account that if replication occurs the HCV-RNA positive-negative ratio is within 10–100-fold, perhaps in these negative cases HCV replication is taking place but at such a low level that detection is beyond the sensitivity of the assay.

In conclusion, our findings indicate that HCV replicates in the PBMC of patients with occult HCV infection. As it has been shown that HCV can propagate in lymphoid cell cultures and that the virus derived is infectious, it should be determined whether these patients (although they do not have detectable circulating viruses) are potentially infectious.

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Conflict of interest: None declared.

REFERENCES

1. Miller RH, Purcell RH. Hepatitis C virus shares amine acid sequence similarity with pestiviruses and flaviviruses, as well as members of two plant virus supergroups. Proc Natl Acad Sci U S A 1987;84:5075–61.
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