

## Original Article

# Presence of Hepatitis C Virus (HCV)-RNA in Peripheral Blood Mononuclear Cells in HCV Serum Negative Patients during Interferon and Ribavirin Therapy

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**SUMMARY:** Identification of hepatitis C virus (HCV)-RNA in blood serum is crucial for hepatitis C diagnosis and for appropriate treatment. Detection of HCV-RNA in blood serum is used for therapy monitoring of patients with hepatitis C. Despite HCV-RNA elimination from blood serum during treatment in some patients, HCV viremia appears again after the completion of therapy. The aim of this study was to assess HCV-RNA in peripheral blood mononuclear cells (PBMCs) of hepatitis C patients in relation to HCV-RNA and antibodies to HCV in the serum. The study involved 71 patients undergoing anti-viral therapy (interferon and ribavirin). RNA isolated from serum and PBMCs was examined for the presence of HCV-RNA by an RT-PCR technique using specific oligonucleotide primers or by commercially available kits. In order to show the possible presence of HCV sequences in PBMCs, molecular DNA probes were constructed with a PCR amplicon and biotin-labelled by nick translation, and FISH and extended chromatin fibers in situ hybridization (ECFs-FISH) techniques were used. A 24-month follow-up study revealed that 34 out of 59 patients (58%) eliminated HCV-RNA from their sera. In the serum negative group, HCV-RNA was detected in PBMCs of 2 patients. The presence of HCV-RNA in PBMCs was confirmed by the FISH technique. In the ECFs-FISH procedure, no signal was found in all examined patients. Our data suggest that PBMCs infected with HCV can serve as a virus reservoir. HCV-RNA serum negative patients who have HCV-RNA in their leukocytes after completion of anti-viral therapy would be at great risk of hepatitis C recurrence. These HCV-RNA serum negative but PBMCs positive patients would be a potential source of HCV spread.

## INTRODUCTION

Infection with the hepatitis C virus (HCV) is a major cause of hepatitis worldwide. The infection rate of HCV in the general population of different countries ranges from 0.8 to 3% (1,2). Standard diagnostic tests for hepatitis C include detection of antibody to HCV and molecular identification of HCV-RNA in blood serum. Determination of HCV molecules is also used for the monitoring the effectiveness of interferon with ribavirin therapy. The effectiveness of interferon with ribavirin therapy varies from 30-60% (3). Clearance of HCV-RNA in the serum is achieved in 60-70% of cases at the end of antiviral treatment. However, over the subsequent 6 months only 41-48% of patients remained HCV-RNA negative (4). Taking into account the presence or even replication of HCV in mononuclear cells, it is possible that the virus may persist in these cells after antiviral treatment (5). Therefore, the persistence of HCV in peripheral blood mononuclear cells (PBMCs) may constitute a reservoir for viral load.

The objectives of our study were to investigate the possible correlation between presence of HCV-RNA in the PBMCs and serum of hepatitis C patients. During a 24-month follow-up study, we monitored HCV-RNA in the PBMCs and serum

at 6-month intervals. We attempted to determine whether serum HCV-RNA negative patients may have HCV-RNA in their PBMCs as a virus reservoir. Finally, based on the results of this analysis, we evaluated whether serum HCV-RNA testing is sufficient for monitoring of antiviral therapy.

## MATERIALS AND METHODS

The study involved 71 children, aged from 3 to 18 years, who had been diagnosed with chronic hepatitis C, and most of whom were hospitalized and treated in the nephrology, cardiology, and gastroenterology departments of the Children's Hospital of the University of Medical Sciences in Poznań, Poland. Informed consent was obtained from the parents of all 71 children. All patients were HCV-antibodies positive, HBsAg negative and serum HCV-RNA positive. Patients were treated for 1 year with anti-viral therapy consisting of interferon 3MU subcutaneously 3 × weekly and ribavirin 15 mg/kg orally daily. We conducted a 24-month follow-up study with HCV-RNA testing at 6-month intervals.

Serum samples from the patients were analyzed for anti-HCV antibodies using a 2nd generation anti-HCV kit (Organon Teknika, Dublin, Ireland). RNA isolated from serum and PBMCs (by kits for genomic or viral RNA; Qiagen GmbH, Hilden, Germany) was examined for the presence of HCV-RNA by an RT-PCR technique using specific oligonucleotide primers (6) and an Amplicor HCV amplification kit (Roche Diagnostics GmbH, Mannheim, Germany). Quantitative

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analysis of HCV-RNA was performed using a commercially available kit (Roboscreen GmbH, Leipzig, Germany). An HCV genotype assay (LIPA) (Versant, Bayer, Pureaux Cedex, France) was used for HCV genotyping.

In order to assay for the possible presence of HCV sequences in PBMC, an HCV-DNA probe was constructed with a PCR amplicon and biotin-labelled by nick translation. HCV sequences were detected by FISH and also on chromatin fibers by extended chromatin fibers in situ hybridization (ECFs-FISH).

Human interphase nuclei were prepared according to Cremer et al. (7) and Trask et al. (8). Briefly, PBMCs isolated on Ficoll/Hypaque gradients were treated with 7 ml hypotonic solution (0.4% KCl), then incubated at 37°C for 20 min. Obtained cell nuclei were treated thrice with 7 ml cold fixative (methanol:acetic acid, 3:1) and centrifuged 10 min at 1,500 rpm. The interphase nuclei pellet was spread on cold microscope slides and dried at room temperature. The slides were then washed in 2 × SSC, dehydrated in 70, 85, 96% ethanol and dried at room temperature.

Preparation of human extended chromatin fibers was conducted according to Haaf and Ward (9) and Florijn et al. (10) with some modifications. The isolated PBMCs were washed with sterile 7 ml PBS buffer three times. The cells were spread on microscopic slides in Cytospin (Shandon Southern, Pittsburgh, Pa., USA) at 600 rpm for 8 min. Then the slides were treated with concentrated salt solution (15 mM NaCl, 2 M Tris, 0.5 M EDTA, 1 mM H<sub>2</sub>PO<sub>4</sub> pH 8.0 + 90% glycerol) at room temperature for 30 min. Chromatin fibers were fixed by incubation of slides in absolute ethanol for 30 min.

The HCV probe was constructed from a PCR amplicon obtained using specific oligonucleotide primers (6). The PCR product was labeled with biotin by nick translation (Boehringer, Mannheim, Germany). Ten microliters of biotin-labeled HCV probe was suspended in a mixture composed of formamide (50 μl), SSCP (20 μl), 50% dextran sulfate (10 μl), and H<sub>2</sub>O (110 μl) and stored at -20°C. Before hybridization, the probe was denatured at 80°C for 12 min and poured on the slides. The edges of the microscope cover glasses were sealed with fixogum. Then, the probes were hybridized with nuclei or chromatin fibers in a moist chamber at 37°C for 48 h.

Post-hybridization washes were conducted in formamide/2 × SSC solution at 40°C. The hybridized probe was detected by an immunological reaction with avidin-fluorescein (FITC) and biotinylated anti-avidin (Vector Laboratories, Burlingame, Vt., USA). The slides were stained by propidium iodide and analyzed under an Axiophat fluorescence microscope (Zeiss, Jena, Germany), and ISIS software (MetaSystems GmbH, Altlusheim, Germany) for digital analysis of microscopic images was used.

To exclude nonspecific binding controls for FISH analysis included hybridization without the HCV-probe and with unlabeled HCV-probe followed by avidin-fluorescein (FITC) and biotinylated anti-avidin antibody treatment.

## RESULTS

Seventy-one patients were selected for interferon and ribavirin therapy as described above. Anti-HCV antibodies were detected before antiviral therapy and were observed in all patients with a median value of 34.23 S/CO (range, 1.2 S/CO - 138.7 S/CO). The duration of HCV infection in the study group ranged from 13 to 120 months. Anti-viral therapy with interferon and ribavirin was conducted for 1 year. At the time of diagnosis, all 71 patients were positive for HCV RNA in serum and 65 were positive for HCV RNA in PBMCs. A majority of the patients were infected with HCV 1b genotype (*n* = 67), but 2 patients were infected with the 1a, 1 patient was infected with the 3a, and 1 patient was infected with the 1b+3a genotypes.

After 6 months of anti-viral therapy, the virus was eliminated from the circulation in more than half of the patients (Table 1). It is interesting that after 6 months of therapy the proportion of patients showing HCV positive serum to patients showing HCV positive PBMCs was changed. That is, more patients were HCV-PBMC positive than serum positive. This trend continued during the 24-month follow-up, where virus presence has been detected more often in PBMCs as compared to standard identification of HCV in blood serum. Six-month antiviral therapy resulted in a sustained response in 39% of patients as assessed by HCV elimination from blood serum. However, when we examined the presence of HCV RNA in PBMCs, it appeared that 52% of patients were still positive. The best antiviral therapy response was achieved after 12 months, when only 31% of patients were found to have the virus in their sera and 44% in their PBMCs. At the end of the 24-month follow-up, 42% of patients had responded effectively to therapy as evidenced by a standard PCR test. However, 2 patients who were serum-negative possessed HCV in their PBMCs.

Laboratory data including titers of alanine transaminase (ALT), serum antibodies against HCV and HCV-RNA at the

Table 1. Presence of HCV-RNA in blood serum and in PBMCs before antiviral therapy and during a 24-month follow-up study

| HCV-RNA | Before therapy | 6 mo       | 12 mo      | 18 mo      | 24 mo      |
|---------|----------------|------------|------------|------------|------------|
| Serum   | 71/71 (100)    | 28/71 (39) | 22/71 (31) | 23/65 (35) | 25/59 (42) |
| PBMCs   | 65/71 (92)     | 37/71 (52) | 31/71 (44) | 24/65 (37) | 27/59 (46) |

The figures in parenthesis indicate the percentage.

Table 2. Titers of ALT, serum antibodies against HCV and HCV-RNA at the beginning of antiviral therapy and after 24-month follow-up study in groups of non-responders and sustained viral responders

| Groups of patients                                | ALT (IU/L)      | ALT (IU/L)      | HCV-AB (S/CO)       | HCV-AB (S/CO)       | HCV-RNA (IU/ml)  | HCV-RNA (IU/ml)  |
|---|-----------------|-----------------|---------------------|---------------------|--|--|
|   | before therapy  | 24 mo           | before therapy      | 24 mo               | before therapy   | 24 mo  |
| Non-responders<br><i>n</i> = 25                   | 157<br>(31-570) | 131<br>(47-349) | 35.4<br>(1.2-138.7) | 31.3<br>(1.7-110.4) | 9.2 × 10 <sup>4</sup><br>(7.1 × 10 <sup>2</sup> -4.8 × 10 <sup>6</sup> ) | 9.7 × 10 <sup>3</sup><br>(3.8 × 10 <sup>2</sup> -6.5 × 10 <sup>6</sup> ) |
| Sustained virological responders<br><i>n</i> = 34 | 112<br>(39-610) | 34<br>(11-41)   | 41.7<br>(2.8-79.3)  | 28.4<br>(1.5-87.4)  | 8.9 × 10 <sup>4</sup><br>(6.3 × 10 <sup>2</sup> -5.1 × 10 <sup>6</sup> ) | negative   |

Median value and a range of the data are presented.

ALT, alanine transaminase; HCV-AB, HCV antibodies.

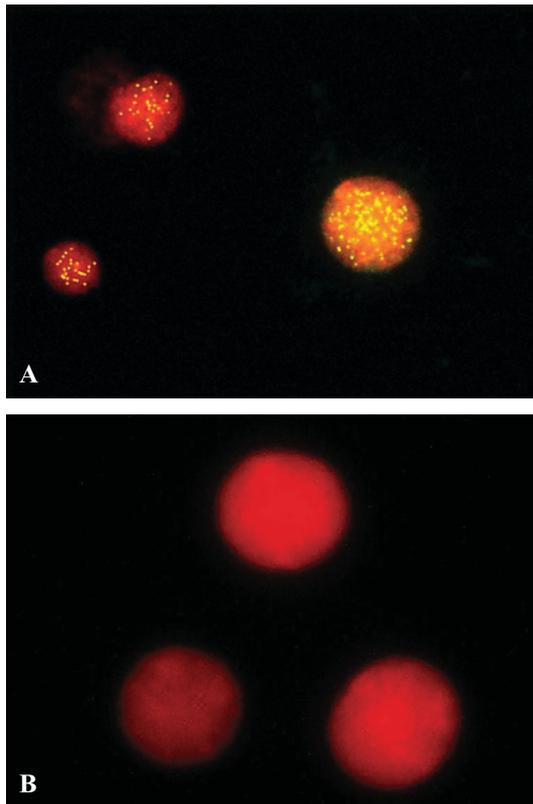


Fig. 1. FISH to PBMCs with HCV probe.  
 (A) Presence of HCV particles in PBMCs of HCV serum negative patient. (B) Lack of HCV particles in HCV-serum and -PBMCs negative patient.

beginning of antiviral therapy and after 24 months in groups of non-responders and sustained viral responders are presented in Table 2. There were no significant differences in any of these parameters between the two groups.

To confirm the presence of HCV in PBMCs, we used standard FISH analysis as well as ECFs-FISH. In all patients in whom PBMCs were positive by PCR, FISH analysis also gave positive results (Figure 1). In some cases we tried to detect HCV on chromatin fibers by ECFs-FISH analysis. The results were always negative. No HCV sequence was detected as being incorporated into chromatin fibers.

## DISCUSSION

The obtained results clearly documented the presence of HCV RNA in PBMCs in HCV-infected patients. PBMCs positive for HCV could be related to viral persistence and possible perturbation of PBMCs function in chronically infected patients (11). In previous studies, the altered function of PBMCs allowed generation of HCV-specific B lymphocytes producing anti-HCV antibodies (11,12). We cannot exclude the possibility that HCV can multiply in PBMCs being extra-hepatic site of virus replication. Our present finding adds further weight to the involvement of PBMCs in the persistence of HCV infection and emphasizes the role of B lymphocytes as an HCV reservoir (13,14). The presence of HCV RNA in PBMCs may partially explain the limited HCV antiviral response rates observed in the studied patients.

Several authors have postulated that the detectability of HCV-RNA in total RNA from PBMCs is dependent on the HCV concentration in serum (3,5,14). They explained that

the presence of HCV in PBMCs was due to contamination or passive adsorption by circulating virus. In our opinion, the reason for the detection of HCV-RNA in PBMCs is most likely its intracellular presence, as evidenced by FISH results (Figure 1A). Walker et al. (15) used in situ RT-PCR with FITC-labeled primers and localized the HCV in 38 liver biopsy specimens from chronically infected HCV patients. They also found positive signals from lymphocytes, strongly indicating the persistence of HCV in these cells. Localization of HCV-RNA was intranuclear in hepatocytes, bile ducts cells and portal lymphocytes. They observed the persistence of HCV within these cells in responders even 1 year after interferon therapy cessation. Other authors have demonstrated the persistence of HCV-RNA in PBMCs or the liver for up to 9 years after a sustained virologic response (SVR) (4). Similar results have been obtained by Xu et al. (16).

Rouille et al. (17) detected HCV structural proteins in human hepatoma cells (Huh-7 line), which efficiently replicate the virus. Electron microscopy analysis showed dense elements suggesting the presence of viral particles.

Replication of HCV has been shown in various hepatoma and HeLa cell lines (18). Some authors have reported that the replication of HCV RNA in PBMCs may be the source of relapse after antiviral treatment in patients with chronic hepatitis C (16).

According to the data in the literature and our present results, PBMCs constitute a virus reservoir. It is very likely that HCV indeed replicates in PBMCs. We have observed that 4 patients negative for serum HCV and positive for PBMCs after 6 months of therapy became HCV serum positive after 18 months. However, another 5 patients who were negative for HCV in serum and positive for HCV in PBMCs showed a complete elimination of HCV from PBMCs after 18 months. Elimination of the virus from circulation after 6 months of therapy is usually called a SVR. Our results indicate that HCV may persist in PBMCs after the SVR.

Based on the above results, we conclude that PBMCs infected with HCV can serve as a virus reservoir. HCV-RNA serum negative patients who have HCV-RNA in their PBMCs after completion of anti-viral therapy would be at great risk of hepatitis C recurrence. In addition, these HCV-RNA serum negative but PBMCs positive patients would be a potential source of HCV spread.

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