HCV-RNA positivity in peripheral blood mononuclear cells of patients with chronic HCV infection: does it really mean viral replication?

Volker Meier, Sabine Mihm, Perdita Wietzke Braun and Guliano Ramadori

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Abstract

AIM  To analyze the association of HCV-RNA with peripheral blood mononuclear cells (PBMC) and to answer the question whether HCV-RNA positivity in PBMC is due to viral replication.

METHODS  HCV-RNA was monitored in serum and PBMC preparations from 15 patients with chronic HCV infection before, during and after an IFN-α therapy using a nested RT/PCR technique. In a second approach, PBMC from healthy donors were incubated in HCV positive plasma.

RESULTS  In the IFN-α responding patients, HCV-RNA disappeared first from total RNA preparations of PBMC and then from serum. In contrast, in relapsing patients, HCV-RNA reappeared first in serum and then in PBMC. A quantitative analysis of the HCV-RNA concentration in serum was performed before and after transition from detectable to non detectable HCV-RNA in PBMC-RNA and vice versa. When HCV-RNA was detectable in PBMC preparations, the HCV concentration in serum was significantly higher than the serum HCV-RNA concentration when HCV-RNA in PBMC was not detectable. Furthermore, at no time during the observation period was HCV specific RNA observed in PBMC, if HCV-RNA in serum was under the detection limit. Incubation of PBMC from healthy donors with several dilutions of HCV positive plasma for two hours showed a concentration dependent PCR positivity for HCV-RNA in reisolated PBMC.

CONCLUSION  The detectability of HCV-RNA in total RNA from PBMC seems to depend on the HCV concentration in serum. Contamination or passive adsorption by circulating virus could be the reason for detection of HCV-RNA in PBMC preparations of chronically infected patients.

INTRODUCTION  Hepatitis C virus (HCV) is an enveloped singlestranded positive-strand RNA virus of the Flaviviridae family[1]. It is the major agent responsible for parenterally transmitted non-A, non-B hepatitis[2]. Acute infection with HCV is often clinically asymptomatic and the majority of patients develop a chronic hepatitis[3]. Treatment with interferon α (IFN-α) in combination with ribavirin, a synthetic guanosine analogue, is now the regimen of choice for patients chronically infected with HCV[4,5]. During drug administration, HCV-RNA may disappear both in serum and in peripheral blood mononuclear cells (PBMC)[6], but the proportion of patients responding to this therapy in terms of a sustained virological response varied around 40%. About 15%-20% of HCV infected patients progress to end-stage liver disease with cirrhosis and also hepatocellular carcinoma[7-10]. After orthotopic liver transplantation, reinfection of the graft with HCV is the rule[11,12]. It has been hypothesized that virus replication takes place at extrahepatic sites. Possible sites are the different cell fractions (PBMC, granulocytes or red blood cells/pellets) of the peripheral blood[13,14] or tissues like lymph nodes, pancreas, adrenal gland thyroid, bone marrow or spleen[15]. In particular, PBMCs have been suggested to function as an important extrahepatic reservoir or as a possible site for extrahepatic HCV replication[16-19]. This assumption was based on the demonstration of negative-strand HCV-RNA, the replicative intermediate of HCV, in association with PBMC from HCV infected patients in whose sera negative-strand HCV-RNA was not detected.
of negative-strand HCV-RNA in extrahepatic compartments based on PCR detection assays has been suggested in many reports with very significantly different detection rates (from 0% to 100%). An extensive artefactual detection of negative-strand HCV-RNA due to self-priming and mispriming events is possibly responsible for these differences. This is especially true when a 5'-noncoding-region primer pair is used\[20,21\].

Other authors have also shown that the presence of HCV sequences in PBMC is compatible with passive virus adsorption via specific receptors or with contamination by circulating virus\[22,23\]. HCV has been found to bind to low density lipoproteins (LDL) and therefore, enter the PBMC via LDL-receptor\[24-26\]. Pileri and colleagues demonstrated the binding of HCV to CD 81, a 25-kD molecule of the tetraspanin superfamily expressed in various cell types including hepatocytes and B-lymphocytes\[27\].

The present study aims to analyse the association of HCV-RNA with PBMC and to answer the question whether HCV-RNA positivity in PBMC is really due to viral replication. Therefore, HCV-RNA detectability was serially monitored in both the serum and PBMC preparations from 15 patients with chronic HCV infection before, during and after IFN-α therapy. In a second approach, PBMC from healthy donors were incubated for a short time in HCV-RNA positive plasma to investigate the possibility of an attachment of HCV to PBMC.

**Materials and Methods**

**Patients**

A total of 15 patients (8 women and 7 men; mean age 57.0 years; age range 41-79 years) (Table 1) infected with HCV as diagnosed by the presence of anti-HCV antibodies and HCV-RNA in serum were studied consecutively. All were anti-HCV and HCV-RNA positive for at least six months. The degree of the liver injury was estimated histopathologically according to established criteria\[28,29\]. Patients with active hepatitis B virus or human immunodeficiency virus infection and those with continued alcohol or drug abuse were excluded. The patients received an IFN-α therapy (3 × 10⁶ to 6 × 10⁶ IU IFN-α₂a three times weekly to 6 × 10⁶ IU IFN-α₂a daily, Roferon A, Hoffmann La Roche, Basel, Switzerland; doses were adapted individually based on well-being and response parameters) over a period of 6 to 17 months. Blood samples were taken twice before the therapy, monthly during therapy and after therapy. Serum samples and PBMC preparations were stored at -80°C. Additionally, blood samples were collected from three healthy donors (VM, NN and BS) for isolation of HCV negative PBMC and from three untreated patients (EZ, SG and UP) with chronic HCV infection for isolation of HCV positive plasma. The study was approved by the local ethics committee of the Georg-August-University, Göttingen, Germany.

**Table 1 Clinical, virological and therapeutic parameters of 12 patients with chronic HCV infection and treated with IFN-α₂a**

| Patient | Age  | Sex | Genotype | ALT  | Therapy                  | Duration | Response |
|---------|------|-----|----------|------|--------------------------|----------|----------|
| 1       | 60   | F   | 1b       | 30   | 3 × 10⁶ IU/week           | 18       | Respond  |
| 2       | 79   | M   | n.d.     | n.d. | 2 × 3 × 10⁶ IU/week       | 19       | Respond  |
| 3       | 51   | M   | 1a       | 34   | 3 × 10⁶ IU/week           | 6        | Relaps   |
| 4       | 62   | M   | 1b       | 36   | 7 × 6 × 10⁶ IU/week       | 6        | Relaps   |
| 5       | 41   | M   | 1b       | 40   | 3 × 3 × 10⁶ IU/week       | 17       | Respond  |
| 6       | 63   | M   | 1b       | 46   | 3 × 6 × 10⁶ IU/week       | 14       | Relaps   |
| 7       | 61   | M   | 1b       | 45   | 7 × 6 × 10⁶ IU/week       | 6        | Respond  |
| 8       | 48   | F   | 1a/1b    | 36   | 3 × 3 × 10⁶ IU/week       | 7        | Relaps   |
| 9       | 63   | F   | 1b       | 20   | 3 × 10⁶ IU/week           | 12       | Relaps   |
| 10      | 60   | M   | 1b       | 36   | 3 × 6 × 10⁶ IU/week       | 12       | Relaps   |
| 11      | 53   | M   | n.d.     | 92   | 3 × 9 × 10⁶ IU/week       | 12       | Respond  |
| 12      | 43   | F   | 1a       | 160  | 3 × 6 × 10⁶ IU/week       | 12       | Relaps   |
| 13      | 53   | F   | 1b       | 9    | 3 × 6 × 10⁶ IU/week       | 12       | Relaps   |
| 14      | 69   | F   | 1b       | 22   | 3 × 6 × 10⁶ IU/week       | 12       | Relaps   |
| 15      | 63   | M   | 1a       | 69   | 3 × 6 × 10⁶ IU/week       | 9        | Relaps   |

ALT: alanine transaminase; M: male; F: female; n.d.: not determined; respond: responder; relaps: relapsed.

**Preparation of PBMC**

Human PBMCs were isolated from peripheral blood by Ficoll density gradient centrifugation\[30\]. Residual red blood cells were hypotonically lysed and cells were washed three times with phosphate buffered saline (PBS) pH 7.3. Cell preparations were routinely assessed for viability (>95%) by trypan blue dye exclusion. Typically, a PBMC preparation consisted of >98% lymphocytes and monocytes and <2% granulocytes as determined by morphology of cells stained according to Papppenheim.

**Isolation of total cellular RNA**

PBMCs obtained from approximately 30 mL peripheral blood were taken up in 3 mL guanidinium isothiocyanate (GTC) buffer\[31\]. The material was subjected to shearing forces by drawing it rigorously through a capillary needle. Subsequently, total cellular RNA was isolated by cesium chloride (CsCl) density gradient centrifugation\[32\] and the RNA concentration was determined photometrically.

**Detection and quantification of serum HCV RNA**

HCV specific RNA was extracted from serum samples (140 µL) using QIAamp viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. For qualitative determination of HCV specific RNA one fifth of the extracted material was subjected to a nested RT/PCR procedure essentially as described\[33\]. For the quantitative measurement of HCV viral RNA the Amplicor HCV Monitor Test Kit (Hoffmann-La
Roche AG, Grenzach-Wyhlen, Germany) was used according to the manufacturer’s protocol. Test results less than 600 copies/mL are below the lower limit of quantification of the test and should be reported as “HCV detected, less than 600 copies/mL.”

Detection of HCV RNA in PBMC by nested RT/PCR-procedure

For the qualitative detection of HCV specific RNA in PBMC 100 ng of total cellular RNA were subjected to a HCV specific nested RT/PCR procedure essentially as described[33].

Incubation of HCV-RNA negative PBMC in HCV-RNA positive plasma

Blood samples were obtained from the three healthy donors (VM, NN and BS) for preparation of HCV-RNA negative PBMC and from the three patients (SG, EZ and UP) chronically infected with HCV for isolation of HCV-RNA positive plasma samples. The PBMCs were isolated as described before. The plasma samples were obtained by centrifugation (10 minutes, 14 000 rpm and 4 °C) of the HCV-RNA positive blood samples. The HCV concentration was then measured in these samples using the Amplicor Monitor Test Kit. Afterwards each of the three different plasma samples were diluted 1:4, 1:16 and 1:64 with HCV-RNA negative plasma from a healthy donor to reduce the virus concentration; one undiluted sample was also used.

PBMC isolated from donor VM were then incubated with the plasma samples from patient UP, from donor NN in the plasma samples of patient EZ and from donor BS in the plasma samples of patient SG. Incubation was performed for 2 hours at 37°C in an air incubator. After incubation the PBMCs were washed three times in PBS as described above. The total cellular RNA was then isolated by CsCl density gradient centrifugation and the HCV-RNA was detected with HCV specific nested RT/PCR technique as described.

RESULTS

HCV-RNA detectability in sera and in total RNA preparations of PBMC from patients with chronic HCV infection before, during and after an IFN-α therapy

The detectability of HCV specific RNA was monitored regularly before, during and after an IFN-α therapy in sera and in total RNA preparation of PBMC in 15 patients with chronic HCV infection by a nested RT/PCR procedure. HCV-RNA was below the detection limit in 102 serum samples taken during the IFN-α therapy. In none of these cases could HCV-RNA be observed in PBMC. In contrast, HCV specific RNA was only detectable in PBMC when HCV-RNA in serum was above the detection limit.

In patients who completely responded to the IFN-α therapy, the number of HCV-RNA copies decreased progressively until they became undetectable in serum samples. HCV-RNA in PBMC fell below the detection limit before positivity disappeared from the serum. In the patients relapsing after cessation of IFN-α therapy, HCV-RNA reappeared first in serum and later in PBMC. Serum HCV-RNA concentrations were measured quantitatively before and after transition from detectable to non-detectable HCV-RNA in PBMC preparation and vice versa. The transition from detectable to non-detectable HCV-RNA in PBMC during drug therapy was associated with a strong and significant decrease of serum HCV-RNA concentration (Table 2 and Figure 1). Vice versa, the transition from non-detectable to detectable HCV-RNA in PBMC, in the case of a breakthrough under therapy, or when the therapy was stopped and a relapse was observed, was associated with a marked and significant increase of serum HCV-RNA concentration (Table 2 and Figure 1). HCV-RNA was undetectable in PBMC if HCV-RNA concentration in serum fell below 2263 copies/mL and became detectable when the HCV-RNA level in serum was higher than 4708 copies/mL (Table 2).

In one patient (pt #15) after therapy and in four patients (pt #3, pt #4, pt #9 and pt #14) relapsing after cessation of therapy, the blood samples taken during this period did not include the moment, when HCV-RNA was detectable in serum and undetectable in PBMC.

![Figure 1](image_url)

Figure 1 HCV-RNA concentration (copies/mL) in sera from patients with chronic HCV infection with regard to HCV-RNA detectability in PBMC before, during and after an IFN-α therapy. The transition from detectable to non-detectable HCV-RNA in PBMC during drug therapy was associated with a strong and significantly decrease of serum HCV-RNA concentration. Vice versa, the transition from non-detectable to detectable HCV-RNA in PBMC, in the case of a breakthrough under therapy, or when therapy was stopped and a relapse was observed was associated with a marked and significantly increase of serum HCV-RNA concentration. Columns A and D: HCV-RNA concentration, if HCV-RNA in serum and in PBMC was detectable. Columns B and C: HCV-RNA concentration, if HCV-RNA in serum was detectable and in PBMC undetectable. (*) Test results less than 600 copies/mL are below the lower limit of quantification of the test and should be reported as “HCV detected, less than 600 copies/mL.”
samples of each patient with chronic HCV infection using the Amplicor HCV Monitor Test Kit were as follows: patient SG 119271 copies/mL, patient UP 337539 copies/mL and patient EZ 77518 copies/mL. The virus concentration of the dilutions steps in each patient is shown in Table 3. The results of incubation of PBMC from healthy donors in HCV-RNA positive plasma with different virus concentration are summarized in Table 3. After a two-hour incubation at 37°C, all PBMC samples incubated in the undiluted plasma samples were HCV-RNA positive. When PBMCs from donor BS were incubated in plasma samples from patient SG, the HCV-RNA was detectable only in the undiluted sample (119271 copies/mL, Table 3). After incubation of PBMC from donor VM in plasma samples of patient UP, the HCV-RNA was detectable in dilution step 1:16 (21096 copies/mL, Table 3). On incubation of PBMC from donor NN in plasma samples of patient EZ, the HCV-RNA was detectable in dilution step 1:4 (19290 copies/mL, Table 3). These results indicate a possible correlation between the detectability of HCV-RNA in PBMC and the HCV concentration in the plasma, since no HCV-RNA could be detected in PBMC when the HCV titer was below 19290 copies/mL.

Table 3 Detectability of HCV-RNA in PBMC of healthy donors after incubation with different dilutions of HCV-RNA positive plasma

| Patients | HCV concentration (copies/mL) | PMBC of healthy donor | Detectability of HCV-RNA in PMBC of healthy donors |
|----------|-----------------------------|-----------------------|-----------------------------------------------|
|          | Undiluted sample | 1:4 | 1:16 | 1:64 | Before incubation | After incubation dilution step (copies/mL) |
| SG       | 119 271          | 29 818 | 7454 | 1863 | BS | n.d. | Undiluted (119271) |
| UP       | 337 539          | 84 385 | 21 096 | 5274 | VM | n.d. | 1:16 (21096) |
| EZ       | 77 518           | 19 290 | 4822 | 1206 | NN | n.d. | 1:4 (19290) |

**DISCUSSION**

In all cases of orthotopic liver transplantation, an HCV reinfection of the graft occurs\(^{11,12}\). Persistence of HCV at extrahepatic sites is considered to be responsible for the reinfection. Therefore, any extrahepatic association of virus, e.g., by productive replication or simply by adhesion to outer membrane structures, appears to be relevant. One important extrahepatic reservoir is possibly the whole blood, which consists of a liquid component (plasma) and different cell fractions (PBMC, granulocytes and red blood cells/platelets). In the literature it has been suggested that PBMC can function as an important extrahepatic reservoir and a possible site for HCV replication. The evidence for this assumption was based on the demonstration of negative-strand HCV-RNA in association with PBMC from HCV infected patients by a RT/PCR technique\(^{16-19}\). Other possible extrahepatic reservoirs were described by Laskus and colleagues in chronically HCV infected patients, additionally infected with the acquired immunodeficiency syndrome. HCV-RNA negative-strand could be detected by a Tth-based reverse transcriptase polymerase chain reaction in lymph nodes, pancreas, adrenal gland, thyroid, bone marrow and spleen\(^{15}\). The value of these findings is however controversial, since the presence of negative-strand HCV-RNA in extrahepatic compartments based on RT/PCR techniques has been described in many reports with a very large range of detection rate (from 0% to 100%). In fact, by using synthetic as well as biological templates, an extensive artefactual detection of negative-strand HCV-RNA, due to self-priming and mispriming events could be documented. This is especially true when a 5’ noncoding region primer pair is used\(^{20,21}\). The
HCV, before during and after an IFN-

preparations of 15 patients infected chronically with

qualitatively analysed in serum and in total RNA

In this work, the presence of HCV-RNA was

was performed for the detection of negative-strand HCV-

RNA in PBMC preparations.

In this work, the presence of HCV-RNA was

was only detectable above the RNase H detection limit of the assay; (b) the transition from detectable to non-detectable HCV-RNA in PBMC was detectable in each cell line by in situ hybridization. HCV-RNA negative-strand, as evidence for replication, was detected in the Hep G2 and Daudi cells, but not in the G4 cells, a B-

lymphocyte cell-line. Therefore, in this study no experiment could be performed in vivo to confirm the presence of HCV-RNA in PBMC.

These findings support the assumption that the presence of HCV sequences in total RNA preparations of PBMC is probably compatible with passive virus adsorption, with endocytosis of the virus or with contamination by circulating virus or virus-protein complexes could also adhere to specific receptors on the cell surface. Bronowicki and colleagues described the SCID mouse model as a possible in vivo model to analyse the issue of HCV-

HCV could bind to \( \beta \)-lipoprotein (LDL) and therefore possibly adhere in this form to specific lipoprotein-receptors on PBMC. Recently, Agnello and colleagues demonstrated that HCV can enter the cells via the LDL-receptor. The endocytosis of HCV by the LDL receptor was mediated both by VLDL or LDL and directly by HCV binding to the cell surface. Three kinds of cell lines (Hep G2, G4 and Daudi cells) were used in this study. After an incubation in HCV positive HCV-RNA serum positive-strand was detectable in each cell line by in situ hybridization. HCV-RNA negative-strand, as evidence for replication, was detected in the Hep G2 and Daudi cells, but not in the G4 cells, a B-

lymphocyte cell-line. These findings are in agreement with our results, that no replication occurs in PBMC. Another possibility for adherence of HCV to PBMC has been shown by Pileri and colleagues, who demonstrated the binding of HCV to CD 81, a 25-kD molecule of the tetraspanin superfamily, expressed in various cell types including hepatocytes and B-lymphocytes. Fluorescence in situ hybridization of HCV-RNA in PBMC showed signals on the cytoplasmatic membrane of the cells. However, this could also be an indication for passive viral adsorption via a specific receptor or for a contamination by circulating virus. Other fluorescent signals appeared in granules in distinct submembrane areas or diffuse in the cytoplasm. A possible explanation for these findings may be the ingestion of virus particles by phagocytosis particularly in macrophages or by endocytosis via the LDL-receptor.

HCV-RNA was not only detected in PBMC but also in other cell fractions of the whole blood such as granulocytes and red blood cells or platelets. Schmidt and colleagues have investigated the distribution of HCV in whole blood and in the different cell fractions. Whole blood contained significantly more HCV-RNA than plasma, which contained more HCV-RNA than PBMC, the lowest level of HCV-RNA was found in granulocytes and in red blood cells/pellets. In the case of granulocytes, the virus may simply be ingested by phagocytosis and in the red blood cells or pellets. The HCV may be present because virus or virus-protein complexes could also adhere to specific receptors on the cell surface.
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