The Origin of Hepatitis C Virus Reinfecting Transplanted Livers:
Serum-Derived versus Peripheral Blood Mononuclear Cell–Derived Virus

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When hepatitis C virus (HCV) infection recurs after liver transplantation, it is unclear whether the liver graft is colonized by virions present in the circulation or by those associated with peripheral blood mononuclear cells (PBMC). In 6 HCV-infected transplant recipients, HCV sequences were analyzed by the single-strand conformational polymorphism (SSCP) assay and direct sequencing in pretransplant-paired PBMC and serum samples and in posttransplant follow-up serum samples. In 2 patients, SSCP patterns for pretransplant PBMC-serum pairs were identical, while in 4 patients they were different. In 3 patients from the latter group, the postransplant viral sequences resembled those found in pretransplant serum samples, whereas in the other patient from that group, viral sequences after transplantation were transiently identical to those found in pretransplant PBMC. In HCV-positive liver transplant recipients, the liver graft is colonized primarily by liver-derived virus remaining in the circulation. However, virus variants of likely extrahepatic origin can be detected in serum early after transplantation.

Hepatitis C virus (HCV) is a common cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma [1–3]. Of importance, chronic hepatitis develops in >80% of acutely infected individuals, and 20%–35% of them could develop cirrhosis [1, 4, 5]. The overall prevalence of anti-HCV in the United States is 1.8%, and ~2.7 million Americans carry HCV [6]. Liver transplantation is currently the only life-saving alternative in end-stage HCV-related liver disease; major American centers report that ~25%–30% of their transplant candidate pools consist of HCV-infected patients, and this number is increasing [7–9]. However, HCV infection almost invariably recurs after transplantation, leading to increased graft loss and mortality, although the individual course varies, ranging from benign to rapid liver insufficiency, chronic hepatitis, and cirrhosis [10].

In the majority of cases, the patients are infected by the same virus strain that was present before transplantation [11–13], and it seems logical to assume that the liver graft is colonized by virions left in the circulation after the explant organ is removed. However, the results of several studies suggest that HCV could replicate at extrahepatic sites, particularly in the cells of bone marrow origin [14–16]. Peripheral blood mononuclear cells (PBMC) were already proposed to be the source of recurrent infection after transplantation [12], although no studies were conducted to support or disavow this hypothesis.

Determining whether the infecting strain is derived from the liver or whether its origins are extrahepatic requires that both involved strains are readily discernible. We recently provided evidence that HCV strains associated with PBMC commonly differ from those circulating in serum in the 5′ untranslated region (5′ UTR), and the presence of these differences correlates with the presence of viral replication within these cells [17, 18]. In the current study, we analyzed 6 patients with end-stage HCV-related liver disease who received uninfected liver grafts. Viral sequences were analyzed in pretransplant-paired PBMC and serum samples, and, when found to be different, sequences amplified from sequential posttransplant serum samples were studied.

Subjects and Methods

The subject group consisted of 6 patients who underwent orthotopic liver transplantation for end-stage HCV-related liver cirrhosis and received a graft from an HCV-negative donor. These subjects were part of a previously published large study on the outcome of liver transplantation in HCV-positive recipients [19]. Neither the patients nor their donors had serologic evidence of hepatitis B infection. The donors were anti-HCV negative, and the liver graft tissue was negative for the presence of HCV RNA by reverse-transcription (RT) polymerase chain reaction (PCR). Paired serum and PBMC samples were obtained from recipients just before transplantation, and the follow-up samples were collected at 1, 2, and 4 weeks. All samples were kept at −80°C until analysis.
PBMC were isolated by standard Ficoll-Hypaque (Pharmacia) density-gradient centrifugation, were washed 3 times with PBS (pH 7.4), and were stored frozen at –80°C. RNA was extracted from $3 \times 10^5$ to $1 \times 10^7$ cells or from 100 μL of serum, using commercially available kits (UltraSpec 2 and UltraSpec 3; Biotex Laboratories), and then dissolved in 30 μL of water. Ten microliters of this RNA solution was used for each RT-PCR.

The analysis was conducted on the stable 5′ UTR for the following reasons: (1) we have previously found that HCV strains associated with PBMC commonly differ in this region from those circulating in serum, thus providing a convenient "signature" for each virus variant [17]; (2) a small number of expected variants within the quasi species allows for a reliable tracking of involved strains; and (3) analysis of the E2 region may be unreliable because PBMC selectively adsorb viral subpopulations that differ in this region [17].

Previously described extensive measures [20, 21] were employed to prevent and detect carry-over contamination. All RT-PCR runs included positive controls consisting of end-point dilutions of synthetic RNA strand, and negative controls included uninfected PBMC and serum samples. The HCV genotypes were determined by analyzing the NS5 region, as described elsewhere [20].

For the purpose of sequence comparison, nested protocols were used to maximize the yield of PCR product. The 5′ UTR was amplified by use of RT-PCR, as described elsewhere [18].

HCV quasi species were compared by the single-strand conformational polymorphism (SSCP) assay, as described elsewhere [18], with minor modifications. This assay is highly sensitive: we were routinely able to detect any minor variant representing ≥3% of the whole population. In brief, PCR products were purified with a DNA binding resin system (Wizard PCR; Promega) and resuspended in 50 μL of water. Next, 2–4 μL of the purified product was diluted in 15 μL of low-ionic-strength solution (10% saccharose, 0.5% bromophenol blue, and 0.5% xylene cyanol), denatured by heating at 97°C for 3 min, immediately cooled on ice, and subjected to non-denaturing 8% PAGE in 1× Tris-borate–EDTA buffer, with 400 V applied for 4–5 h at a constant temperature of 25°C. The bands were visualized with silver staining (Silver Stain; Promega). All analyzed products were sequenced directly in both directions, using an automatic sequencer (ABI 377; Perkin-Elmer).

To lower the risk of artificial polymorphism, the above SSCP and sequence analysis was duplicated in an independent experiment using a new RNA template: the results of this control experiment were consistent with the initial findings. Since the analysis of posttransplantation samples required that the pretransplantation PBMC and serum sequences be different, patients 5 and 6 were excluded from further analysis.

In the next step, sequential follow-up serum samples from patients 1–4 were compared with respective pretransplantation serum samples and PBMC samples by SSCP assay (figure 3). The posttransplant 5′ UTR band pattern for patients 2–4 was more similar to that found in pretransplant serum samples than to that associated with pretransplant PBMC samples, suggesting that serum-derived virions colonized the new liver in these patients. In patient 3, in whom the pretransplant "master" serum and PBMC sequences were different, infection of liver graft by virus variants present in serum was confirmed by direct sequencing (figure 2).

In patient 1, the SSCP band pattern 2 weeks after transplantation was identical to that observed in pretransplantation PBMC but was clearly different from that found in pretransplant serum. After another 2 weeks, the band pattern changed (figure 2). Direct sequencing confirmed that the 5′ UTR consensus sequence at 2 weeks was identical to that amplified from pretransplant PBMC. However, the consensus sequence amplified 4 weeks after transplantation closely resembled the pretransplant serum sequence and was different from PBMC-associated sequences (figure 2).

Sequence analysis of the NS5 region revealed that patients 5 and 6 were infected by genotype 1a strains, patients 2 and 4 were infected by genotype 1b, patient 1 was infected by genotype 2b, and patient 3 was infected by type 4 strain.

**Results**

Viral 5′ UTR sequences were successfully amplified from all analyzed PBMC and serum samples, except for the serum obtained from patient 1, a week after transplantation. When analyzed by the SSCP assay, the RT-PCR products amplified from paired pretransplant PBMC and serum samples had dissimilar band patterns for patients 1–4, whereas the band patterns were virtually indistinguishable for patients 5 and 6 (figure 1). However, when RT-PCR products were sequenced directly, differences in pretransplant PBMC- and serum-derived consensus sequences were observed for only patients 1 and 3 (figure 2). This was not unexpected, because major SSCP bands, representing dominant virus variants, were different in patients 1 and 3 but not in patients 2 and 4 (figure 1).

**Discussion**

The current study represents the first attempt to determine if the graft in HCV-positive patients undergoing liver transplantation is reinfected by the viruses circulating in serum or by the virions associated with PBMC. In 2 of 6 patients studied,
the source of reinfection could not be determined because pre-
transplant PBMC- and serum-derived viral sequences were
identical. In 3 of the remaining 4 patients, the posttransplant
viral sequence matched pretransplant serum sequences, sug-
gest that the liver graft was colonized by liver-derived viri-
ons left in the circulation. However, in 1 case, the posttransplant
viral sequence matched the pretransplant PBMC-derived se-
sequence at 2 weeks but was almost identical to the pretransplant
serum sequence at 1 month.

The origin of the strain detectable in patient 1 at 2 weeks after
transplantation is unclear. One possibility is that it originated at
extrahepatic sites and did not represent virus replicating in the
liver. HCV titers fall precipitously within the first few days after
transplantation [23–25], and any virus that didn’t originate in
the liver could become dominant during that time. The fact that
the virus titer was low (<10^3 viral Eq/mL) and serum collected
at 1 week was negative for the presence of HCV RNA supports
the notion of a nonhepatocyte origin of this virus. Contribution
of extrahepatic virus to the circulating virus pool seems to be
small: in our previous study, we did not detect PBMC-derived
viral sequences in the background of liver-derived virus [17],
and removal of infected organ resulted in lowering of the virus

![Figure 2](https://academic.oup.com/jid/article-abstract/185/4/417/849639)

**Figure 2.** Nucleotide sequence alignment of the 5’ untranslated region of hepatitis C virus (HCV) recovered from serum and peripheral blood mononuclear cells (PBMC) in patients 1 and 3. Sequences are compared with the prototype sequence of HCV-1 published by Choo et al. [22], shown on the top line. Hyphens (-) indicate sequence identity with HCV-1, and underlines (_) indicate gap introduced to preserve se-
sequence alignment. In patient 1, the consensus sequence at 2 weeks was identical to that amplified from pretransplant PBMC; however, the con-
ensus sequence amplified from serum 4 weeks after transplantation closely resembled pretransplant serum sequence. In patient 3, all posttrans-
plant viral sequences were identical to that found in pretransplant serum. C, pretransplant PBMC; Pt, patient; S, pretransplant serum; 1w, 2w, and
4w, follow-up serum samples collected 1, 2, and 4 weeks, respectively, after transplantation.

![Figure 3](https://academic.oup.com/jid/article-abstract/185/4/417/849639)

**Figure 3.** Analysis by single-strand conformational polymorphism (SSCP) assay of the 5’ untranslated region of hepatitis C virus (HCV) se-
quences amplified from follow-up serum samples from 4 HCV-infected liver transplant recipients in whom the pretransplant SSCP patterns for
serum- and peripheral blood mononuclear cell (PBMC)–derived viral sequences were different. Serum samples obtained 1, 2, and 4 weeks (1w, 2w, and 4w, respectively) after transplantation were compared with pretransplant PBMC (C) and serum (S) samples. The posttransplant band pattern
for patients 2–4 was more closely related to that found in pretransplant serum than to that associated with PBMC, suggesting that serum-derived vi-
rions colonized the liver graft in these patients. In patient 1, the SSCP band pattern 2 weeks after transplantation was identical to that observed for pre-
transplant PBMC, but it changed after another 2 weeks. The results of direct sequencing in patients 1 and 3 are shown in figure 2.
titer by several logs [23]. Another possibility is that the virus detectable 2 weeks after transplantation originated in the liver, but, since it was adapted to lymphoid tissue, its initial replication was inefficient. Thus, the variant detectable at 1 month could represent the pretransplant liver-derived virus, which finally caught up and overgrew the PBMC-associated variant. Obviously, because of sensitivity limitations of the techniques used, it cannot be excluded that a minor variant present in PMBC was in fact responsible for liver infection.

Some studies have implicated genotype 1b in poor prognosis following liver transplantation [25], and it remains possible that different genotypes recur differently. However, this problem could not be properly addressed because the patients studied were infected with different genotypes.

The possibility that a transplanted liver might be infected by extrahepatic strains associated with PBMC may be seen as counterintuitive, since the graft is initially submerged in a large pool of circulating, liver-derived virus particles. However, since the free, circulating virus has a very short half-life, virus titers immediately fall precipitously after the removal of infected liver [23–25], thus possibly increasing the relative proportion of any virus that did not originate in the liver. Infected cells could be an efficient mode of spreading infection by other viruses; for example, macrophage-tropic variants of human immunodeficiency virus type 1 are likely to be responsive for perinatal and sexual transmission [26, 27]. In liver transplant recipients, lymphoid and macrophage cells enter the transplanted organ soon after transplantation [28], and cells trafficking to and from the liver graft can carry HCV [29].

The presence of extrahepatic replication in PBMC is still widely debated because negative-strand HCV RNA, which is a viral replicative intermediate, is generally not detected in these cells when proper strand-specific assays are employed [21, 30, 31]. However, strand-specific assays are relatively insensitive, and, in healthy immunocompetent subjects, any HCV replication in PBMC may be below the detection limit of the method. Studies supporting these assumptions are demonstrating active extrahepatic HCV replication in immunosuppressed subjects [15, 18] and in human hematopoietic cells inoculated into mice with severe immunodeficiency [14]. HCV replication in PBMC can also become detectable once the cells are stimulated with phytohemagglutinin, and, even in immunocompetent subjects, PBMC often contain virus variants differing from those circulating in serum [15, 17]. The pathogenic implications of extrahepatic HCV replication in the lymphoid system are still unknown and could be related to such extrahepatic manifestations of infection as mixed cryoglobulinemia and non-Hodgkin’s lymphoma [32]. However, the current study shows for the first time, to our knowledge, that this replication is productive, because viremias enter the circulation and could play a role in transmission of infection.

In summary, our study suggests that, in HCV-positive liver transplant recipients, the liver graft is primarily colonized by liver-derived virus remaining in the circulation. However, virus variants of extrahepatic origin can be detected in serum early after transplantation, although their role in recurrent infection is unclear.

References


