Hepatitis C Virus in Lymphoid Cells of Patients Coinfected with Human Immunodeficiency Virus Type 1: Evidence of Active Replication in Monocytes/Macrophages and Lymphocytes

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It has been reported that hepatitis C virus (HCV) may be lymphotropic in the setting of human immunodeficiency virus type 1 (HIV-1) coinfection. The present study was undertaken to determine the phenotype of lymphoid cells harboring replicating HCV in HIV-1–positive subjects. By means of highly strand-specific thermostable enzyme Tth–based reverse-transcriptase polymerase chain reaction, the presence of viral RNA–negative strand was sought in different subpopulations of peripheral blood mononuclear cells from 10 HIV-positive patients. HCV RNA–negative strand was most commonly present in monocytes/macrophages (4 cases), followed by CD8+ and CD4+ lymphocytes (2 cases) and CD19+ cells (1 case). In 2 cases that were further analyzed, viral-negative strand remained detectable in monocytes/macrophages cultured for 3 weeks. Moreover, monocyte/macrophage- and serum-derived viral sequences differed in the 5′ untranslated region. These findings imply that, in HIV-infected subjects, HCV may replicate in the same cells as HIV-1, which raises the possibility of direct interactions between these pathogens.

Hepatitis C virus (HCV) is a common etiologic agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma [1, 2]. The overall prevalence of anti-HCV in the United States is 1.8%, and it is estimated that 2.7 million Americans carry the virus [3]. The HCV population consists of complex and dynamic distributions of closely related but nonidentical genomes, termed “quasispecies” [4–6]. The presence of this dynamic mutant reservoir is an important determinant of virus adaptability and could be directly related to its persistence, because constantly emerging viral escape mutants could evade extracellular neutralization [7]. Additionally, it could also facilitate the virus’s adaptation to replication in various cells.

One of the most controversial aspects of HCV infection is the presence of extrahepatic replication sites. In support of such a possibility, a number of studies report the relatively common detection of HCV RNA–negative strand, which is a viral replicative intermediate, in peripheral blood mononuclear cells (PBMC) [8–11]. This evidence, however, has been questioned, because commonly used techniques are limited in their ability to discriminate between RNA-positive and -negative strands [12]. Importantly, in several studies that used assays carefully optimized for strand specificity, HCV RNA–negative strand was not detected in PBMC [13–15].

Nevertheless, although the presence of extrahepatic viral replication in immunocompetent subjects remains the subject of controversy, there is evidence that it does occur in the setting of infection with human immunodeficiency virus type 1 (HIV-1). We have recently reported the detection of HCV RNA–negative strand in lymph nodes and PBMC from 40%–60% of subjects coinfected with HIV-1 [16]. However, PBMC represent heterogeneous cell populations, and the phenotype of infected cells was not determined. Here we report the positive identification of lymphoid cell populations harboring actively replicating HCV.

Material and Methods

Biologic samples. Subjects for the study were recruited from consecutive HIV-1–positive patients who presented for clinical care in September and October 1998 at a specialized AIDS center in Warsaw. The inclusion criteria were HCV RNA positivity in serum and no anti-HCV or anti-HIV antiviral therapy before the study. Ten patients, all of them intravenous drug addicts, fulfilled the above criteria. Some laboratory data on studied subjects are presented in Table 1. PBMC were isolated from 40–50 mL of blood by centrifugation over density gradient (Ficoll-Paque; Pharmacia, Uppsala, Sweden). Cells were removed from the upper band located at the plasma and resolving medium interface and were washed 3 times with PBS (pH 7.4). The typical yield of PBMC was 2–4×10^7; these cells were determined to be free of granulocytes.
and platelets. About 10%–15% of total PBMC were stored unfractoned, whereas the remaining cells were incubated in plastic petri dishes at 37°C for 30 min. The fraction of adhering cells, which was composed exclusively of monocytes/macrophages, was washed and extracted for RNA as described later, whereas non-adhering cells were divided into 3 equal parts. The latter cells were subsequently sorted and purified by the immunomagnetic separation technique with use of monoclonal antibodies against specific surface antigens: CD4 (MT310; Dako, Carpinteria, CA), CD8 (DK25; Dako), and CD19 (B-C3; Biosource International, Camarillo, CA). Each cell fraction was incubated for 20 min at 4°C with 1 of the above antibodies and then with magnetic beads coated with goat anti-mouse IgG (Dynabeads M-450; Dynal, Oslo). The cells were further separated and washed in a magnet (MPC; Dynal), as suggested by the manufacturer.

The viability of the isolated cells was ascertained by trypan blue exclusion, and the purity of each subset was determined by flow cytometry (Cytoron Absolute; Ortho Diagnostics, Raritan, NJ) with specific antibodies. Each fraction contained 1–5 × 10⁶ cells, and the purity was ≥98%. All cells were stored frozen at −80°C until analysis. RNA was extracted from cells and 100 μL of serum by means of a modified guanidinium thiocyanate-phenol-chloroform technique with a commercially available kit (Ultraspec 2; Biotex Laboratories, Houston) and was dissolved in 20 μL of water. Of this RNA solution, 4–5 μL was reverse-transcribed as further described.

Fifteen HIV-negative patients with chronic hepatitis C served as a control group. Their PBMC were collected as described for the studied HIV-positive subjects. However, because unfractoned cells were persistently negative for the presence of HCV RNA-negative strand, they were not sorted into different phenotypes.

**Table 1.** Detection and titers of positive and negative strands of hepatitis C virus RNA in serum and different subsets of peripheral blood mononuclear cells (PBMC) of 10 human immunodeficiency virus (HIV)-infected subjects.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Serum</th>
<th>PBMC</th>
<th>CD4⁺ cells</th>
<th>CD4⁺ cells</th>
<th>CD8⁻ cells</th>
<th>CD19⁺ cells</th>
<th>Monocytes</th>
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<th>HIV RNA</th>
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**NOTE.** The titers of the positive (+str) and negative (−str) strands were determined by Tth-based strand-specific assays. Data are as follows: serum, eq/mL; PBMC, eq/10⁶ cells; CD4⁺, CD8⁻, and CD19⁺ cells, monocytes, and macrophages after culture, genomic eq/10³–10⁶ cells; HIV RNA, copies/mL. N, negative; ND, not determined.

Titors were determined by analyzing 10-fold serial dilutions of the RNA template. The titers were calculated by assuming that the end point dilution contains 10³ genomic eq when tested with the Tth-based assay. Appropriate measures, described elsewhere [14, 17], were used to prevent and detect contamination. All RT-PCR runs included positive controls, consisting of end point dilutions of synthetic RNA strands, and negative controls, including sera and PBMC from HCV-negative subjects.

Culture of monocytes/macrophages. To separate monocytes/macrophages from other cells, PBMC were collected from 20 mL of blood, suspended in RPMI, and incubated in plastic petri dishes at 37°C for 30 min. Nonadhering cells were removed by washing, and adhering cells (~10⁶ cells) were maintained in RPMI (Life Technologies Gibco BRL, Gaithersburg, MD) with 10% fetal bovine serum. After a 3-week culture, RNA was extracted from macrophages and stored at −80°C until analysis.

Culture of PBMC. PBMC from 15 control patients were cultured with or without polyclonal activators by use of standard procedures [18]. Briefly, ~5 × 10⁶ cells were resuspended at 10⁶/mL of RPMI either alone or in the presence of phytohemagglutinin (10 μg/mL; Wellcome Diagnostics, Research Triangle Park, NC) or pokeweed mitogen (1% v/v; Life Technologies Gibco BRL). After 72 h of incubation, the cells were pelleted, washed several times, and stored at −80°C until final analysis.

Analysis of HCV quasispecies. The analysis was conducted on the stable 5' untranslated region, because a small number of expected quasispecies allows for reliable comparison, and we previously found that variations in this region may correlate with extrahepatic replication [19]. For the purpose of sequence comparison, nested or heminested protocols were used to maximize the yield of PCR product [14, 19].

HCV sequences were compared by the single-strand conformation polymorphism (SSCP) assay, as described elsewhere [20]. In addition to its simplicity, the assay is highly sensitive: it can detect a minor variant admixture representing ≥3%/5% of the whole population. In brief, PCR products were purified with a DNA binding resin system (Wizard PCR; Promega, Madison, WI); 0.1 μg of the purified product in 9 μL of alkali denaturing buffer (50 mM NaOH, 1 mM EDTA) was mixed with 1 μL of SSCP loading buffer (95% formamide, 0.5% bromophenol blue, 0.5% xylene cy-
anal) and was subjected to nondenaturing 8% PAGE in 1× Tris borate-EDTA buffer with 400 V applied for 4–6 h at a constant temperature of 25°C. The bands were visualized with silver staining (Promega).

To lower the risk of artifactual polymorphism, the analysis was duplicated in an independent experiment with use of new RNA template. Both reactions had to be identical before the experiment was considered to be valid. To lower the risk of sampling error due to low copy number of the template [19], analysis of samples showing different SSCP band patterns was repeated after adjusting the amount of viral template to be about the same in each compared reaction.

All SSCP-analyzed products were sequenced directly in both directions by the Sanger dideoxy chain-termination method with a modified T7 DNA polymerase (Sequenase version 2.0 kit; United States Biochemical, Cleveland). To rule out incorporation errors by Taq polymerase, direct sequencing was routinely repeated from a new amplification reaction.

The RNA secondary structures were predicted by means of the program RNA Structure, version 2.52 (University of Rochester, Rochester, NY) [21, 22]. HIV-1 load was measured with a commercially available quantitative assay (bDNA assay 3.0; Chiron Diagnostics, Emeryville, CA).

**Results**

All 10 subjects were positive for the presence of HCV RNA–plus strand in serum and PBMC (table 1). HCV RNA–negative strand was not detected in any of the serum samples. However, PBMC samples from 6 patients were positive for the presence of HCV RNA–negative strand when tested with the Tth-based strand-specific assay in 2 independent experiments (table 1). When individual subpopulations of PBMC were studied, virus-negative strand was most commonly present in the monocyte/macrophage fraction (4 cases), followed by CD8 and CD4 lymphocytes (2 cases) and CD19 cells (1 case). These reactions were highly unlikely to represent false-positive results, because nonspecific detection of the incorrect strand might be expected when the latter is present at high number, at least 10⁸ genomic eq/reaction, which was not encountered in any of the samples studied (table 1). For 4 patients in whose unsorted PBMC HCV RNA–negative strand was not detected, all of the analyzed cell fractions were negative as well.

When 4 control liver tissue samples were tested, HCV RNA–positive strand was detected in all livers in titers ranging from 10⁴ to 10⁸ genomic eq/μg of RNA, and HCV RNA–negative strand was present at a titer 1–2 logs lower (not shown).

To exclude the possibility that the HCV RNA–negative strand present in monocytes/macrophages was associated with phagocytosed liver debris, additional control experiments were undertaken. First, monocytes were collected from 4 patients without evidence of HCV replication in monocytes/macrophages (patients 5–8) and from 2 patients in whom HCV RNA–negative strand was detected in these cells (patients 1 and 2). The monocytes/macrophages were then cultured in vitro for 3 weeks, after which RNA was extracted and tested for the presence of HCV RNA–negative strand with the strand-specific assay. HCV-negative strands were detected in cultured macrophages from patients 1 and 2 but not in macrophages from the remaining 4 patients (table 1).

In the next step, viral RNA–positive and –negative strands were amplified from cells before and after culture and compared by SSCP with one another and with the positive strand in serum. In both cases, the band patterns for positive strands derived from serum and monocytes/macrophages were initially indistinguishable but became dissimilar after culture (figure 1). However, virus-negative strand in the monocytes/macrophages was different from the serum-derived fraction from the beginning. This initial lack of difference between serum- and macrophage-derived positive strands most likely reflected virions

![Figure 1](image)
Figure 2. Nucleotide sequence alignment of 5' untranslated region fragment of hepatitis C virus (HCV) recovered from serum and monocytes/macrophages in patients (Pt) 1 and 2. Sequences are compared with the prototype sequence of HCV-1 published by Choo et al. [23], shown on top line. Dots (.), sequence identity with HCV-1; +, positive strand; −, negative strand; a, uncultured monocytes/macrophages; b, monocytes/macrophages cultured for 3 weeks. Both positive- and negative-strand “master” sequences recovered from cultured macrophages differed by several nucleotide substitutions from the serum consensus sequences, whereas positive-strand viral sequences recovered from monocytes/macrophages before culture were identical to serum sequences. In Pt 2, the pre- and post-culture negative-strand sequences differed by a single nucleotide.

The 5' untranslated region was reported to be very conserved structurally; the location of identified differences between the serum- and macrophage-derived sequences in the proposed conserved stem-loop structure [24, 25] is presented in figure 3. As seen, the predicted secondary structure was generally not affected, because the differences either were localized in the non-base-paired terminal loop or were compensatory, and therefore the base pairing was maintained. Similarly, other differences between the pairs of sequences outside the stem-loop did not appear to affect the stability of postulated secondary structures.

All 15 HIV-negative controls were positive for the presence of HCV RNA–plus strand in serum and PBMC. When tested with the Tth-based strand-specific assay, HCV RNA–negative strand was not detected in any of these samples. However, after 72 h of culture in the presence of phytohemagglutinin, cells from 2 patients became positive for the presence of virus-negative strand, whereas all pokeweed mitogen–stimulated cell cultures remained negative.

Discussion

This is the first study identifying the phenotype of HCV-infected lymphoid cells from HIV-1–positive subjects by means of a highly strand-specific Tth-based assay. In our previous report, we provided evidence for the common presence of active HCV replication in lymphoid tissue from HIV-infected subjects. Although the phenotype of infected cells was not determined at that time, circumstantial evidence pointed to the cells of the monocyte/macrophage lineage [16]. The current study con-
Figure 3. Predicted secondary structures of consensus 5’ untranslated region sequences derived from serum (S) and cultured macrophages (M) in patients (Pt) 1 and 2 (see also figure 2). Sequence differences are indicated in bold. In Pt 2, sequence recovered from cultured macrophages differed by single substitution (arrow) from initial negative-strand sequence. Free energy (ΔG) is expressed in kJ/mol.

confirmed this conjecture, because the presence of viral replicative forms could be documented in monocytes/macrophages from 4 of 6 patients in whom HCV RNA–negative strand was present in unfractioned PBMC. Importantly, we showed that these cells can retain HCV RNA–negative strand for up to 3 weeks in cell culture, and strains infecting monocytes/macrophages may be different from those circulating in serum. The latter evidence is crucial, as the presence of viral sequences in phagocytic cells could come, at least theoretically, from liver debris entrapped inside these cells. Infection of monocytes/macrophages is not unexpected: they are known to be permissive to a wide range of viruses, including some other flaviviruses [26]. Importantly, HCV replication was also detected in T and B lymphocytes.

In contrast to findings for HIV-positive patients, we did not detect HCV replication in PBMC from HIV-negative subjects either in the current or in our previous study [14], which used exactly the same Tth-based assay. Similarly, other studies [13, 15] failed to demonstrate the presence of HCV RNA–negative
strands in PBMC from HIV-negative subjects when they were sought with highly strand-specific RT-PCR assays. The reason for this discrepancy is currently unclear but is likely to be multifactorial. One obvious possibility is that the impairment of containment effects of the immune system enhances extrahepatic replication in HIV-positive subjects to higher and detectable levels. This view is supported by the finding that HCV RNA–negative strand may be found in PBMC from some liver transplant recipients after, but not before, transplantation [27] and by a recent study showing an occasional presence of HCV replication in hematopoietic cells inoculated into mice with severe combined immunodeficiency [28]. It is also likely that HCV replication is more efficient in activated cells. In support of this concept are our observations that after stimulation with phytohemagglutinin, PBMC from 2 of 15 HIV-negative subjects with chronic hepatitis C became positive for the presence of HCV RNA–negative strand. Although the phenotype of infection in patients with chronic hepatitis C became positive for the presence of HCV RNA–negative strand, although the phenotype of infection in patients with chronic hepatitis C became positive for the presence of HCV RNA–negative strand. Nevertheless, all of these scenarios are compatible with active replication of both viral agents in monocytes/macrophages remaining to be determined.

The biologic meaning of the described sequence differences between macrophage- and serum-derived sequences is unclear, one of the possibilities being that they represent adjustments by the virus to specific compartments. The 5′ untranslated region contains an internal ribosomal entry site [38, 39], and similar structures in the picornaviruses are likely to determine the host range of individual viruses [40]. Alternatively, 5′ untranslated region variations could identify virus subpopulations replicating with different dynamics or even defective viruses. Nevertheless, all of these scenarios are compatible with active replication in monocytes/macrophages.

In summary, we documented the presence of active HCV replication in monocytes/macrophages and also in T and B lymphocytes in HIV-infected patients. This implies that in HIV-infected subjects, HCV may replicate in the same cells as HIV-1, which raises the possibility of direct interactions between the 2 pathogens.

References