Quantification of Hepatitis C Virus–Infected Peripheral Blood Mononuclear Cells by In Situ Reverse Transcriptase-Polymerase Chain Reaction

By Luigi Muratori, Davide Gibellini, Marco Lenzi, Michela Cataleta, Paolo Muratori, Maria Cristina Morelli, and Francesco B. Bianchi

Hepatitis C virus (HCV) is known to infect peripheral blood mononuclear cells (PBMC) of patients with chronic hepatitis C, but the proportion of HCV-infected circulating cells is not detectable by conventional reverse transcriptase-polymerase chain reaction (RT-PCR) and the pathogenic significance of HCV lymphotropism is still unclear. Therefore, we have devised an in situ RT-PCR technique using fluorescein-labeled HCV-specific primers revealed by flow cytometry. PBMC were isolated from 28 patients with chronic HCV-related liver disease; of these, 6 had previously received an orthotopic liver transplantation (OLT) and were on immunosuppressive treatment. Fourteen patients (50%) were found positive for HCV genome within PBMC by in situ RT-PCR, the proportion of HCV-infected cells ranging from 0.2% to 8.1%. All 6 OLT patients tested positive. The fluorescent signal, corresponding to the HCV-specific 340-bp amplicon, was confined to part of the cytoplasmatic compartment of scattered PBMC. Of these 14 patients, 12 had also negative-strand HCV RNA within PBMC detected by "tagged" RT-PCR. We conclude that HCV may infect a significant proportion of PBMC in chronic hepatitis C patients, especially immunosuppressed OLT cases, and that viral replication within PBMC is a common occurrence. Over time, the persistence of HCV-infected immune system cells might interfere with normal immunologic mechanisms and play a role in the pathogenic processes leading to extrahepatic disorders such as mixed cryoglobulinemia and B-cell malignant lymphoma.

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MATERIALS AND METHODS

Patients. HCV genome was searched for within PBMC isolated from 28 patients (10 women; median age 44 years; range, 23 to 68) with HCV-related chronic liver disease. Twenty-two were consecutive patients referred to our observation because of anti-HCV positivity. All of them had a history longer than 6 months of persistently elevated or fluctuating alanine aminotransferase (ALT) levels, anti-HCV positivity (Ortho HCV 3.0 ELISA; Ortho Diagnostic Systems, Milan, Italy) and serum HCV RNA, detected as previously described.15 After clinical, biochemical, virological, and histological assessment, they were diagnosed as having chronic hepatitis C (16 cases) or HCV-related cirrhosis (6 cases), of whom 2 with concomitant hepatocellular carcinoma. The remaining 6 patients had previously received an orthotopic liver transplantation (OLT) for HCV-related end-stage liver disease and were on standard immunosuppressive therapy. All of them tested positive for serum HCV RNA, by a conventional "nested" RT-PCR technique.12 Patients with symptomatic HCV-related mixed cryoglobulinemia were excluded from this study. None of the patients was on interferon therapy at the time of blood sample drawing. Patient no. 16 was HBsAg positive (EIA kit; Abbott Laboratories, North Chicago, IL) and patient no. 24 had markers of ongoing HBV and hepatitis delta virus infection (DELTA-IGMK; Sorin Biomedica, Saluggia, Italy).

Table 1 summarizes the main clinical, histological, biochemical, and virological features of the patients studied.

As controls, PBMC were obtained from 15 patients with chronic liver disease without markers of HCV infection, namely chronic hepatitis B (7 cases), alcoholic cirrhosis (3 cases), primary biliary cirrhosis (5 cases), and from 15 healthy blood donors.

In situ RT-PCR. The protocol described by Embleton et al12 for the identification of mRNAs within single cells was followed with some modifications. Cells were separated from EDTA-treated peripheral venous blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation; PBMC were carefully washed in sterile saline, suspended in ice-cold 10% buffered formaldehyde solution (Carlo Erba, Milan, Italy) and kept for 2 hours at 4°C. After further washing, PBMC were stored in 50% ethanol at −20°C and tested within 3 weeks. For each set of experiments, 2 × 10^6 cells were used.
IN SITU RT-PCR TO DETECT HCV RNA WITHIN PBMC

aliquoted in 0.5-mL Eppendorf tubes and treated with a 1% sterile saline solution of Triton-X-100 (Sigma, St Louis, MO) at 4°C for 10 minutes with gentle shaking. Permeabilized PBMC were then resuspended in 20 μL of RT buffer (50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L MgCl₂) containing 10 mmol/L dithiotreitol, 200 U of cloned Moloney murine leukemia virus RT (GIBCO-BRL, Gaithersburg, MD), 40 U of RNAse inhibitor (RNAguard; Pharmacia), 1 mmol/L each dGTP, dATP, dTTP, dCTP, and 50 pmol of HCV-specific antisense primer (NCR2) (Table 2). The tubes were incubated at 37°C for 1 hour, boiled for 10 minutes to inactivate residual RT activity, and immediately chilled on ice. PCR was performed following the “hot start” modifications outlined by Nuovo.22 After the RT step, PBMC were washed again with ice-cold sterile saline, spun down, and resuspended in 25 μL of PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, 2.5 mmol/L MgCl₂) containing 1 mmol/L each dGTP, dATP, dTTP, dCTP, and 25 pmol of both antisense (NCR2) and sense (NCR1) primers. After preheating the tubes for 7 minutes at 82°C, 1 U of Taq DNA polymerase (Perkin Elmer, Nonvalk, CT) was added to each sample. Premelting at 95°C for 3 minutes was followed by 20 cycles of 1 minute each at 94°C (DNA denaturation), 55°C (primer annealing), and 72°C (primer extension). A final extension step of 7 minutes at 72°C followed the last PCR cycle. After the first 10 PCR cycles, 10 pmol of both NCR1 and NCR2 primers labeled with fluorescein were added to each sample. At the end of in suspension PCR, PBMC were carefully washed in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.4), with gentle agitation at 37°C. Between 2 and 4 × 10⁶ cells were spread on a glass slide, counterstained with propidium iodide, covered with a coverslip, and sealed at the edges with nail polish. PBMC were then examined on a fluorescence microscope (Orthoplan, Leitz, Wetzlar, Germany). Each PBMC sample was tested at least twice, in a blinded fashion.

Table 1. Clinical, Histological, and Biochemical Features of the 28 Patients Studied

<table>
<thead>
<tr>
<th>Pt</th>
<th>Sex</th>
<th>Age</th>
<th>Liver Histology</th>
<th>ALT (IU/L)</th>
<th>Serum HCV RNA</th>
<th>PBMC/mL</th>
<th>% HCV-infected PBMC</th>
<th>PBMC Negative-Strand HCV RNA*</th>
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<td>1</td>
<td>M</td>
<td>29</td>
<td>PI</td>
<td>54</td>
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<td>+</td>
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<td>3</td>
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<td>+</td>
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<td>-</td>
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<tr>
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<td>M</td>
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<tr>
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<td>F</td>
<td>41</td>
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<td>+</td>
<td>-</td>
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<td>+</td>
<td>-</td>
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<td>49</td>
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<td>M</td>
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<td>0.2 ± 0.1</td>
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<td>50</td>
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<tr>
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<td>Cirrhosis</td>
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<td>+</td>
<td>2,013</td>
<td>1.9 ± 0.4</td>
<td>+</td>
</tr>
<tr>
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<td>M</td>
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<td>NA</td>
<td>110</td>
<td>+</td>
<td>2,088</td>
<td>1.9 ± 0.4</td>
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<td>56</td>
<td>AHGR</td>
<td>366</td>
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<td>2,220</td>
<td>4 ± 0.5</td>
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<td>454</td>
<td>818</td>
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<td>8.1 ± 1</td>
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<td>M</td>
<td>36</td>
<td>PI</td>
<td>88</td>
<td>2,003</td>
<td>+</td>
<td>8.1 ± 1</td>
<td>+</td>
</tr>
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</table>

Abbreviations: PI, portal inflammation; CAH, chronic active hepatitis; HCC, hepatocellular carcinoma; ML, minimal lesions; AHGR, acute hepatic graft rejection; NA, not available; ALT, upper normal limit, 40 IU/L; PBMC, peripheral blood mononuclear cells.

* The negative-strand HCV RNA was evaluated by tagged RT-PCR, according to Lanford et al.20
† Immunosuppressed OLT patients.

Table 2. Primers’ Sequences

- HCV-specific primers
  - NCR1 (sense, outer): 5'-GTAACAGGGGACGACATGCAATAGAGT3'
  - NCR2 (antisense, outer): 5'-ATACTCGAGTTGACAGGCTCATAGGACC3'
  - NCR3 (sense, inner): 5'-CACCATAGATGCTCCACCTG3'
  - NCR4 (antisense, inner): 5'-CAGCTTCGAGACCACCTACTACGCGAC3'
- PT1 (sense, inner): 5'-CCTTCATGTAGTGCTCATGCTG3'
- PT2 (antisense, inner): 5'-CGGTTACTCCAGGGTTC3'
- tagged-NCR3 (sense, inner): 5'-TCAATCGTGGCGGAAATACACCCATAGATCCTTCTCCCGT3'
- tagged-NCR3 (sense, inner): 5'-TCAATCGTGGCGGAAATACACCCATAGATCCTTCTCCCGT3'
- bcr-abl mRNA-specific primers
  - (sense): 5'-GTTCGAGGAACTTCTGCTTCC3'
  - (antisense): 5'-CCATTGTGGTTGCTGCTTCCACCGCCATCC3'
- β-Actin mRNA-specific primers
  - (sense): 5'-ACACGTCGCGCCATACGAGGG3'
  - (antisense): 5'-ATGATGAGTTGACAGG3'
**Flow cytometry.** The remaining cells were analyzed by an independent investigator with a FACScan (Becton Dickinson, Baltimore, MD) flow cytometer. Argon laser excitation was 15 mW at 488 nm. Instrument sensitivity was standardized before each experiment with calibration beads (Becton Dickinson). Cells were gated for forward and side scatter and the percentage of fluorescent positive cells was determined by integration over a range of 0.1% positive counts on identically treated negative controls or on cell samples where in situ RT-PCR had been performed with fluorescein-labeled nonspecific primers. At least 5,000 to 10,000 events were analyzed for each sample.

**Detection of the HCV replicative intermediate.** The detection of the negative-stranded HCV genome, which is considered the HCV replicative intermediate, can be falsely achieved by conventional RT-PCR as a consequence of self-priming or nonspecific priming. Therefore, in this study we used the “tagged” RT-PCR method described by Lanford et al. which allows the specific detection of the HCV RNA negative strand. Briefly, tagged-NCR3, the positive-sense, forward primer for cDNA synthesis, contains a sequence (the tag, shown in boldface type in Table 2) unrelated to HCV at the 5' end and an HCV-specific sequence at the 3' end. RT reaction was performed on total RNA extracted from approximately 10^7 PBMC and, as control, from 100 μL of the final wash of each sample. The cDNA obtained was amplified for 40 cycles using NCR4 as antisense primer and the tag sequence alone as sense primer. One microliter of the first PCR product was then amplified for 30 cycles in a second PCR round, using PT1 and PT2 as inner primer pair. The final 82-bp “nested” PCR product was visualized by agarose gel electrophoresis and ethidium bromide staining, transferred to a nylon filter, and hybridized with a digoxigenin-labeled HCV-specific internal probe (probe sequence: 5'AGAGCCA-TAGTGGTCTG3').

**RESULTS**

We developed an in situ RT-PCR revealed by flow cytometry to evaluate the presence of the HCV genome in circulating mononuclear cells. With our technique we analyzed PBMC obtained from 28 patients with chronic hepatitis C and, as negative controls, 15 chronic liver disease patients with no evidence of HCV infection and 15 healthy blood donors. In a proportion of cases, direct evidence for HCV replication within PBMC was also found by “tagged” RT-PCR.

A green fluorescent signal, corresponding to accumulated HCV-specific 340-bp amplicon, was detected in 14 (50%) of the 28 HCV-positive PBMC samples and in none of the controls. Fluorescence microscopy analysis showed that positive PBMC exhibited a fluorescent signal surrounding the nucleus and confined to part of the cytoplasmic compartment. Differences between positive and negative cells were unequivocal (Fig 1). PBMC were also analyzed by flow cytometry and positive cells were quantified (Fig 2). The proportion of PBMC positive for the HCV genome ranged from 0.2% to 8.1% and is reported in Table 1.

To assess the assay specificity, the following controls were performed in each set of experiments: (1) omission of the RT step; (2) withdrawal of Tag DNA polymerase; (3) use of fluorescein-labeled primers unrelated to the HCV genome (specific for the chimeric bcr-abl messenger RNA and peculiar to chronic myelogenous leukemia cells only [Table 2]). As expected, all controls tested negative. Furthermore, we successfully amplified the DNA extracted from positive PBMC samples after the in situ RT-PCR procedure with a second round “nested” PCR, using NCR3 and NCR4 as internal primers (Table 2).

To rule out the possibility that the fluorescent amplicon might be caused by HCV RNA merely attached to the external membrane of the cells, we performed a control experiment where approximately 10^7 PBMC isolated from a healthy blood donor were mixed for 5 minutes with 100 μL of plasma known to contain HCV RNA: in situ RT-PCR performed on samples of these cells failed to give any HCV-specific fluorescence.

Because established cell lines expressing known amount of HCV RNA are lacking, the sensitivity of our in situ RT-PCR assay was evaluated targeting β-actin mRNA, which is constitutively present in PBMC. The same procedure outlined above was followed with β-actin mRNA specific fluorescein-labeled primers (Table 2) and up to 99% PBMC tested positive (data not shown).

Using the “tagged” RT-PCR approach, the negative-stranded HCV RNA was specifically detected within PBMC of 12 of the 28 patients, of whom all tested positive by in situ RT-PCR for the positive strand of the HCV genome (Table 1 and Fig 3), whereas the final wash of each sample was negative.

**DISCUSSION**

The presence and active replication of HCV within PBMC was previously reported in a proportion ranging from 53% to 100% of patients with chronic hepatitis C. However, by conventional RT-PCR the percentage of HCV-infected PBMC and the precise cellular location of the viral sequences were not determined as yet, and contamination due to infected serum particles attached to the external cell membrane could not be ruled out completely.

A significant breakthrough in the field of molecular research has recently occurred thanks to the development of in situ PCR, a technique that couples the extremely high sensitivity of PCR with the advantages of in situ hybridization, allowing the preservation of cell morphology and the localization of the positive signal within intact cells. In addition to the morphological examination, the in situ PCR assay performed in suspension on isolated cells allows further analysis by flow cytometry. Thus, we have developed an assay that, combining in situ RT-PCR with flow cytometric analysis, permits the unambiguous identification and localization of the amplified HCV genome at single-cell resolution. As expected, in situ RT-PCR greatly enhances the chances of detecting HCV-infected PBMC with respect to in situ hybridization, and clearly identifies PBMC cytoplasm as the cellular compartment where the HCV genome is located. Among patients with chronic hepatitis C, those with detectable HCV-infected PBMC do not differ from the others with regard to age, gender, and liver disease activity, evaluated as ALT levels and hepatic histology. However, it is tempting to hypothesize that HCV, via lymphoid cells infection, might interfere with the normal immunologic mechanisms of the host. Only a close clinical and immunologic follow-up, therefore, will indicate whether those patients in which HCV infection of PBMC is conspicuous are more likely to develop over time HCV-related extra-...
Fig 1. Fluorescence microscopy evaluation of PBMC after in situ RT-PCR. (A) Positive sample. At low magnification, scattered brightly fluorescent cells are clearly visible. Nuclear counterstaining is propidium iodide. (B) Positive sample. At higher magnification it is possible to appreciate the HCV-specific amplicon confined to part of the PBMC cytoplasmic compartment and surrounding the nucleus, which is counterstained with propidium iodide. (C) Negative sample. No accumulation of the fluorescent signal is observed. Nuclear counterstaining is propidium iodide. A similar negative pattern was also obtained with normal PBMC mixed with HCV RNA before performing the assay (for details, see Results).
hepatic disorders, such as mixed cryoglobulinemia and low-grade B-cell malignant lymphoma. Furthermore, in association with other quantitative techniques, the determination of HCV-infected PBMC could be useful to acquire quantitative information on the PBMC compartment, which, added to the other two sites where HCV is known to accumulate (plasma and liver), would allow to evaluate the global viral load and, possibly, to elucidate the HCV kinetic between different tissues and cell types. In addition, in situ RT-PCR could be a promising approach, at least theoretically, to also identify which PBMC cell type is preferentially infected by HCV.

Interestingly, all 6 OLT patients had HCV-infected PBMC detectable with our technique: the immunosuppressive regimen, already known to enhance plasma viremia, is likely to favor the spread of HCV also within PBMC, which in this particular subset of immunosuppressed patients might represent an important additional site of viral persistence and replication. The 2 OLT patients who were developing acute liver graft rejection (nos. 26 and 27) had the highest proportion of HCV-infected PBMC: whether a shift of the virus from the hepatocellular to the PBMC compartment may take place as a consequence of massive cytolitic damage of the liver remains to be clarified.

The observation that HCV replication does occur within PBMC in a significant proportion of our HCV-infected patients suggests that the infection of PBMC is an active process, whose long-term effects are at present only speculative.

In conclusion, our findings not only support the notion that PBMC may act as an extrahepatic viral ‘‘reservoir,” but also favor the hypothesis that HCV, penetrating and actively replicating inside immune system cells, might be able to modulate or even derange immune system functions. Because circulating lymphocytes, at any given time, represent only about 2% of the total lymphocyte pool, most of which is in the lymphoid organs (ie, BM, spleen, lymph nodes) to better elucidate the pathogenic pathways of interaction between HCV and immune system cells.

Fig 3. Detection of negative-stranded HCV RNA within PBMC by “tagged” RT-PCR. (A) “Nested” PCR products of the expected size (82 bp) are visualized in lanes 3, 4, 6, 7, and 12 after 3.5% agarose gel electrophoresis and ethidium bromide staining. Boehringer VI molecular weight markers were run at both sides, along the external lanes. (B) Hybridization of the “nested” PCR products with an internal HCV-specific probe (see text for details).
IN SITU RT-PCR TO DETECT HCV RNA WITHIN PBMC

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