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

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**Subject headings** hepatitis C like viruses; hepatitis C, chronic; RNA, viral/blood; virus replication; monocytes; interferon alpha/therapeutic use; polymerase chain reaction

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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.**

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## 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. The presence

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-á therapy ($3 \times 10^6$ to $6 \times 10^6$ IU IFN-$\alpha_{2a}$ three times weekly to $6 \times 10^6$ IU IFN-$\alpha_{2a}$ 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℃. 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, G-ttingen, Germany.

**Table 1 Clinical, virological and therapeutic parameters of 12 patients with chronic HCV infection and treated with IFN-$\alpha_{2a}$**

| Patient No. | Age (yrs) | Sex | Genotype (U/L) | ALT | Therapy regiment (IFN-α) | Duration of therap (month) | Response |
|---|---|---|---|---|---|---|---|
| 1 | 60 | F | 1b | 30 | $3 \times 3 \times 10^6$ IU / week | 18 | Respond |
| 2 | 79 | M | n.d. | n.d. | $2 \times 3 \times 10^6$ IU / week | 19 | Respond |
| 3 | 51 | F | 1a | 34 | $3 \times 3 \times 10^6$ IU / week<br>800 mg Ribavirin / day | 6 | Relaps |
| 4 | 62 | M | 1b | 36 | $7 \times 6 \times 10^6$ IU / week | 6 | Relaps |
| 5 | 41 | M | 1b | 40 | $3 \times 3 \times 10^6$ IU / week | 17 | Respond |
| 6 | 63 | F | 1b | 46 | $3 \times 6 \times 10^6$ IU / week | 14 | Relaps |
| 7 | 61 | M | 1b | 45 | $7 \times 6 \times 10^6$ IU / week | 6 | Respond |
| 8 | 48 | F | 1a / 1b | 36 | $3 \times 3 \times 10^6$ IU / week<br>800 mg Ribavirin / day | 7 | Relaps |
| 9 | 63 | F | 1b | 20 | $3 \times 4.5 \times 10^6$ IU / week | 12 | Relaps |
| 10 | 60 | M | 1b | 36 | $3 \times 6 \times 10^6$ IU / week | 12 | Relaps |
| 11 | 53 | M | n.d. | 92 | $3 \times 9 \times 10^6$ IU / week | 12 | Respond |
| 12 | 43 | F | 1a | 160 | $3 \times 6 \times 10^6$ IU / week | 12 | Respond |
| 13 | 53 | F | 1b | 9 | $3 \times 6 \times 10^6$ IU / week<br>800 mg Ribavirin / day | 12 | Respond |
| 14 | 69 | F | 1b | 22 | $3 \times 6 \times 10^6$ IU / week | 12 | Relaps |
| 15 | 63 | M | 1a | 69 | $3 \times 6 \times 10^6$ IU / week | 9 | Relaps |

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

### 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 Pappenheim.

### 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℃) 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℃ 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 these period did not include the moment, when HCV-RNA was detectable in serum and undetectable in PBMC.

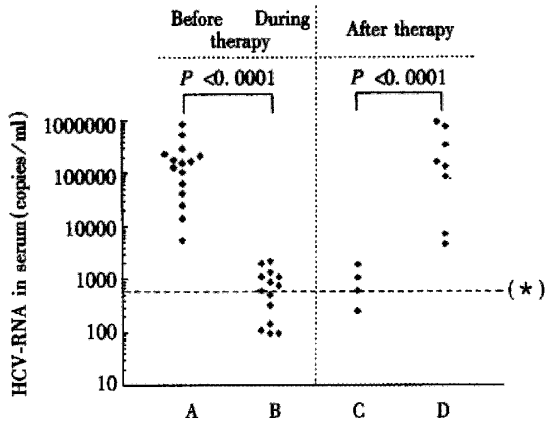


**Figure 1** HCV-RNA concentration (copies / mL) in sera from patients with chronic HCV infection with regard to HCV-RNA detectability in PBMC before, during and after an IFN-α therapy. The transition from detectable to non-detectable HCV-RNA in PBMC during drug therapy was associated with a strong and 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".

**Table 2 Detectability of HCV-RNA in serum and PBMC during an IFN-$\alpha_{2a}$ therapy**

| Patient No. | Before therapy HCV-RNA concentration (copy/mL) (HCV-RNA: serum pos./ PBMC *pos.*) | During therapy HCV-RNA concentration (copy/mL) (HCV-RNA: serum pos./ PBMC *n.d.*) | After therapy HCV-RNA concentration (copy/mL) (HCV-RNA: serum pos./ PBMC *n.d.*) | After therapy HCV-RNA concentration (copy/mL) (HCV-RNA: serum pos./ PBMC *pos.*) |
|---|---|---|---|---|
| 1 | 64717 | <600 | 1) | 1) |
| 2 | 14300 | <600 | 1) | 1) |
| 3 | 132224 | <600 | 2) | 167 898 |
| 4 | 107684 | 624 | 2) | 135 242 |
| 5 | 161718 | 1404 | 1) | 1) |
| 6 | 5620 | 903 | 1108 | 938 861 |
| 7 | 183091 | 2041 | 1) | 1) |
| 8 | 172345 | <600 | 1960 | 86 679 |
| 9 | 29542 | 775 | 2) | 770 810 |
| 10 | 219018 | 1138 | <600 | 7240 |
| 11 | 42601 | <600 | 1) | 1) |
| 12 | 237335 | <600 | 1) | 1) |
| 13 | 25097 | 2263 | 1) | 1) |
| 14 | 553901 | 1144 | 2) | 4706 |
| 15 | 868996 | 2) | 627 | 349 918 |

*Abbreviations*: 1) responder; 2) no sample available.

### *Incubation of HCV-RNA negative PBMC from healthy donors in HCV-RNA positive plasma from patients infected chronically with HCV*

The results of the measurement of the HCV concentrations in the undiluted positive plasma 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℃, 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 PBMC 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 acqured 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

mispriming artefacts were directly correlated to the titer of positive strand and depend on the RT/PCR technique used[21]. Therefore, in this study no experiment was performed for the detection of negative-strand HCV-RNA in PBMC preparations.

In this work, the presence of HCV-RNA was qualitatively analysed in serum and in total RNA preparations of 15 patients infected chronically with HCV, before during and after an IFN-α therapy. During the observation period after beginning IFN-α therapy, it was noted that HCV-RNA disappeared first from total RNA preparations of PBMC and then from serum. In the case of a breakthrough under therapy or of a relapse at the end of therapy, HCV-RNA reappeared at first in serum and then in the total RNA preparation of PBMC. Therefore, we performed a quantitative analysis of the HCV titer at these points of transition, and obtained the following results: (a) HCV specific RNA in total RNA preparations of PBMC was only detectable when HCV-RNA in serum was above the detection limit of the assay; (b) the transition from detectable to non-detectable HCV-RNA in PBMC during drug administration was associated with a marked decrease of serum HCV-RNA concentration; (c) the transition from non-detectable to detectable HCV-RNA in PBMC in the case of a breakthrough or a relapse was associated with a marked increase in serum HCV-RNA concentration; (d) when HCV-RNA in PBMC was detectable, the HCV concentration in serum was significantly higher than when HCV-RNA in PBMC was not detectable (Figure 1): and (e) it was possible to contaminate PBMC of healthy donors with HCV by incubation for a short time (2 hours) at body temperature (37℃) in HCV positive plasma samples. These results are in agreement with those published by Cribier and colleagues (1998). These authors could show that HCV-RNA become detectable in the PBMC of healthy donors after an incubation of the cells with HCV-RNA positive serum with high virus concentration[34].

HCV-RNA in PBMC become undetectable in the *in vivo* study when HCV-RNA concentration in serum falls below 2263 copies/mL (Table 2) and in the *in vitro* experiments when the HCV-RNA level falls below 19290 copies/mL (Table 3). One explanation for this discrepancy could be that for the *in vitro* experiments we choose a short period of incubation, which however, better shows the adherence of HCV to blood cells.

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[22,23]. In 1992 Thomssen and colleagues described a possible mechanism of how HCV could bind to the surface of PBMC. They found, that HCV could bind to β-lipoprotein (LDL) and therefore possibly adhere in this form to specific lipoprotein-receptors on PBMC[25,26]. Recently, Agnello and colleagues demonstrated that HCV can enter the cells via the LDL-receptor[24]. 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* hybridiziation. 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[24]. 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[27]. 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[35,36]. 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 pasma, which contained more HCV-RNA than PBMC, the lowest level of HCV-RNA was found in granulocytes and in red blood cells/pellets[13,14]. 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. Bronowicki and colleagues described the SCID mouse model as a possible *in vivo* model to analyse the issue of HCV-RNA persistence in mononuclear cells[37]. In their study, they injected PBMC from patients infected chronically with HCV into SCID mice. To exclude the possibility that just virus contamination and not true infection of the cells occurred, they incubated as a control sheep PBMC and human fibroblasts in HCV-RNA positive serum and injected them into

SCID mice. After injection of human PBMC, HCV-RNA was detected in 30% and 23% of blood cells and serum samples respectively of SCID mice. On the other hand, in the control mice, HCV-RNA sequences were not detectable either in serum samples or in PBMC. Therefore, they postulated the possibility of a replication of HCV in PBMC. In fact, the detection of no HCV-RNA in the control mice would argue against an adsorption of virus particles. The hypothesis was strengthened by detection of HCV negative-strand in two cell fraction samples of two SCID mice. However, no data were available showing that the sheep PBMC or human fibroblasts become HCV-RNA positive after the incubation in HCV-RNA positive serum.

Taken together, our findings could be explained as follows. During the IFN-α treatment, the HCV level is lowered by the inhibition of the hepatic virus replication. When the virus concentration decreases below a not yet exactly known upper limit, contamination of the PBMC becomes undetectable. At this time point, HCV is only detectable in serum for a short period and then disappears. On the other hand, it is also possible that IFN-α inhibits virus replication in both PBMC and the liver, and consequently, the HCV concentration in serum decreases in parallel with that of PBMC, although in lower amounts, the HCV-RNA continues to be detectable in the serum even when PBMC are negative. This indicaties that liver is the major site of replication. This could also explain that in the case of a relapse, the virus replication goes on in the liver and that the virus become detectable in the serum and then in the PBMC. The only approach to prove a true virus replication in PBMC is the detection of non-structural proteins (proteases, helicase or RNA-dependent RNA polymerase), which are important for virus replication, but this topic has not yet been investigated.

Our *in vitro* study, however, suggests that HCV-RNA positivity in PBMC may be due to the binding of the virus to blood cells and not to the true virus production. This would mean that PBMCs do not function as an extrahepatic reservoir for HCV when the diseased liver is explanted. 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. This could happen even in the patients who are complete responders under IFN-α therapy immediately before transplantation.

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