Peripheral Blood Mononuclear Cells Serve as a Possible Extrahepatic Site for Hepatitis C Virus Replication

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Hepatitis C virus (HCV) is thought to have a strong tropism for hepatic tissue but also replicative intermediates are found in extrahepatic tissues suggesting active viral replication in these cells. The aim of the study is to confirm that HCV can infect and replicate in the peripheral blood mononuclear cells (PBMCs) as one of the possible sites of extrahepatic hepatitis C virus replication during the natural course of infection which may serve as a source of virions causing reinfection. Reverse transcriptase Polymerase chain reaction (RT-PCR) for detection of positive and negative strands of HCV-RNA in PBMCs was done for 30 patients with chronic HCV infection admitted to Ain Shams University Hospitals and the results were compared with those from 20 healthy controls matched in age and sex. Our results revealed statistically significant association between both HCV-RNA positive strand and negative strand and both serum HCV-RNA and HCV-Antibody (Ab) \( (P<0.01) \). Moreover the association between the presence of HCV positive- and negative-strand RNA in PBMCs was statistically significant \( (P<0.01) \). Finally the correlation between HCV positive and negative stranded RNA in PBMCs and extrahepatic complications was statistically significant \( (P<0.01) \). In conclusion our results support previous observations indicating that HCV can infect PBMCs. This is reflected by a higher frequency of extrahepatic complications and diseases associated with chronic HCV infection.

Hepatitis C virus (HCV) is responsible for most cases of post-transfusion hepatitis (Alter & Seeff, 2000) with a chronic course in up to 60% of individuals, leading to cirrhosis and eventual hepatocellular carcinoma in 20% of individuals (Trends, 2004). The liver is the main site of virus replication but it can also replicate at extrahepatic sites such as peripheral blood mononuclear cells (PBMCs) (Change et al., 1996). It was suggested that PBMCs could be a site for viral replication of HCV during the natural course of infection and may represent a reservoir for hepatitis C virions. In addition, it has been proposed that PBMCs could be the source of recurrent HCV infection after liver transplantation (Laskus et al., 2007). It is assumed that virus replications involve the synthesis of a negative strand RNA molecule that acts as a template for production of positive strand or genomic HCV-RNA (Clarke, 1997). Thus detection of HCV RNA positive strand confirm the existence of HCV infection but detection of HCV-RNA negative strand is indicative of viral replication (Wu et al., 1999; Castillo et al., 2006). HCV can be detected in the serum and liver tissue using reverse transcriptase and subsequent amplification of the cDNA by polymerase chain reaction (PCR). Strand-specific oligonucleotide primers were used in the reverse transcriptase reaction for detection of genomic viral RNA and the presence of minus-stranded RNA of HCV in serum, liver tissue, PBMCs and different lymphocyte sub-populations from chronically HCV-infected patients (Nishguchi et al., 2003; Castillo et al., 2006). It was found that the detection of viral RNA in PBMCs is related to a chronic carrier status. However, it is not known at which time extrahepatic cells such as PBMCs are infected and whether they stay permanently infected (Muller et al., 1993). The aim of the present study is to confirm that HCV can infect and replicate in PBMCs as
one of the possible sites of extrahepatic hepatitis C virus replication during the natural course of infection which may serve as a source of virions causing reinfection.

**Subjects and Methods**

**Subjects**
This study was conducted on 30 patients with chronic HCV infection admitted to Ain Shams University Hospitals. Patients included in this study were divided into two groups: The first group (21 patients) was positive for HCV-Ab by ELISA and positive for HCV RNA in the serum by Real time PCR (Stratagene’s Real time PCR Mx3000P). The second group (9 patients) was positive for HCV-Ab by ELISA and negative for HCV RNA in the serum by Real time PCR.

Twenty healthy controls matched for age and sex, were also included in this study.

**Methods**
All patients included in this study were subjected to the following:
- Full history taking and thorough clinical examinations.
- ALT and AST for each subject were done as a routine investigation.

**Analytical Methods**
A venous blood sample of 10 ml were drawn, from each participant, under aseptic condition. Of these, aliquots of 5 ml were collected into vacutainer tube with no additive, centrifuged and the sera were collected and stored at -20°C for assessment of anti-HCV antibodies by ELISA using ORTHO 3rd generation ELISA kits (ORTHO Clinical Diagnostic, inc., a Johnson & Johnson Company, Raitan, New Jersey 08869 USA).

The other 5 ml were drawn into heparinized tube for peripheral blood mononuclear cell (PBMC) separation prior to be subjected to the RT-PCR assay.

**RT-PCR (reverse transcriptase or conventional PCR) procedure**
- **Sample Preparation**
  A) Separation of PBML: Peripheral blood mononuclear cells were separated as a layer (PBML) by the Ficoll-Hypaque sedimentation gradient method, and then the cells were suspended in 500μl phosphate buffer saline (PBS). RNA was extracted in the same day of PBML separation.

B) RNA extraction was done using the RNA Isolation Kit (SV Total RNA Isolation System, Promega) according to the manufacturer’s instructions.

**Reverse Transcription of the Target RNA to Generate Complementary DNA (cDNA) was Performed Using the Fermentas kits (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas) According to the Manufacturer’s Instructions**

**Procedure**
Two separate test tubes were used for each sample. The first tube was used for synthesis of +ve HCV strand. The second tube was used for the –ve HCV strand. The same protocol was used for both except for the primers. Briefly, 5μl of the RNA extract and 3μl (50 pmole) of antisense oligonucleotide primer (primer 2 for synthesis of +ve strand and primer 1 for synthesis of -ve strand) where denatured at 65 °C for 5 min then annealed at 37 °C for 5 min, then transcribed to cDNA utilizing Revert Aid<sup>™</sup> M-MuLV Reverse Transcriptase enzyme (Roche Molecular System, USA) after incubation for 60min at 42°C using a Perkin-Elmer thermal cycler (Perkin-Elmer Corporation, Gene Amp PCR System 9700). The reaction was stopped by heating at 90°C for 7min.

**PCR Amplification of Target cDNA Using HCV Specific Complementary Primers**
PCR amplification of target cDNA was done by adding 5 μl of Taq Master Mix (Taq Master/high yield Ready-to-use Master Mix of thermostable DNA polymerase LAROVA, Germany) and 1.5μl from each primer 1 & 2 (Table 1) to 5 μl of the cDNA in each reaction tube. The PCR was performed according to the following protocol: denaturation at 95°C for 1 min, primer annealing at 50°C for 1min and extension at 72°C for 3 min, then final elongation at 72°C for 2 min. This process was repeated for 35 cycles. A second PCR with nested primers was performed using the same protocol as described above with substitution of primer1, 2 by primer 3 and 4 (Table 1). Finally the PCR products (amplicons) were separated on a 1% agarose gel and revealed under ultraviolet light after staining with ethidium bromide and compared to 100 bp DNA ladder marker (BIORON, Germany) (Figure 1).

**Statistical Analysis**
SPSS statistical software package (V. 17.0, Echosoft Corp., USA, 2008) was used for data analysis. The following tests were done: Comparison between qualitative variables was carried out by using Chi
square test. Correlation between two quantitative variables was carried out by Ranked Sperman correlation test. The probability of error at $\leq 0.05$ was considered significant.

Table 1. Nested primer sets used for amplification of HCV RNA

<table>
<thead>
<tr>
<th>No.</th>
<th>Sense</th>
<th>Position</th>
<th>Sequence (5'→3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>14 to 33</td>
<td>TGGGGGCAGACACTCCACC</td>
<td>576</td>
</tr>
<tr>
<td>2</td>
<td>_</td>
<td>567 to 590</td>
<td>CCAAGGTACCCGGCTGAGCCA</td>
<td></td>
</tr>
<tr>
<td>3 (1N)</td>
<td>+</td>
<td>59 to 82</td>
<td>TGTCTTCAGCAGAAGCGTGCTG</td>
<td>355</td>
</tr>
<tr>
<td>4 (1N1)</td>
<td>_</td>
<td>394 to 414</td>
<td>GGAACCTGACGTCCTGTCGGC</td>
<td></td>
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</tbody>
</table>

All primers were diluted with distilled water to give a final concentration of 100 pmole/µl.

Figure 1. (a): Ethidium bromide stained gel showing the PCR products from genomic HCV RNA (positive- and negative-strand) obtained with RT-PCR derived from PBMNCs RNA of several anti-HCV positive patients, lane (1-10) which represent sample no. 2, 5, 11, 24 and 29. Lane (11): Negative anti-HCV (control). (b): Ethidium bromide stained gel showing the PCR product from genomic HCV RNA (positive- and negative-strand) obtained with nested PCR derived from PBMNCs RNA of several anti-HCV positive patients, lane (2,3,4,5,7,8,9,10) which represent sample no.6, 2, 27, and 22. Lane (1, 6, 11): Negative anti-HCV (control), Lane L: 100bp DNA ladder, C: control.
Results

The results of the present study are shown in tables (2 & 3) and figures (2 & 3). Concerning the presence of HCV positive-strand RNA in PBMCs in relation to serum HCV-RNA and HCV-Ab, our results revealed a significant association between HCV-RNA positive strand and both serum HCV-RNA and HCV-Ab ($P<0.01$). On the other hand, HCV positive-strand RNA was not detected in the PBMCs in the 9 cases who were negative for HCV-RNA in the serum nor in the controls (Table 2).

<table>
<thead>
<tr>
<th>HCV-RNA PBMC +ve strand N (%)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive HCV Ab (total=30)</td>
<td></td>
</tr>
<tr>
<td>10(33.3%)</td>
<td>20(66.7%)</td>
</tr>
<tr>
<td>HCV-RNA +ve serum (total=21)</td>
<td></td>
</tr>
<tr>
<td>1(4.8%)</td>
<td>20(95.2%)</td>
</tr>
</tbody>
</table>

Similarly, HCV negative-strand RNA in PBMCs showed significant association with both serum HCV-RNA and HCV-Ab ($P<0.01$ & 0.05 respectively). Also we could not detect negative strand in the 9 patients with HCV-RNA negative serum nor in the controls (Table 3).

<table>
<thead>
<tr>
<th>HCV-RNA PBMC -ve strand N (%)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive HCV Ab (total=30)</td>
<td></td>
</tr>
<tr>
<td>23(76.7%)</td>
<td>7(23.3%)</td>
</tr>
<tr>
<td>HCV-RNA +ve serum (total=21)</td>
<td></td>
</tr>
<tr>
<td>14(66.7%)</td>
<td>7(33.3%)</td>
</tr>
</tbody>
</table>

There was a significant association ($P<0.01$) between the presence of HCV positive- and negative-strand RNA in PBMCs as shown in (Figure 2). Finally we investigated the prevalence of extrahepatic complications among HCV-RNA positive and negative strand positive cases in PBMCs. We observed a significant positive correlation between HCV positive and negative stranded RNA in PBMCs and extrahepatic complications ($P<0.01$) (Figure 3).
Figure 2. Association between HCV positive- and negative-strand RNA in PBMCs.

Figure 3. Prevalence of extrahepatic complications among HCV positive strand and negative strand in PBMCs.
Discussion

Although the mechanism of HCV replication is not fully understood, it is assumed that virus replication involves the synthesis of negative-strand RNA molecule that act as a template for the production of positive-strand or genomic HCV-RNA (Clarke, 1997). Thus detection of the HCV-RNA negative-strand is indicative of active viral replication.

The liver is the main site of HCV replication but it can also replicate at extrahepatic sites (Chang et al., 1996). In particular, PBMCs have been suggested to function as an important extrahepatic reservoir or as a possible site for extrahepatic HCV replication (Laskus et al., 2002). This assumption was based on the demonstration of negative-strand HCV-RNA, the replicative intermediate of HCV, in association with PBMCs from HCV infected patients (Mihm et al., 2001). Hence, it has been proposed that PBMCs could be the source of recurrent HCV infection after liver transplantation, as the reinfection of the graft is the rule (Castillo et al., 2006).

In the current study, we investigated HCV replication in PBMCs of patients with HCV infection, as a possible reservoir of virions causing reinfection, by detection of HCV RNA positive- and negative-strands.

Our results showed that HCV RNA positive-strand was found in PBMCs from 66.7% of the sero-positive chronic patients which indicated a significant association between the presence of HCV RNA positive-strand and the presence of HCV-Ab and serum HCV RNA in sero-positive chronically infected patients. ($P < 0.01$).

Also our results could detect the presence of negative-strand HCV RNA in the PBMCs from 7 out of the 30 sero-positive patients (23.3%). The presence of HCV negative-strand RNA was significantly associated with HCV-Ab positive serum from chronically infected patients ($P < 0.05$).

These results are in accordance with the observations of, Chang et al. (1996) and Gong et al. (2003). These findings suggest that the replication of HCV in PBMCs may play an important role in the process of chronicity. The mechanism could be that HCV in PBMCs can escape from clearance resulting from host immunity, and make the infection of HCV persistent. On the other hand, the dysfunction of the HCV infected PBMCs could lead to decline of the immune function or disorder, making it more difficult for the host to clear intrahepatic HCV, so that the injury of hepatocytes persists. A safe conclusion is that PBMCs could be the viral "reservoir" responsible for viral chronicity.

In the current study, the presence of both positive and negative-strand HCV-RNA was significant ($P<0.01$), as the HCV-RNA negative-strand was detected in PBMCs from 7 out of 20 patients (35%) whose PBMCs contained HCV-RNA positive-strand. On the other hand, no HCV-RNA negative-strand was found in patients negative for HCV-RNA positive-strand. Several studies, done by Falcón et al. (2005), Castillo et al. (2006), and Muratori et al. (2008), have reported similar findings.

A study done by Meier et al. (2001) was in contrast to our results. They concluded that the presence of HCV- RNA in PBMCs is probably compatible with passive virus adsorption via specific receptors endocytosis of the virus or with contamination by circulating virus but not due to the true virus replication and production. In their opinion, this would mean that PBMCs do not function as an extrahepatic reservoir for HCV when the diseased liver is explanted and in these cases, small amounts of HCV-RNA may still be present in the serum or attached to blood cells and be responsible for reinfection of the graft.
In the present study, we did not detect positive- and negative-stranded RNA in 10 sero-positive patients. Nine of them were negative for HCV-RNA in serum and only one was seropositive with HCV-RNA positive serum. This agrees with the study done by Kaiser et al. (2006) and Flavien et al. (2008), they found no evidence for cell-associated HCV RNA in aviremic seropositive subjects. Also they found strong association between the detection of plasma virus and the detection of PBMC-associated HCV RNA. This opinion was supported by Crovatto et al. (2000) who evaluated viral replication in PBMCs.

On the other hand, these results were in contrast with that studies done by Falcón et al. (2005) and Castillo et al. (2006), who described the existence of HCV infection and replication in PBMCs in the absence of detectable serum HCV-RNA. However, it has to be taken in consideration that the difference between these results and our results may be attributed to the different techniques used.

Finally our results showed a significant association between the presence of HCV negative-stranded RNA in PBMCs as an evidence for extrahepatic replication and extrahepatic complications ($P<0.01$). Among the 7 patients positive for both positive- and negative-strand RNA, there was 5 patients had extrahepatic complications (71.4%) including mixed cryoglobulinaemia, glomerulonephritis and neuropathy.

Our results are in accordance with others who found association between HCV RNA negative and positive strand and mixed cryoglobulinaemia (Disdier et al., 1995), glomerulonephritis, (Cacoub et al., 2005) and neurological manifestation (Ramos et al., 2006).

However, Ferri et al. (2004) found that in a 30% of mixed cryoglobulinaemia patients, HCV infection has also been found without any clinical evidence of liver damage. Their observation suggested that mixed cryoglobulinaemia was not simply an extrahepatic manifestation of HCV liver infection, but primarily a systemic lymph proliferative disorder. Also, Zignego et al. (1998) showed that HCV is both a hepatotropic and lymphotropic virus. On this basis, they could suppose that in mixed cryoglobulinaemia patients, chronic HCV infection could be responsible for the lymphoproliferative disorder undergoing clinical manifestations of the disease as HCV infection in mixed cryoglobulinaemia may produce a monoclonal or polyclonal B-cell expansion.

In conclusion, our results support previous observations indicating that HCV can infect PBMCs. This is reflected by a higher frequency of extrahepatic complications and diseases associated with chronic HCV infection. Thus, PBMCs not only act as possible sites of extrahepatic replication but can also serve as a source for virions causing reinfection of hepatocytes. Additional studies are needed to identify the response of the patients with HCV RNA negative-strand to interferon both biochemically and virologically in comparison with those without negative-strand. Detection of HCV replication in PBMCs in occult HCV infection by using other assay methods as FISH would be another avenue of required research.

References


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