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Sporadic Reappearance of Minute Amounts of Hepatitis C Virus RNA After Successful Therapy Stimulates Cellular Immune Responses

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Abstract

BACKGROUND & AIMS—Several studies have reported hepatitis C virus (HCV) RNA sequences in the circulation after treatment-induced or spontaneous recovery. We investigated whether the HCV RNA represents persistence of HCV infection or reinfection.

METHODS—We studied 117 patients who recovered from HCV infection (98 following therapy and 19 spontaneously). A reverse-transcription polymerase chain reaction assay was used to detect the 5′-untranslated region of HCV. T-cell responses were studied by enzyme-linked immunospot for interferon-γ.

RESULTS—Plasma samples from 15% of treatment-recovered patients and no spontaneously recovered patient tested positive for HCV RNA. Lymphocytes from 3 patients who responded to therapy and 1 who recovered spontaneously tested positive. The frequency of HCV RNA detection in plasma correlated inversely with the time after the end of treatment. Post-treatment HCV 5′ - untranslated region sequences matched pretreatment sequences in 85% of cases. T-cell responses were significantly greater at time points with detectable trace amounts of HCV RNA than at time points without detectable HCV RNA ($P = .035$) and were primarily against nonstructural HCV antigens. The immune hierarchy was preserved over 5 years in patients whose post-treatment HCV RNA sequences matched pretreatment sequences, indicating HCV RNA persistence. An altered immune hierarchy with dominant immune responses, shifting from nonstructural to structural antigens, was observed in a single patient whose post-treatment HCV genotype differed from that of the pretreatment genotype, indicating HCV reinfection.

CONCLUSIONS—Trace amounts of HCV RNA of pretreatment sequence persisted and reappeared sporadically in the circulation within 8 years after recovery from hepatitis C but not thereafter, indicating that patients are cured of HCV infection. Reappearance of HCV RNA induced HCV-specific T-cell responses.

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Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.10.048.

Conflicts of interest

The authors disclose no conflicts.

Keywords

Interferon; IFN; Liver Disease; Virology

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is one of the most common viruses worldwide. Approximately 25% of infected patients clear the infection spontaneously within the first 12 months, and 75% remain chronically infected. Significant improvements in antiviral therapy for hepatitis C have occurred in the past 10 years. The current combination of pegylated (PEG) interferon (IFN) (PEG-IFN) and ribavirin (RBV) achieves a sustained virologic response (SVR) in 40%-70% of treated patients depending on genotype (reviewed in Feld and Hoofnagle[1]) with an SVR being defined as undetectable HCV RNA in the serum 6 months after the end of treatment. Patients are typically not followed for years after mounting an SVR.

Reports by several groups describe that HCV persists at low levels in individuals who had been diagnosed as having recovered from hepatitis C either spontaneously or after treatment.[2-7] These reports raised significant concerns among patients about the potential consequences of this state of infection. Whereas some investigators claimed that low-level HCV RNA persistence is associated with low-level necroinflammatory activity and steatosis in the liver and with an increased risk of hepatocarcinogenesis,[8] others reported that treatment-induced recovery from hepatitis C results in histologic improvement of liver disease[9,10] and in a reduction in liver-related mortality.[11,12] Likewise, it is unclear whether low-level HCV RNA is perpetually kept under check by the immune response or whether it predisposes to viral breakthrough. Even though only few case reports of high-titer HCV relapse have been reported in sustained treatment responders,[13,14] the true incidence of HCV relapse may not be known because the vast majority of treatment-recovered patients is not followed long-term, and HCV infection is typically clinically asymptomatic. Finally, it remains unknown whether the detected HCV RNA sequences reflect replication-competent, infectious virus and whether patients with low-level HCV RNA can transmit infection. The public health impact, eg, the possibility of HCV spread given the number of treatedpatients and the significance for blood and organ donation, are therefore not clear.

Reports on low-level HCV persistence have also aroused the interest of basic immunologists and virologists because it has been reported that other viruses such as hepatitis B virus and Epstein Barr virus are not completely eradicated but persist at minute levels.[15,16] Low levels of persisting antigen continue to stimulate virus-specific B and T cells and thereby help to maintain virus-specific immune memory.[15] However, in contrast to hepatitis B virus and Epstein Barr virus, HCV is an RNA virus that does not integrate into the host genome and does not exist in a latent form. It is therefore unclear how HCV may achieve low-level persistence. Furthermore, it has not been possible to differentiate between HCV persistence and de novo HCV infection because the sequence of the detected HCV RNA was either not determined or not compared with the pretreatment sequence[2,4,17] and because many of the studied subjects were injection drug users with an increased risk of re-exposure.[2]

This prompted us to look for evidence of low-level HCV RNA persistence and its impact on humoral and cellular immune responses in a pedigreed, large cohort of patients who had recovered from hepatitis C and were followed for up to 20 years in the Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases. We qualify the frequency of persisting minute levels of HCV RNA sequences both cross sectionally and prospectively and try to understand its significance virologically via sequence comparison with the pretreatment virus and immunologically via analysis of the concurrent humoral and cellular immune responses. The data provide reassurance that, although HCV RNA can

persist at minute levels, its presence in the peripheral circulation is limited to the initial few years after spontaneous and treatment-induced recovery. It is likely controlled by the immune responses that it triggers and does not appear to present a long-term risk.

Materials and Methods

Study Design and Population

Treatment-recovered patients (n = 98) had been treated either with IFN or PEG-IFN as monotherapy or PEG-IFN/RBV combination therapy for 24 or 48 weeks according to the standard of care at the time of treatment initiation. Sustained virologic responders tested negative by qualitative COBAS Amplicor HCV Test 2.0 (lower limit of detection: 100 IU/mL [270 copies/mL] serum [Roche Diagnostics, Branchburg, NJ]). Spontaneously recovered patients (n = 19) were anti-HCV positive by Abbott HCV EIA 2.0 (Abbott, Princeton, NJ) and HCV RNA negative. For spontaneously recovered patients, the year of HCV infection was estimated based on risk factors, and it was assumed that they recovered the same year as typical for spontaneous recovery. The cross-sectional analysis was performed between November 2004 and February 2009 with patients who returned to the clinic and donated a blood sample for our study. In a second part of the study, 85 patients with blood and/or peripheral blood mononuclear cells (PBMC) samples from multiple time points were followed longitudinally. All patients displayed normal alanine aminotransferase values, tested negative for HBV and human immunodeficiency virus, and gave written informed consent for research testing under protocols approved by the Institutional Review Board of National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. Plasma and PBMC from healthy, HCV-uninfected blood donors served as negative controls.

HCV RNA Extraction, Reverse Transcription, Amplification, and Sequencing

Plasma was separated from acid citrate dextroseanticoagulated blood tubes by centrifugation at 400*g* for 10 minutes and stored at −80°C. The remaining blood was diluted with an equal amount of phosphate-buffered saline. PBMC were isolated via density gradient centrifugation, washed thrice with phosphate-buffered saline, and cryopreserved with 70% fetal bovine serum, 20% RPMI, and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen. For some experiments, 200 μL fresh plasma was incubated in 800 μL AVL buffer (Qiagen, Valencia, CA) at 25°C for 10 minutes, and 10^6 PBMC were incubated in 200 μL of RNA*later* (Ambion, Austin, TX) at 4°C overnight prior to freezing.

RNA was extracted from plasma using the QIAmp Viral RNA kit (Qiagen) and from PBMC after homogenization with QIAshredders (Qiagen) using the RNeasy minikit (Qiagen). Total RNA was eluted in 50 μL RNase-free water, and 2 μL of 40 U/μL RNaseOUT (Invitrogen, Carlsbad, CA) were added. One fifth of the RNA was reverse transcribed with 4 pmol of the gene-specific primer 5′ -GCGGTTGGTGTTACGTTT (antisense; RTU1), 1 μL of a 10-mmol/L solution of each deoxyribonucleotide triphosphate, and 200 U of SuperscriptII (Invitrogen) in a final volume of 20 μL. A 243-base pair fragment of the HCV 5′ -untranslated region (UTR) was amplified by polymerase chain reaction (PCR) with the first round primers 5′ -CTGTGAGGAACTACTGTCTTC (sense; UTR1) and RTU1, and the nested primers 5′-GCAGAAAGCGTCTAGCCATGGC (sense; UTR2) and 5′-CTGCAAGCACCCTATGAGGCAGT (antisense; RTU2), which annealed to the 5′-UTR of all HCV genotypes. Primary and nested PCR conditions were as follows: 94°C for 2-minute initial denaturation followed by 40 cycles (94°C, 35 seconds; 60°C, 45 seconds; 72°C, 55 seconds) and 72°C, 5-minute final extension. Five microliters of the primary reaction product was used for the nested PCR. The sensitivity of the nested reverse transcription (RT)-PCR was determined using serial dilutions of 2 plasma samples of known

viral titer from patients with chronic HCV infection. An amplification efficiency of 95% was achieved with an input of < 40 RNA copies/mL in the reverse transcription step. PCR products were analyzed on 2% agarose gels containing ethidium bromide. Both strands of purified PCR products were directly sequenced using the ABI Prism Big-Dye Terminator Cycle Sequencing Reaction kit (Applied Biosystems, Foster City, CA).

Each sample was analyzed twice in independent experiments, and each experiment included alternating test samples and negative controls, ie, samples from anti-HCV negative blood donors without any history of HCV infection. Plasma and PBMC from chronic HCV patients served as positive controls.

IFN-γ Enzyme-Linked Immunospot Assay

Enzyme-linked immunospot (ELISpot) assays were performed exactly as previously described[18] using PBMCs that were stimulated in duplicate cultures of 3×10^5/well in RPMI 1640 containing 5% fetal bovine serum and 2 mmol/L L-glutamine for 42-48 hours with either (1) 600 fifteen-mer peptides (Mimotopes; Clayton, Australia), overlapping by 10 amino acids, covering the complete HCV genotype 1 polyprotein sequence and arranged in 18 pools of up to 42 peptides/pool[18]; (2) 1 μg/mL phytohemagglutinin (Sigma, St Louis, MO), or (3) DMSO. IFN-γ spots were counted using an AID ELISpot Reader Version 3.5 (Autoimmun Diagnostika GmbH, Straβberg, Germany). Average background (DMSO) responses were 27 spots/300,000 PBMC; responses against a pool of irrelevant HDV peptides were less. The number of HCV-specific spots was determined by subtracting the number of spots in the absence of HCV peptides (DMSO control) from the number of spots in the presence of HCV peptides.

Neutralizing Antibody Assay

Serial dilutions (from 1:200 to 1:3200 in duplicates) of heat-inactivated sera from patients and healthy individuals were incubated for 1 hour at 37°C with 100 focus-forming units (ffu)/well of either HCV (JFH1 strain) with genotype 2a structural proteins[19] or HCV (chimeric H-NS2/NS3-J virus) with genotype 1a structural proteins.[20] Cell of the human hepatoma line Huh7.5.1 were seeded in 96-well plates at 10^4 cells/well 24 hours prior to a 4 hour-infection with JFH1 or at 8×10^3 cells/well 24 hours prior to a 4 hour-infection with H-NS2/NS3-J virus. After removal of unbound virus in a single wash and addition of fresh Dulbecco's modified Eagle medium with 3% fetal calf serum cultures were incubated for 72 hours (JFH1 virus) or for 96 hours (chimeric H-NS2/NS3-J virus), respectively, followed by methanol fixation and HCV NS5A staining.[21] The number of ffu/well was determined with a light microscope. One ffu was defined as 1 or more infected cells, separated from other infected cells by at least 2 uninfected cells. The percentage of neutralization was determined by comparing infectivity in the presence of test sera with infectivity in the presence of control sera at the same dilution. The reciprocal 50% neutralization titer was calculated by linear interpolation using the formula [(50% – low percentage)/(high percentage - low percentage)] × (high dilution – low dilution) + low dilution.[22]

Statistical Analysis

Fisher exact test (2-tailed) was used to compare gender and ethnicity between groups. The nonparametric Mann-Whitney test was used to compare the median length of recovery. Analyses were performed with Prism software (version 3; GraphPad Software Inc, La Jolla, CA). A 2-sided *P* value of < .05 was considered significant.

Results

One hundred seventeen patients were included in this study and had been diagnosed as having cleared HCV either spontaneously ("spontaneously recovered") or, in response to state-of-the-art antiviral therapy ("treatment recovered") based on their medical history, the presence of HCV-antibodies and the absence of HCV RNA by Cobas Amplicor HCV Test. The female-to-male ratio was approximately 1:1 in both groups, and the median age did not significantly differ (Table 1). Most patients were of white ethnicity, and most had been infected with HCV genotype 1.

HCV RNA Is Not Detected in Plasma and Rarely Detectable in PBMC of Spontaneously Recovered Patients

Plasma and PBMC of spontaneously recovered patients were tested at a median of 20.8 years (interquartile range [IQR], 13.0-31.9 years) after HCV clearance. None of the 19 spontaneously recovered patients had detectable HCV RNA in plasma when a sensitive nested RT-PCR (sensitivity, <40 copies/mL) specific for the HCV 5′-UTR was employed (Table 2). Cryopreserved PBMC were available for 14 of the 19 patients and subjected to the same nested RT-PCR. As shown in Table 2, a single patient (1/14, 7%) tested positive for HCV RNA in PBMC but not in plasma. At this time point, HCV RNA had been undetectable in the plasma for more than 11 weeks. As shown in Figure 1, HCV-specific, T-cell responses were vigorous and multispecific targeting both structural and nonstructural HCV sequences. A second PBMC sample, collected 28 weeks later, tested positive by nested RT-PCR in the same assay, but the PCR band exhibited lower intensity (Figure 1*A*). Interestingly, a similar decrease in intensity was observed for the HCV-specific, T-cell response. HCV RNA was finally cleared from the PBMC compartment by week 93 and HCV-specific, T-cell responses decreased further (Figure 1*B*). These results suggest that low-level HCV RNA persistence is not a common feature of spontaneous HCV clearance.

Trace Amounts of HCV RNA Are Detected in Plasma and PBMC of a Subgroup of Treatment-Recovered Patients

Next, cryopreserved plasma and PBMC samples of treatment-recovered patients were analyzed for the presence of HCV 5′-UTR sequences with the same nested RT-PCR. Most treatment-recovered patients (83/98, 85%) were studied within the first 10 years after cessation of treatment. Fifteen of 98 (15%) patients tested positive for HCV RNA in the plasma, and 3 of 76 (4%) tested positive in the PBMC compartment (Table 2).

HCV RNA was just above the limit of detection for 14 of these 15 patients, ie, detectable in the nested, but not in the primary round of the RT-PCR reaction. One of the 15 patients (patient 75) displayed high HCV RNA levels that were quantifiable as 40,500,000 copies/mL (15,000,000 IU/ mL). This patient, a 58-year-old African-American male, had been treated for HCV genotype 1a infection with a 48-week course of IFN/RBV. He tested negative by Cobas Amplicor HCV Test 3, 4, and 6 months and 1.1 years after the end of treatment then did not return until 6.4 years after the end of treatment, the date of the current study. At this time, the patient remained clinically asymptomatic with normal alanine aminotransferase values, but HCV RNA titers remained > 13,000,000 copies/mL (>5,000,000 IU/mL) for at least 5 months of follow-up consistent with a virologic relapse between 1.1 and 6.4 years after the end of treatment.

Table 3 shows the clinical, virologic, and immunologic characteristics of all patients who tested HCV RNA positive. The sequences of the amplified PCR products differed among patients, and, except for patients 43 and 45, the post-treatment HCV genotype matched the pretreatment HCV genotype, which suggested that pretreatment HCV RNA persisted in

most patients. Patients who tested positive for HCV RNA did not differ in gender, race, or age from those who tested negative (not shown). Also, they did not differ in risk factors of infection. Most had acquired their initial HCV infection by transfusion of blood products, a 1-time, not recurrent event. Furthermore, they did not differ in treatment regimen or duration. In fact, most HCV RNA positive patients had received the optimal treatment for hepatitis C, a 48-week PEG-IFN/RBV combination therapy, and most had low pretreatment HCV RNA levels, a positive predictor for a treatment response.

Interestingly, however, the length of time that had passed since the cessation of therapy differed between those patients with detectable HCV RNA and those without (median of 3.1 years [IQR, 1.4-4.2] vs 5.6 years [IQR, 2.6-8.5], $P = .0073$ Mann-Whitney test, Figure 2*A*). Whereas 15 of 77 plasma from patients within 8.5 years after cessation of therapy tested positive for HCV RNA, all 21 samples obtained at later time points tested negative ($P = .035$, Fisher exact test). Finally, there was a decreasing trend in the prevalence of HCV RNA positive results when the first 8 years after end of therapy were split into 2-year intervals in this cross-sectional part of the study (Figure 2*B*). Collectively, these results suggest that the detection of HCV RNA becomes less frequent with increasing time after the end of treatment.

Trace Amounts of HCV RNA Persist in the Face of Neutralizing Antibodies

To evaluate the presence of neutralizing antibodies, we tested the capacity of patient plasma to block infection of human hepatoma cells by HCV with either genotype 2a (JFH1 virus)[19] or genotype 1a structural proteins (H-NS2/NS3-J virus).[20] Plasma samples from all patients with detectable HCV RNA blocked infection in this assay. Plasma samples from 6 patients (patients 45, 67, 43, 95, 26, and 61) neutralized HCV if the genotype of its structural sequences matched the genotype of the detected HCV RNA. Plasma samples from 5 other patients (patients 31, 41, 55, 40, and 13) neutralized HCV with either genotype 1a or genotype 2a structural proteins, which indicated the presence of cross-neutralizing antibodies. Thus, the SVR patients in this study maintain a humoral immune response that has been described as typical for patients with chronic HCV infection.[23]

Trace Amounts of HCV RNA Stimulate HCV-Specific T cells

HCV RNA, antibodies, and T-cell responses were analyzed prospectively for those patients for whom multiple samples were available. HCV RNA positive time points were interspersed with HCV RNA negative time points when individual patients were studied prospectively (Figure 3). PBMC were available for a subgroup of these patients (Figure 4). Interestingly, HCV-specific, T-cell responses were stronger at time points when trace amounts of HCV RNA were detected than at time points when they were not ($P = .039$, Fisher exact test, paired testing). This pattern was not only observed for patients with low-level HCV RNA recurrence (Figure 4*A*) but also for patient 75, who experienced a relapse with high HCV RNA titers (Figure 4*B*). In contrast, antibody responses did not differ in strength or specificity at HCV RNA positive and negative time points (not shown), and immune responses remained relatively constant in those who tested repeatedly HCV RNA negative (Figure 4*D*). The strength of the T-cell response was comparable with that of 10 patients with chronic HCV infection who were tested for comparison (median, 111 IFN-γ producing cells per 300,000 PBMC [IQR, 31-214]; Supplementary Figure 1).

Interestingly, the dominant T-cell responses targeted nonstructural HCV sequences when pretreatment HCV RNA sequences were detected (Figure 4*A* and *B*). This preferential targeting of nonstructural HCV sequences was preserved over years and maintained at those time points at which HCV RNA became detectable and T-cell responses increased. Because nonstructural HCV proteins are not part of the HCV particle and expressed only in infected

cells, this result suggests that increased HCV-specific T-cell responses may have been triggered by increased HCV translation. The preserved immune hierarchy also suggests that persisting HCV RNA was the source of the increased HCV protein generation. In contrast, for patient 45, the dominant immune response switched from nonstructural proteins to predominantly structural HCV antigens when HCV RNA became detectable in the blood (Figure 4*C*). Interestingly, this was the sole patient for whom the post-treatment HCV RNA genotype differed significantly from the pretreatment HCV genotype (genotype 3 to 1a switch), which is consistent with a new HCV infection inducing new T-cell responses and altering the immune hierarchy.

Discussion

This study detected HCV RNA in the plasma of a small proportion of treatment recovered patients (15/98, 15%) by an in-house nested RT-PCR even though they had been classified as sustained treatment responders based on undetectable plasma HCV RNA by Cobas Amplicor HCV Test. Because plasma samples from 14 of 15 HCV RNA positive patients tested positive only in the nested round of the PCR, the HCV RNA concentration was below the detection limit of the Cobas Amplicor HCV Test. Furthermore, HCV RNA positive bleed dates were interspersed with HCV RNA negative bleed dates for each patient, which suggests that the frequency of HCV RNA detection may be underestimated if only a single bleed date per patient is studied as typical for a cross-sectional study. The true prevalence of HCV persistence is likely higher than reported in this study because (1) patients were not tested at close intervals, (2) the liver was not studied, and (3) the sensitivity of the assay was limited to 40 copies/mL. Detection of HCV RNA was most frequent in the early years after cessation of treatment, and one of the patients in this group experienced high level HCV relapse in this time period similar to case reports of HCV relapse in 3 immune suppressed patients 0.5 years, 2 years,[14] and 8 years[13] after cessation of therapy. Whereas 15 of 77 plasma samples from patients within 8.5 years after cessation of therapy tested positive for HCV RNA, all 21 samples obtained at later time points tested negative ($P = .035$, Fisher exact test), which supports the notion that HCV is eventually completely cleared. This is consistent with results by Morishima et al, who reported that HCV RNA level declined from transcription mediated assay (TMA) positive to TMA discordant to TMA undetectable.[24] It is further supported by the observation that plasma samples from all spontaneously recovered patients, the majority of whom were studied more than 8 years after HCV infection, tested HCV RNA negative as well.

HCV RNA positive patients did not differ from HCV RNA negative patients in terms of age, gender, or HCV genotype. Also, treatment duration and regimen did not explain the persistence of low levels of HCV RNA. In fact, the majority of HCV RNA positive patients had received a 48-week course of PEG-IFN/RBV therapy, the optimal therapy according to the current state of the art. The post-treatment PCR results were unlikely to represent false positives because of the stringent precautions to prevent intersample contamination. In addition, the heterogeneity among the viral sequences obtained from patients precludes the presence of a common, contaminating laboratory strain. Finally, the HCV genotype after interferon-based treatment matched the pretreatment genotype in 11 of 13 (85%) of all patients, indicating persistence of autologous HCV RNA.

The topic of HCV RNA persistence has been controversially discussed in the literature. Several studies suggested that HCV is completely eradicated from serum and PBMC of spontaneously recovered or successfully treated patients.[25,26] At the other extreme, 2 laboratories reported HCV RNA in more than 90% of serum and PBMC samples from spontaneously recovered[2,17] and treatment-recovered patients.[2,3] Whereas we employed RNA extraction, RT, and PCR techniques identical to the ones used by Pham et al,[2] we

detected HCV RNA only in a minority of samples. This discrepancy may be explained by the fact that some of the previous studies included a high proportion of injection drug users[7] and subjects with unknown risk factors[17] in whom the risk of reinfection/re-exposure is increased. In contrast, the majority of HCV RNA positive patients of this cohort acquired the initial HCV infection via transfusion and, thus, was less likely to have been exposed a second time. Also, in contrast to our study, neither Pham et al[7] nor Radkowski et al[17] confirmed that the detected HCV sequence represented persisting HCV RNA, ie, that it matched the sequence in the initial HCV infection. The percentage of HCV RNA positive test results in our study is more consistent with the results obtained by Morishima et al,[24] Hoare et al,[27] and George et al,[28] who detected HCV RNA in a smaller proportion of treatment responders by nested RT-PCR or TMA, respectively, in approximately 6% of the studied patients.

What can be the source of the detected HCV RNA? We propose that PBMCs are not the source for the HCV RNA detected in plasma. First, HCV RNA was less frequently detected in the PBMC compartment than in plasma samples in our samples. Second, whereas HCV may bind to and be phagocytized by B cells and dendritic cells (reviewed in Timpe and McKeating[29]), these cells do not support HCV replication in vitro[30] and do not express claudin-1, an obligatory receptor for HCV infection that is solely expressed in the liver.[31] Instead, we favor localized foci of HCV replication in the liver as the most likely source for the detected HCV RNA. This theory derives support from reports that HCV sequences were found in the liver even when they were not detectable in the blood.[3,26,32] Studies to determine the infectious nature of the residual RNA are currently in progress in our laboratory.

Several interesting aspects should be noted about the T-cell response. First, the vigor of HCV-specific, T-cell responses was greater at HCV RNA positive time points than at HCV RNA negative ones. This result is reminiscent of a correlation between increased antiviral T-cell responses and low-level persisting virus in patients who had spontaneously recovered from acute hepatitis B.[15] Second, the strongest increase in immune responsiveness in our study was observed against nonstructural proteins, which are not part of the virus particle and expressed solely when the virus infects cells and viral RNA is translated into proteins. This observation suggests that the increase in HCV-specific, T-cell responsiveness was because of an antigenic boost, derived from newly translated HCV RNA. Third, the preserved immune hierarchy within the HCV-specific, T-cell responses and the preserved pretreatment viral sequences support the notion that pretreatment HCV RNA persists. This interpretation is consistent with findings in other models that show persistent virus rarely induces new T-cell responses,[33] whereas infection with a new virus of different sequence induces new T-cell responses and changes the hierarchy in the existing memory T-cell repertoire by stimulating and expanding some memory T-cell populations and contracting others.[34] The observation that the sole patient who exhibited a change in immune hierarchy in our study also exhibited a change in viral genotype therefore suggests that this patient experienced a new infection.

In conclusion, HCV RNA may persist for a limited but not indefinite time after successful therapy and may sporadically reappear in the circulation, triggering T-cell responses. This may be missed in standard clinical evaluations, which typically assess the presence of HCV RNA at a single time point 6 months after cessation of therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

DMSO	dimethyl sulfoxide
ELISpot	enzyme-linked immunospot
ffu	focus-forming units
IFN	interferon
IQR	interquartile range
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PEG	pegylated
RT	reverse transcription
RT-PCR	reverse transcription-polymerase chain reaction
RBV	ribavirin
SVR	sustained virologic response
TMA	transcription mediated assay
UTR	untranslated region

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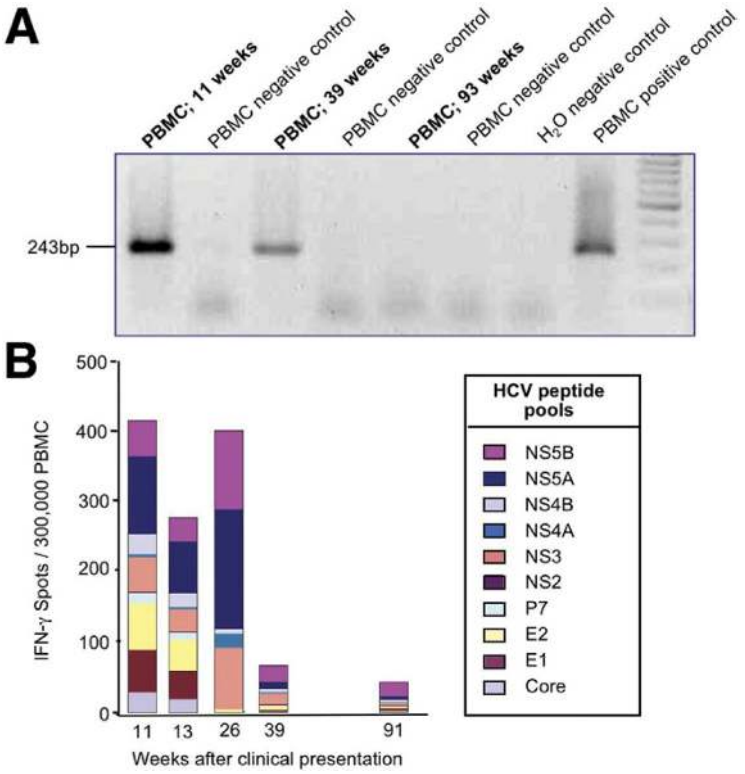

Figure 1.

HCV persists for a limited time after spontaneous recovery from hepatitis C. (*A*) Gel electrophoresis of nested HCV 5′UTR PCR products from PBMC of a spontaneously recovered patient. Weeks represent time after initial clinical presentation. Serum samples tested negative for HCV RNA at all time points. Each PBMC sample was followed by PBMC from a healthy, HCV-uninfected blood donor during the RNA extraction, RT, and PCR steps. (*B*) The frequency of HCV-specific, IFN-γ-producing T cells as determined in ELISpot assays with pools of overlapping HCV peptides decreases with clearance of HCV RNA.

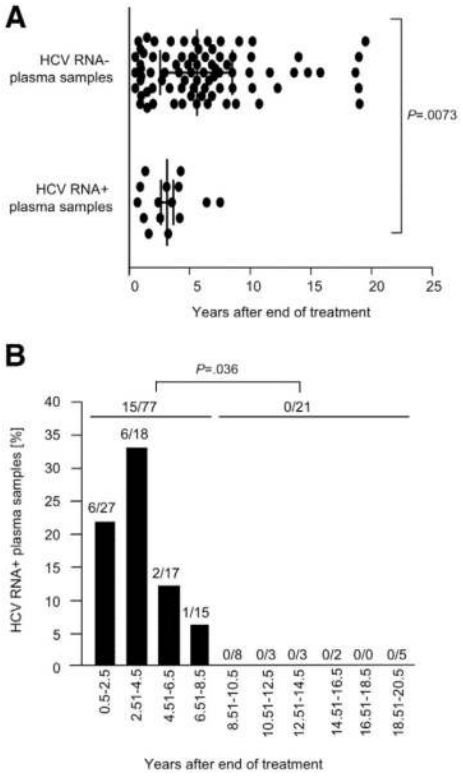

Figure 2.

The prevalence of detectable HCV RNA decreases with time after cessation of therapy. Cross-sectional analysis of plasma samples from 95 patients by nested RT-PCR with primers specific for the HCV 5′ UTR (sensitivity, < 40 copies/mL). (*A*) HCV RNA is detected solely in the first 8 years after cessation of therapy. Mean and standard error of mean are indicated. (*B*) Step-wise decrease in the prevalence of HCV RNA positive results within 2-year periods after the end of treatment.

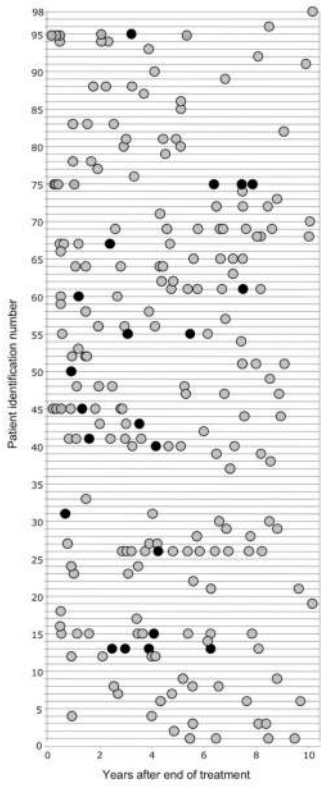

Figure 3.

HCV RNA positive time points (*solid circles*) are interspersed with HCV RNA negative time points (*open circles*). Longitudinal analysis of prospectively collected plasma samples after cessation of treatment by nested RT-PCR (assay as in Figure 2). Patients 5, 10, 11, 19, 20, 25, 32, 34, 35, 36, 46, 84, and 97 were studied at 1–3 time points more than 10.5 years after recovery (not shown). Patient 75 experienced high-titer HCV recurrence (see legend to Table 3 for details). For patient 75, the depicted results less than 1.1 years after end of treatment are derived from serum samples tested by COBAS Amplicor HCV Test 2.0.

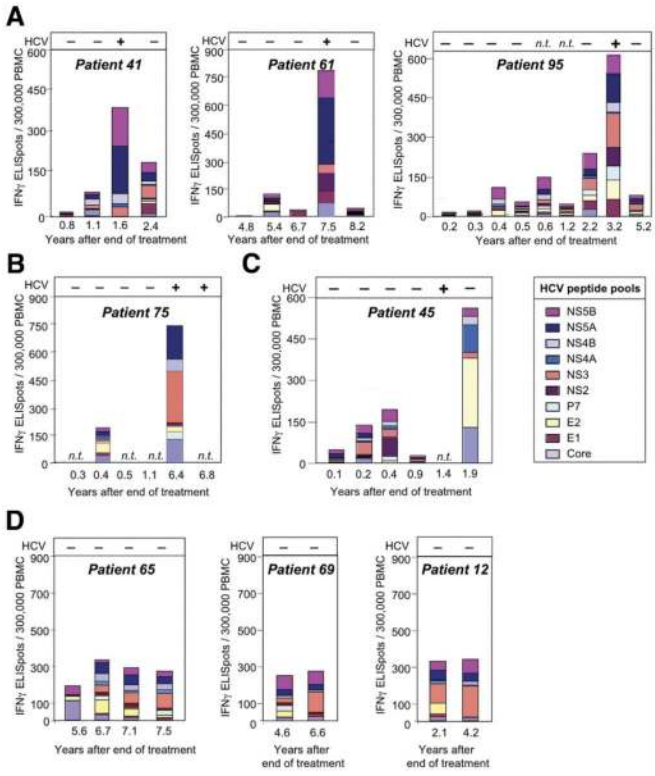

Figure 4.

HCV-specific T-cell responses are stronger at time points with detectable HCV RNA than at those without. Prospective analysis of plasma by nested RT-PCR (sensitivity, < 40 copies/mL) and PBMC by IFN-γ ELISpot. Immune hierarchy with dominant responses against nonstructural HCV antigens was preserved in patients, for which the persisting HCV RNA sequence matched the pretreatment sequence (*A* and *B*). Immune hierarchy changed from nonstructural proteins to predominantly structural HCV antigens in subject 45 (*C*) whose HCV RNA sequence (genotype 3) differed from the pretreatment HCV sequence (genotype 1a). Patients depicted in *panels A* and *C* had HCV RNA detectable only in the nested round of an RT-PCR, whereas patient 75 (*B*) had high-level HCV recurrence (see legend to Table 3 for details). (*D*) Immune responses of patients who tested HCV RNA negative at all studied time points.

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Table 1

Characteristics of Studied Patients

	Spontaneous recovery (n = 19)	Treatment-induced recovery (n = 98)	*P* value
Age at treatment initiation, median (IQR), *y*	n.a.	47.4 (40.6–51.8)	
Age at time of testing, median (IQR), *y*	57.1 (49.0–69.0)	53.6 (47.4–57.6)	n.s.[a]
Years between HCV clearance and current study, n			
Median (IQR)	20.8 (13.0–31.9)	5.1 (2.0–7.6)	<.0001
0.5–2	2	24	
2–4	1	14	
4–6	0	19	
6–8	0	18	
8–10	0	8	
10–12	1	5	
12–14	2	3	
14–16	1	2	
16–18	1	0	
18–20	0	5	
>20	11	0	
Male sex, n (%)	9(47)	56 (57)	n.s.[b]
Ethnicity, n (%)			
White	17 (89)	83 (85)	n.s.[b]
African American	1 (5)	5 (5)	n.s.[b]
Asian	0 (0)	7 (7)	n.s.[b]
Hispanic	1 (5)	3 (3)	n.s.[b]
HCV serotype/genotype, n (%)[c]			
1	5	50 (51)	
2	2	26 (27)	
3	1	18 (18)	
4	0	1 (1)	

	Spontaneous recovery (n = 19)	Treatment-induced recovery (n = 98)	*P* value
5	0	1 (1)	
Unknown	11	2 (2)	

IQR, inter-quartile range; n.a., not applicable; n.s., not significant.

[a]Mann–Whitney test; only statistically significant findings with a 2-sided $P < .05$ are indicated.

[b]Fisher exact probability test; only statistically significant findings with a two-sided $p<0.05$ are indicated.

[c]Viremic plasma samples prior to recovery were only available for the treatment recovered group and not for the spontaneously recovered group. HCV genotypes are therefore indicated for the treatment-recovered patients, and HCV serotypes are indicated for the spontaneously recovered patients.

Table 2

Detectable HCV RNA in Plasma and PBMC

Recovered	Detectable HCV RNA n (%)
Spontaneous recovery	
Plasma	0/19 (0)
PBMC	1/14 (7)
Treatment-induced recovery	
Plasma	15/98 (15)
PBMC	3/76 (4)

PBMC, peripheral blood mononuclear cells.

Table 3

Clinical, Virologic, and Immunologic Characteristics of Treatment Recovered Patients With Detectable HCV RNA

Time since end of treatment	Patient number	Sex	Age, y	Route of infection	HCV titer prior to treatment *(copies/mL)*	Treatment		HCV genotype		Neutralizing antibodies [reciprocal 50% neutralization titer] against	
						Regimen	Duration (wk)	Prior to treatment	After treatment	HCV with genotype 2a structural proteins	HCV with genotype 1a structural proteins
HCV RNA positive plasma samples											
Year 1: n = 2	31	Female	51	Transfusion	1.3×10^6	PegIFN + RBV	48	1b	1b	993	833
	50	Female	53	Transfusion	0.02×10^6	PegIFN + RBV	24	3a	3a	49	343
Year 2: n = 3	60	Male	46	Tattoo	n.d.	PegIFN + RBV	48	u.t.g.[a]	u.t.g.	2163	200
	41	Female	58	Transfusion	0.7×10^6	PegIFN + RBV	48	1b/1a	1b/1a	597	415
	45	Male	46	IVDU, many sexual partners	1.0×10^6	PegIFN + RBV	49	3	1a	<10	1429
Year 3: n = 1	67	Female	62	Transfusion	0.5×10^6	PegIFN+RBV	24	2	2b	3333	22
Year 4: n = 3	43	Male	54	Transfusion	n.d.	PegIFN+RBV	48	1a	1b	897	2604
	55	Female	49	Cocaine	2.2×10^6	IFN+RBV	24	2b	2b	443	808
	95	Female	57	Needlestick	0.4×10^6	PegIFN+RBV	48	1b	1b	<10	856
Year 5: n = 3	15	Male	56	Transfusion	0.3×10^6	PegIFN+RBV	48	5[a]	u.t.g.	<10	577
	40	Male	53	IVDU, many sexual partners	n.d.	PegIFN+RBV	48	1b	1b	906	379
	26	Female	65	Transfusion	n.d.	PegIFN+RBV	48	1a/1b	1a/1b	<10	203
Year 7: n= 2	13[d]	Male	57	IVDU	0.6×10^6	IFN+RBV	48	1a/1b	1a/1b	599	421
	75	Male[c]	58	IVDU	2.5×10^6	IFN+RBV	48	1a	1a	n.d.	n.d.
Year 8: n = 1	61	Female	52	Transfusion	2.6×10^6	IFN+RBV	24	2b	2b	1499	30
HCV RNA positive PBMC samples											
Year 1: n = 1	24	Male	48	IVDU, many sexual partners	53×10^6	PegIFN+RBV	24	2b	2b	768	475
Year 3: n = 1	8	Female	53	Transfusion, tattoo	0.9×10^6	PegIFN+RBV	48	1b	1b	2104	223
Year 7: n = 1	13[d]	Male	57	IVDU	0.6×10^6	IFN+RBV	48	1a/1b	1a/1b	599	421

NOTE. The reciprocal neutralization titer represents the serum dilution that results in a reduction of HCV JFH1 infectivity (genotype 2a structural proteins) or HCV H-NS2/3-J infectivity (genotype 1a structural proteins) of at least 50% compared with antibody-negative serum.

IVDU, intravenous injection drug use; n.d., not done; PegIFN, pegylated interferon; RBV, ribavirin.

[a] u.t.g., unable to genotype by INNO-LiPA HCV II assay (Innogenetics, Ghent, Belgium) and 5′-untranslated region sequencing. All indicated HCVgenotypes were determined by 5′-untranslated region sequencing.

[b] HCV genotype was determined using the INNO-LiPA HCV II assay (Innogenetics).

[c] This patient had a high post-treatment HCV titer of 40,500,000 copies/mL (15,000,000 IU/mL) at the time of this analysis (6.4 years after the end of treatment) and at 2 consecutive time points 7.4 years (19,224,000 copies/mL; 7,100,000 IU/mL) and 7.8 years (13,200,000 copies/mL; 5,000,000 IU/mL) after the end of treatment. The patient had tested HCV RNA negative month 5, 6, 9, and 12 of treatment and month 1, 2, 3, 4, 5, 6, and 13 after the end of treatment.

[d] [d] This patient tested HCV RNA positive both in plasma and PBMC.