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Investigation of Residual Hepatitis C Virus in Presumed Recovered Subjects

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Recent studies have found hepatitis C virus (HCV) RNA in peripheral blood mononuclear cells (PBMCs) of the majority of presumed recovered subjects. We investigated this unexpected finding using samples from patients whose HCV RNA and anti-HCV status had been serially confirmed. HCV RNA was detected in PBMCs from 66 of 67 chronic HCV carriers. Subpopulation analysis revealed that the viral load (log copies/10^6 cells) in B cells (4.14 ± 0.71) was higher than in total PBMCs (3.62 ± 0.71; $P < 0.05$), T cells (1.67 ± 0.88; $P < 0.05$), and non-B/T cells (2.48 ± 1.15; $P < 0.05$). HCV negative-strand RNA was not detected in PBMCs from any of 25 chronically infected patients. No residual viral RNA was detected in total PBMCs or plasma of 59 presumed recovered subjects (11 spontaneous and 48 treatment induced) using nested real-time polymerase chain reaction with a detection limit of 2 copies/μg RNA (from ~1 × 10^6 cells). PBMCs from 2 healthy HCV-negative blood donors became HCV RNA positive, with B-cell predominance, when mixed *in vitro* with HCV RNA–positive plasma, thus passively mimicking cells from chronic HCV carriers. No residual HCV was detected in liver or other tissues from 2 spontaneously recovered chimpanzees. *Conclusion:* (1) HCV RNA was detected in PBMCs of most chronic HCV carriers and was predominant in the B-cell subpopulation; (2) HCV detected in PBMCs was in a nonreplicative form; (3) HCV passively adsorbed to PBMCs of healthy controls *in vitro*, becoming indistinguishable from PBMCs of chronic HCV carriers; and (4) residual HCV was not detected in plasma or PBMCs of any spontaneous or treatment-recovered subjects or in chimpanzee liver, suggesting that the classic pattern of recovery from HCV infection is generally equivalent to viral eradication. (HEPATOLOGY 2013;57:483-491)

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It is estimated that of the 170 million individuals infected with hepatitis C virus (HCV) worldwide,[1] approximately 20% will develop cirrhosis, and 1%-5% of patients with cirrhosis will progress to hepatocellular carcinoma (HCC).[2] B-cell abnormalities, including mixed cryoglobulinemia and non-Hodgkin's lymphoma, may also occur.[3,4] Spontaneous recovery from acute HCV infection, or treatment-induced recovery from chronic HCV infection, has been considered to represent a probable cure.[5,6] However, other studies have reported finding residual HCV RNA in the serum or peripheral blood mononuclear cells (PBMCs) of recovered subjects[7,8] and have cast doubt on the complete eradication of virus in presumed recovered subjects. This is a critical issue, because if residual HCV exists, there is the potential for reactivation in the setting of organ transplantation, chemotherapy, or other acquired immunosuppressive states, as reported in occult HBV infection.[9] Although HCV reactivation is limited to a few case reports,[10-12] the potential for residual HCV in persons whose serum tests HCV RNA negative raises concern regarding the use of organs from presumed HCV-

Abbreviations: Abs, antibodies; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NIH, National Institutes of Health; n-RTD, nested real-time detection; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PHA, phytohemagglutinin; SN/SNs supernatant/s; SVR, sustained virological response; TMA, transcription-mediated amplification.

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recovered subjects in the transplant setting, and raises the potential that such individuals would remain at risk for the subsequent development of HCC. It is thus essential to determine whether residual HCV infection exists in persons whose serum markers suggest full recovery.

We sought to investigate the presence of residual HCV in PBMCs and plasma by (1) measuring HCV RNA in the PBMCs and mononuclear cell subsets of chronically infected and recovered subjects using a sensitive nested real-time detection (n-RTD) polymerase chain reaction (PCR) with a detection limit of 2 copies/μgRNA, (2) determining whether PBMC-associated HCV RNA is in a replicative form by negative-strand PCR and cell culture, and (3) determining whether HCV might bind to PBMCs without infecting them. In addition, we examined tissue samples from the liver and other organs of 2 spontaneously recovered chimpanzees for the presence of HCV RNA.

Patients and Methods

Patients. Consenting blood donors identified as anti-HCV positive by enzyme immunoassay at the time of routine blood donation at the Department of Transfusion Medicine, National Institutes of Health (NIH), or the Greater Chesapeake and Potomac Region of the American Red Cross were enrolled in an NIH prospective study of the natural history of HCV infection.[13] Approximately 750 participants were enrolled. Of these, 67 chronic HCV carriers and 36 presumed recovered subjects (11 spontaneously recovered and 25 sustained virologic treatment responders) were randomly selected for the study. In addition, 23 treatment-recovered patients from Japan were included in the study, bringing the total number of recovered subjects to 59. From the total population, 43 chronic carriers as well as 3 spontaneously recovered and 13 treatment-recovered subjects had their PBMCs studied in cell culture. Selection of donors for PBMC studies depended on the availability of fresh PBMCs from donors in active follow-up; the small number of spontaneously recovered subjects providing fresh PBMCs reflected the low proportion of recovered subjects who remained in active clinical follow-up.

Demographic and clinical characteristics of patients are shown in Table 1. The chronic group and the recovered group had similar characteristics, except for viral load and serum transaminase levels. Study protocols were reviewed and approved by the appropriate institutional review boards, and all subjects gave written informed consent to participate in the study.

Methods.

Serologic and molecular assays. A detailed description of methods used for HCV detection, including serologic assays for anti-HCV, molecular assays for HCV RNA, and assays for HCV negative-strand detection, as well as HCV RNA isolation, can be found in the Supporting Materials.

PBMCs and PBMC subset separation. After separation of plasma from whole blood, PBMCs were isolated by Ficoll-Paque, then washed twice in Dulbecco's phosphate-buffered saline (PBS) and two times in complete RPMI medium. The B-cell subset was purified by positive selection using magnetic-activated cell sorting magnetic beads coated with anti-CD19 antibodies (Abs) (Miltenyi Biotec Inc., Auburn, CA), and the T-cell subset was purified from the non-B-cell fraction by using beads coated with anti-CD3 Abs. Efficiency of separation was >85% for B cells and >95% for T cells, as confirmed by fluorescence-activated cell sorting analysis (BD Biosciences, San Diego, CA).

PBMC purification for cell-culture studies. PBMCs were isolated by Ficoll-Paque PLUS density gradient from acid citrate dextrose anticoagulated blood. Cells were washed with 1× PBS and complete RPMI 1640 medium and suspended in culture medium at 1×10^6 cells/mL. Two culture methods were followed. Twelve patients' samples (3 recovered and 9 chronic) were cultured without exogenous stimuli according to the previously described method.[14] Briefly, 2 million cells were suspended in 2 mL of complete RPMI 1640 medium using round-bottomed 5-mL polystyrene tubes and left undisturbed in a 5% CO_2 incubator. For the rest of the patients, after isolation and cell washes, 4 mL of culture medium plus 5 μg/mL of phytohemagglutinin (PHA-L) was added to 4×10^6 cells and incubated at 37°C in a 5% CO_2 incubator. At day 3 or 4, 3 mL of the medium was replaced by complete RPMI plus 1 μg/mL of PHA-L and 20 U/ml of interleukin-2. Subsequently, for both techniques, half of the supernatant was replaced twice a week with fresh medium. Supernatants (SNs) and cell samples were collected once- or twice-weekly during 3 weeks of culture.

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Additional Supporting Information may be found in the online version of this article.

Table 1. Demographic and Clinical Characteristics of Patients

(A) Patients for HCV RNA Negative-Strand Detection and PBMC Subsets Studies

Characteristics	Chronic (n = 29)	Recovered (n = 26)	*P* Value
Age, years	52.8 ± 10.7	52.1 ± 9.4	NS
Sex, male/female	16/13	12/14	NS
HCV viral load, log(IU/mL)	6.1 ± 0.7	ND	
ALT, U/L	64.2 ± 44.4	22.7 ± 10.9	<0.001
AST, U/L	48.4 ± 27.9	24.3 ± 5.3	<0.001
T-Bil, mg/dL	0.8 ± 0.4	0.8 ± 0.3	NS
Alb, g/dL	3.8 ± 0.3	4.1 ± 0.3	NS
Platelet, K/μL	225.5 ± 58.8	229.0 ± 53.8	NS
PT, seconds	12.6 ± 0.7	12.6 ± 0.4	NS

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; T-Bil, total bilirubin; Alb, albumin; PT, prothrombin time; ND, not detectable; NS, not significant.

(B) Patients for Cell-Culture Studies

Characteristics	Chronic (n = 43)	Recovered (n = 16)	*P* Values
Age, years	57 ± 7	55 ± 10	NS
Sex, male/female	26/17	8/8	NS
HCV viral load, log(IU/mL) (range)	6.3 ± 0.7 (4.4-7.6)	ND	
ALT, U/L	62 ± 30	29.4 ± 9.3	<0.0001
AST, U/L	40 ± 22	17.4 ± 7.4	<0.0001
T-Bil, mg/dL	0.53 ± 0.23	0.58 ± 0.34	NS
Alb, g/dL	4 ± 0.3	4.1 ± 0.3	NS
Platelet, K/μL	226 ± 66	228 ± 66	NS
PT, seconds	13.3 ± 0.8	13.5 ± 0.6	NS

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; T-Bil, total bilirubin; Alb, albumin; PT, prothrombin time; ND, not detectable; NS, not significant.

(C) Group of Recovered Patients from Japan

Characteristics	Recovered (n = 23)
Age, years	52 ± 13
Sex, male/female	11/12
HCV viral load, log(IU/mL)	ND
ALT, U/L	26.4 ± 41.4
AST, U/L	21.4 ± 5.7
T-Bil, mg/dL	0.71 ± 0.2
Alb, g/dL	4.54 ± 0.4
Platelet, K/μL	183 ± 48
PT, seconds	11.8 ± 0.5

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; T-Bil, total bilirubin; Alb, albumin; PT, prothrombin time; ND, not detectable.

HCV detection. We followed the RTD-PCR method designed by Takeuchi et al.[15]; however, to obtain better sensitivity, n-RTD-PCR was performed, as reported previously for the detection of HCV (see Supporting Materials for details).[16,17]

HCV negative-strand detection. For negative-strand HCV RNA detection, the rTth-based method[8,18] was used with minor modification, as described in detail in the Supporting Materials.

In vitro HCV particle attachment to PBMCs from healthy donors. PBMCs from 2 healthy donors and plasma from 4 chronic carriers were mixed in four separate experiments. In each experiment, healthy cells and RNA-positive plasma were incubated for 2 hours at room temperature, and cells were then washed three times in medium. Washed PBMCs and their fractions were tested for the presence of HCV RNA by nRTD-PCR. CD19 separation was performed by positive selection, and total PBMCs were separated into B-cell and non-B-cell fractions.

Statistical Analysis. Student *t* test and Mann-Whitney's U test were used for comparison of continuous variables. Fisher's exact probability test was used for frequency comparisons. Spearman's rank-order correlation was used to evaluate the correlation between HCV viral load in serum and HCV viral load in PBMCs. A *P* value of 0.05 or less was considered significant. Statistical analyses were performed using STATA (version 7.0; StataCorp LP, College Station, TX). Data analysis and graphs from the cell-culture section were performed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

Results

Relationship Between HCV Viral Load in Serum and Uncultured PBMCs. A comparison between serum HCV viral load and the viral load in PBMCs was performed on samples from 28 chronically infected patients. Viral loads in these blood compartments

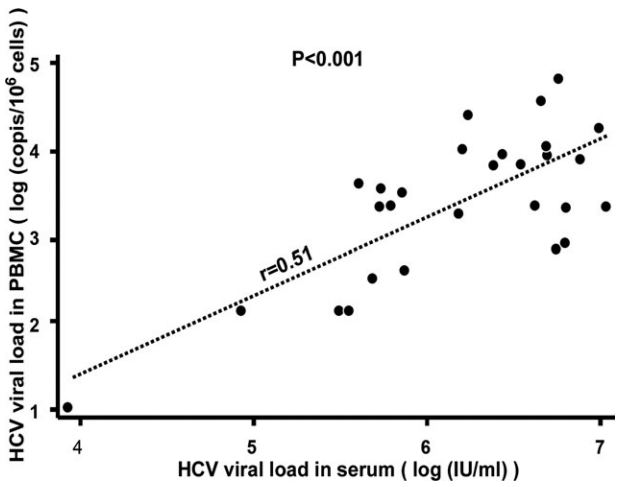

Fig. 1. Correlation between serum and PBMC HCV viral load.Moderate correlation was observed between serum HCV viral load and viral load in PBMCs. HCV viral load from plasma and PBMCs was performed using 28 chronic patients.

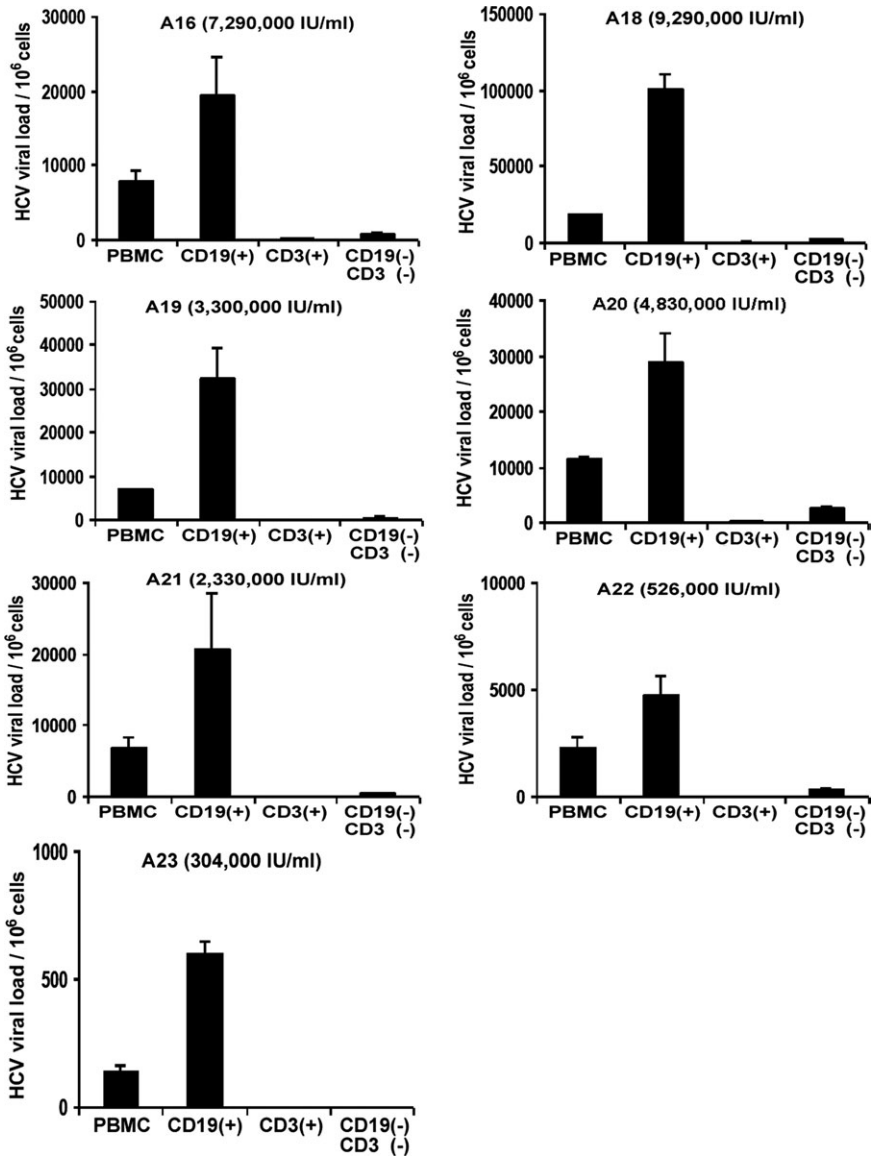

Fig. 2. HCV viral load in PBMC subpopulations: B cells (CD19 [+]), T cells (CD3 [+]), and non-B/T cells (CD19 [−] CD3 [−]). Results of 7 of 8 patients for whom HCV RNA was detectable.

showed moderate correlation ($P < 0.001$; $r^2 = 0.51$) (Fig. 1). A more comprehensive assessment was performed in 8 patients in whom HCV viral load was measured in total PBMCs, CD19-positive B cells, CD3-positive T cells, and CD19, CD3-negative subsets. In 7 of 8 patients, viral load (log copies/10^6 cells) in the B-cell subset (4.14 ± 0.71) was significantly higher than in total PBMCs (3.62 ± 0.71; $P < 0.05$), T cells (1.67 ± 0.88; $P < 0.05$), and non-B, non-T cells (2.48 ± 1.15; $P < 0.05$) (Fig. 2). In 1 patient, virus was not detected in any cell fraction.

Detection of Negative-Strand HCV RNA in PBMCs and PBMC Subpopulations. Using the negative-strand–specific nRTD-PCR, HCV negative strand was assayed in total PBMCs of 25 HCV-infected chronic carriers and in the B-, T-, and non-B-, and non-T-cell subsets from 8 of these carriers. No negative-strand HCV RNA was detected in any of 25 total PBMC samples or 8 PBMC subsets (see Table 2). As a control, negative-strand HCV RNA was detected (3,400 copies/μg total cellular RNA) in liver tissue from 1 chronically infected chimpanzee.

In Vitro Incubation of Plasma From Chronic HCV Carriers and PBMCs From Healthy Donors. To investigate whether the higher viral load in the B-cell subpopulation reflected the specific attachment of HCV virions to B cells, plasma from 4 chronically infected patients were mixed with PBMCs from 2 HCV-negative healthy donors and incubated for 2 hours. PBMC subsets were then separated and washed. After this *in vitro* incubation, PBMCs and PBMC subsets of the healthy donor became HCV RNA positive and the distribution of RNA was similar to that shown in chronically infected patients, being

Table 2. Summary of Results Obtained From Chronic Carriers and Presumed Recovered Subjects

	Number of HCV$^+$ Results of Total Samples Analyzed	
Tests Performed	Chronic Carriers	Presumed Recovered Subjects>
HCV RNA in plasma	67/67	0/59
HCV RNA in total PBMCs	66/67	0/59
HCV RNA in PBMC subsets		
CD19$^+$	7/8	0/21
CD3$^+$	7/8	0/21
CD19$^-$ CD3$^-$	7/8	0/21
HCV negative-strand in total PBMCs	0/25	NA
HCV negative-strand in PBMC subsets		
CD19$^+$	0/8	NA
CD3$^+$	0/8	
CD19$^-$ CD3$^-$	0/8	
PBMC cultures	34/43	0/16

Abbreviation: NA, not applicable.

greatest in the B-cell fraction (Fig. 3). Hence, it appeared that HCV RNA could passively adsorb to healthy PBMCs and preferentially adsorb to B cells.

Residual Virus in Recovered Subjects. A total of 59 samples from recovered patients, including 36 from the United States and 23 from Japan, were included in the study. Samples were first confirmed as HCV RNA negative by qualitative COBAS AMPLICOR assays (Roche Diagnostic Systems, Inc., Somerville, NJ). Then, residual HCV in plasma from 26 of the 36 U.S. recovered patients was measured with our more-sensitive method using RNA extraction purification and nRTD-PCR. Even though the detection limit of the method was as little as 10 IU/mL, as confirmed by diluted World Health Organization International HCV control, no HCV RNA was detected in any of these plasma samples, each tested in four replicates. In addition, residual HCV was sought in total PBMCs from 59 patients and from the B-cell subpopulations of 21 patients. No HCV RNA was detected in any of the PBMC samples or B-cell subset samples from these patients whose serum tested anti-HCV positive, HCV RNA negative (presumed recovered subjects), as shown in Table 2.

HCV Detection in PBMC Cultures. Because previous reports described the finding of HCV replicative intermediates in stimulated cultured PBMCs of sustained virologic responders or chronic carriers,[7,8,19] we cultured PBMCs from 59 patients, including 43 chronic carriers and 16 recovered patients. Culture SNs and cells were evaluated at different time points during 3 weeks of culture. An average of 5 culture SNs and 4 cell samples were analyzed per patient.

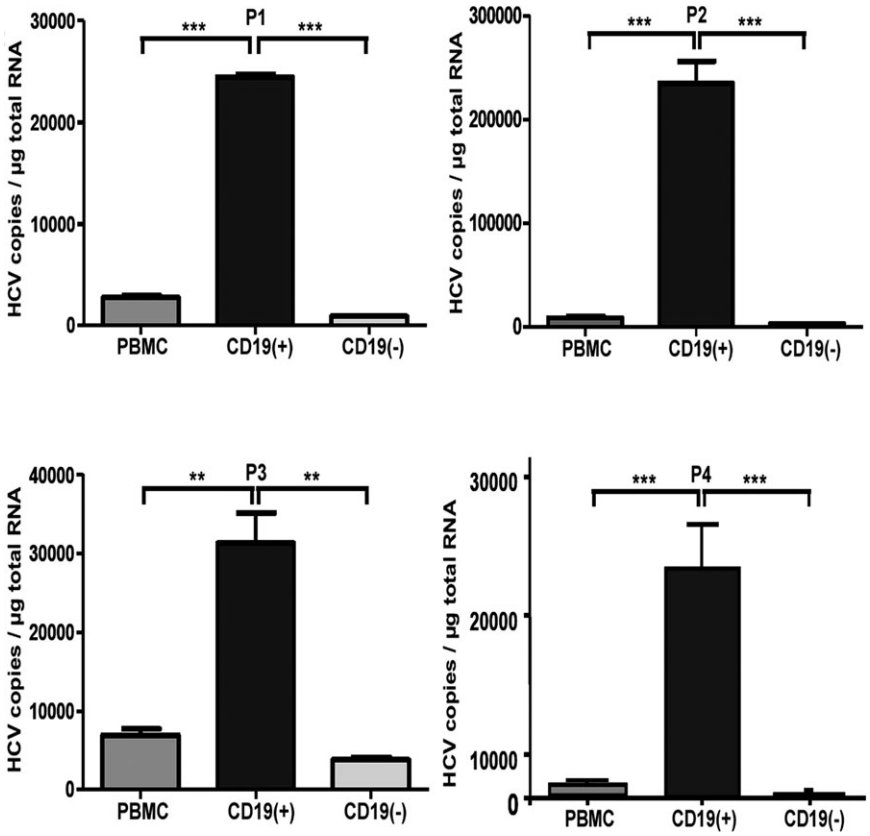

Fig. 3. HCV particle binds to surface of PBMCs. PBMCs from 2 healthy donors were mixed with plasma from 4 chronic HCV carriers (P1-P4) and incubated for 2 hours. Then, cells were washed and CD19 separation was performed. HCV viral loads in total PBMC, CD19-positive, and CD19-negative cells were quantified. ***$P < 0.001$; **$P < 0.01$.

Table 3. HCV Viral Load and Percentage of Chronic Carriers With HCV$^+$ Cultures From Cells and Cell-Free SNs Along the PBMC Culture Period

			Day 0	Week 1	Week 2	Week 3
PBMCs	% patients with HCV$^+$ cultures (n)		100 (38/38)	76 (19/25)	78 (25/32)	61 (23/38)
	VL_1	Mean ± SD	2,898 ± 4,029	15.5 ± 36.0	5 ± 10	4.2 ± 10
		Range	52-17,025	0-196	0-48	0-73
Culture SN	% patients with HCV$^+$ cultures		NA	81 (30/37)	14 (5/37)	3 (1/39)
	VL_2	Mean ± SD	NA	38 ± 79	2 ± 7	0.1 ± 0.8
		Range	NA	0-490	0-45	0–7

Abbreviations: VL_1, viral load in cultured cells expressed in copies/μg total RNA; SD, standard deviation; VL_2, viral load in culture SN expressed in copies/mL SN; NA, not applicable.

None of the 16 recovered patients demonstrated the presence of HCV genome in their SN or cultured cells. Conversely, 34 of 43 (79%) chronic carriers had detectable HCV RNA in their PBMC cultures (cells and/or SN) (Table 2). Calculating the number of HCV$^+$ SNs of the total SNs analyzed per culture, the frequency of HCV$^+$ detection had a median value of 33% and mean of 35%.

In culture, the HCV viral load diminished over time and the majority of HCV positive cultures were observed during week 1 (Table 3; Fig. 4). The maximum viral load reached during week 1 of culture was 490 copies/mL for SN fractions and 196 copies/μg total RNA for cells (Table 3). Viral levels decreased rapidly and progressively in culture, reaching very low levels by week 3, at which time there was only one positive SN with a viral load of 7 copies/mL (Fig. 4; Table 3). However, cell fractions maintained their positivity with 61% (24 of 39 patients) of cell samples still positive at week 3, though the mean copy number diminished from 15.5 at week 1 to 4.2 at week 3 (Table 3). No correlation was found between the viral load in plasma or uncultured PBMCs and the HCV RNA level observed during cell cultures. Overall, it appeared that HCV$^+$ PBMCs lost reactivity in culture over time and showed no evidence for *in vitro* replication.

Residual HCV in Tissue Samples from Recovered Chimpanzees. Tissue samples from 2 chimpanzees who had recovered from acute HCV infection were available for study. The first chimpanzee (4X417) recovered from a single episode of acute HCV infection, and the second chimpanzee (4X355) recovered from two separate episodes of acute HCV infection. Because chimpanzee 4X355 died of unrelated causes after recovery from the second HCV infection, necropsy samples of spleen, kidney, digestive organs, lymph nodes, and liver were available for assay. No residual HCV RNA was detected in the liver or other tissue samples from this chimpanzee, and no HCV RNA was detected in the liver biopsy sample from recovered chimpanzee 4X417.

Discussion

The assumption that persons who recover from HCV infection, either spontaneously or after treatment, have eradicated the virus has been challenged,[7,8] but data are conflicting and the persistence of virus in

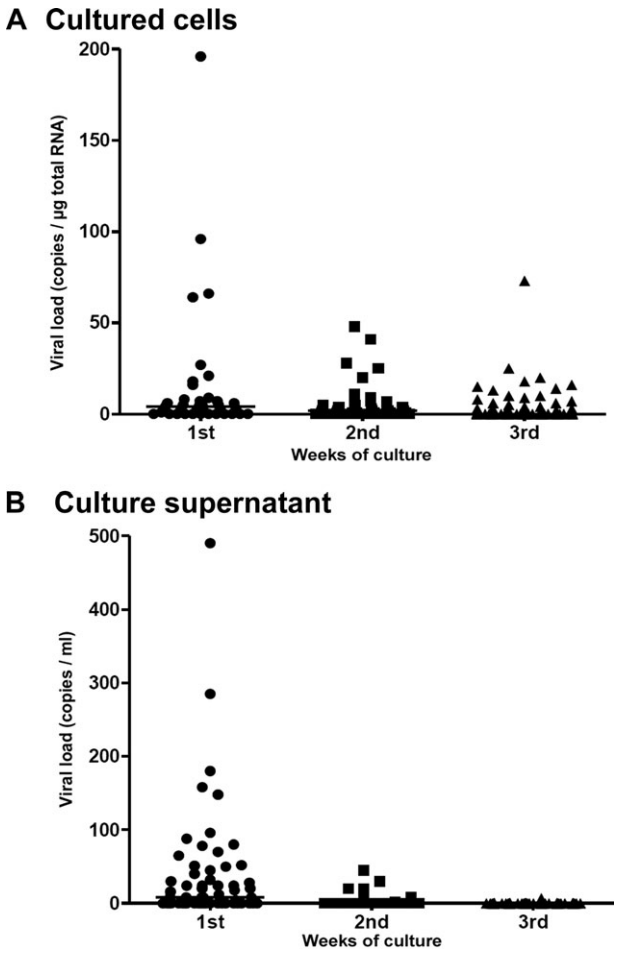

Fig. 4. HCV viral loads in cultured PBMCs and cell-free SN from HCV chronic carriers during 3-week *in vitro* culture period. Decreasing viral levels were observed as cultures progressed.

presumed recovered subjects remains controversial.[5,20] Reports of finding HCV RNA in the leukocytes of the majority of recovered subjects has raised the possibility that HCV infection generally is incurable and that it might exacerbate from residual foci in the setting of malignancy, transplantation, or other states of immune suppression. This is a vital issue, because the number of presumed recovered subjects is large and the lifetime likelihood that some of these individuals will become immunosuppressed by virtue of age, disease, or medication is high. In the United States, where it has been estimated that 4 million persons have been infected with HCV,[21] it can be estimated further that approximately 1 million (25%) have spontaneously recovered or recovered by virtue of treatment. These are probably underestimates of both the number of infected and recovered subjects because they are based on select counties in the United States and not on the highest risk populations, such as drug addicts and prisoners. Whatever the exact number of subjects who meet the serologic and molecular criteria for HCV recovery, it is critical to resolve whether such individuals are truly cured or whether they harbor residual virus that could later reactivate under specific circumstances. Studies from Canada[7,20] suggest that 75%-86% of presumed recovered subjects have HCV RNA in their PBMCs, even in the absence of HCV RNA in plasma. Furthermore, some case reports of HCV reemergence and relapse after sustained virological response (SVR) demonstrated identical sequences during viral reappearance, suggesting that the original infection had persisted at undetectable serum levels, rather than HCV reinfection.[10,12] These reports support the existence of viral reservoirs from which HCV could reemerge and suggest that PBMCs may be an important viral reservoir. In contrast, other investigators have failed to detect HCV RNA in the PBMCs of presumed recovered subjects using sensitive transcription-mediated amplification (TMA) and nested PCR assays optimized for detection of virus in mononuclear cells.[16,17,22,23] Overall, although there are case reports of HCV reappearance in recovered patients who are subsequently immunosuppressed,[10,11] such occurrences are unusual, suggesting full eradication of the virus in most patients who achieve a spontaneous or treatment-induced recovery. New light has been shed on this subject by Veerapu et al.,[24] who studied 117 subjects who had recovered from HCV infection as assessed by standard criteria. They found no evidence of residual virus in the 19 subjects who had spontaneously recovered and in only 15% of 98 patients who had a SVR to antiviral therapy. In these 15%, HCV RNA was intermittently detected in serial samples using a sensitive nested PCR, but not detected by commercial real-time PCR. Thus, the level of virus was very low, was detected only sporadically, and, in all subjects, disappeared over time, usually in the first years after cessation of therapy. Phylogenetic analysis showed that the emergent virus was identical to the virus present before treatment. The investigators conclude that a small minority of patients classified as SVR continue to replicate HCV, and they postulate that replication resides in the liver and not in PBMCs. Interestingly, the reemergence of virus was accompanied by the reappearance of an HCV-specific T-cell response that presumably would control this low-level infection, consistent with the absence of clinical exacerbation and the transient nature of the viremia. The study of Veerapu et al. is consistent with the findings from several groups,[25,26] which show that approximately 6% of SVRs have transient residual virus when tested by a highly sensitive TMA or nRTD-PCR. The controversy in the literature could be the consequence of patient selection that differed as to the source of infection, the type of treatment, and especially the interval, because an SVR was achieved. Another important element of the disparity could reside in the sensitivity and specificity of the assays used to detect positive- and negative-strand RNA in plasma and PBMC samples. The technologic challenges of demonstrating HCV replication in extrahepatic sites are well summarized by Blackard et al.[27]

On this background of uncertainty, we conclude that in our patient cohort, there is no evidence for ongoing HCV replication in presumed recovered subjects and no evidence that HCV replicates in PBMCs of chronically infected patients. Rather, we propose that in chronically infected patients, HCV binds to the surface of PBMC subsets, particularly B cells, but does not replicate within these cells.

These conclusions are supported by the following findings. First, although HCV RNA was found in the PBMCs of 66 of 67 chronic HCV carriers tested, it was not found to be present in a replicative form in 25 consecutive patients tested using an rTth-based assay for negative-strand HCV RNA capable of detecting 50 copies of negative-strand RNA and readily detecting negative-strand RNA in the liver of a chronically infected chimpanzee. Second, in PBMC culture, virtually all the HCV RNA present in PBMCs at time zero was lost within week 1 of culture (Table 3) and the minute residual RNA (0.6%) further decayed over time, suggesting that HCV in PBMCs is not in a replicative form, but rather follows a pattern of rapid,

and then asymptotic, decay in culture. However, other studies, particularly in HCV/human immunodeficiency virus–coinfected patients,[14] have provided evidence for HCV replication in PBMC culture, including detection of viral genotypes that were not present in the patient's plasma.[28] Third, we confirmed that HCV RNA in the PBMCs of chronic carriers was differentially distributed among leukocyte subsets, being highly concentrated on $CD19^+$ B cells, as reported previously.[29] In a unique aspect of this study, we demonstrated that HCV RNA-negative PBMCs, derived from healthy blood donors, became HCV RNA positive when mixed with plasma from chronic HCV carriers, and that, in this *in vitro* setting, HCV RNA was also concentrated on B cells. Hence, healthy PBMCs and their subsets could be made to mimic PBMCs from HCV carriers by mixing them with RNA-positive plasma, suggesting simple adsorption, rather than intracellular replication. Thus, the finding of HCV RNA in PBMCs or their subsets in HCV carriers does not necessarily imply viral replication or an occult site of infection, but rather that PMBCs may be a passive carrier of HCV that is replicated in other sites. There is a logical explanation for this observation, in that most HCV in chronic carriers circulates as immune complexes[30] and that PBMCs, particularly B cells, are rich in immunoglobulin receptors. Alternatively, unbound HCV could attach to known HCV receptors present on PBMCs, such as CD81, sheep anti-BubR1, and claudin-1, without truly supporting HCV replication *in vitro*.[31] Determining the precise mechanism for HCV attachment to PBMCs will require additional study.

Full recovery from HCV infection is indicated by several observations: (1) In this study, we were unable to detect HCV RNA in the PBMCs from 59 presumed recovered subjects using a sensitive nested PCR that measured down to 10 IU. In addition, none of the recovered patients had HCV-positive cultures, even after activation, making it unlikely they harbored an occult reservoir of HCV in their mononuclear cells; (2) Bernardin et al.,[16] using a highly sensitive TMA assay and a relatively large input volume (0.5 mL of plasma and 2.5×10^6 PBMC), failed to detect HCV RNA in the plasma or PBMCs of 69 aviremic blood donors who were anti-HCV and recombinant immunoblot assay positive and presumed to have spontaneously recovered; (3) Operkalski et al.[32] demonstrated that of 12 recipients of blood from donors who had the pattern of HCV recovery (anti-HCV^+, HCV RNA negative by TMA), only 1 (8%) seroconverted for anti-HCV, compared to 83 of 85 (98%) who received an HCV RNA–positive blood unit; (4) we did not detect HCV RNA in the liver tissue of 2 chimpanzees who showed the serologic and molecular pattern of HCV recovery nor was HCV RNA found in any nonhepatic tissue of 1 recovered animal that was necropsied; and (5) several studies that examined liver biopsies after treatment-induced recovery showed the general absence of HCV RNA in liver tissue and only very low levels in those who had not yet totally cleared the virus.[22,33]

Thus, the bulk of evidence suggests that HCV can be cleared not only from plasma, but also from tissue and that PBMCs are an unlikely source of residual replicating virus. The disparity between finding HCV RNA in the PBMCs of the vast majority of recovered subjects[7,8,19] and not finding HCV RNA in any subjects in this study and others[16,17,22,23] cannot be fully explained at this time. Nonetheless, we feel the data presented herein strongly support the concept of absolute HCV clearance in most patients with spontaneous or treatment-induced recovery. Furthermore, this study demonstrates that the finding of HCV RNA in the PBMCs of chronically infected patients does not necessarily imply replication within PBMCs. Rather, our data suggest that PBMCs are passive carriers of HCV that is derived from other replication sites, predominantly, if not exclusively, the liver. These findings and the general absence of documented HCV reactivation in presumed recovered subjects provide support for the concept of "curing" HCV infection. Nonetheless, our study is not sufficiently large to exclude the possibility that a minority of patients who are classified as "recovered" by standard criteria may still harbor small amounts of virus for a limited time, as documented by Veerapu et al.[24] Although, in any given patient, documentation of viral eradication will require serial testing over time, presumed recovered patients can be reassured that the reproducible loss of detectable HCV RNA in plasma generally denotes viral eradication, and that any low-level residual virus can probably be contained by their reinvigorated cellular immune response to HCV that is no longer stunted by an overwhelming viral load.

References

1. World Health Organization. Wkly Epidemiol Rec 1997;72:65-69.
2. Cohen J. The scientific challenge of hepatitis C. Science 1999;285: 26-30.
3. Mele A, Pulsoni A, Bianco E, Musto P, Szklo A, Sanpaolo MG, et al. Hepatitis C virus and B-cell non-Hodgkin lymphomas: an Italian multicenter case-control study. Blood 2003;102:996-999.
4. Giordano TP, Henderson L, Landgren O, Chiao EY, Kramer JR, El-Serag H, et al. Risk of non-Hodgkin lymphoma and lymphoproliferative precursor diseases in US veterans with hepatitis C virus. JAMA 2007;297:2010-2017.

5. Welker MW, Zeuzem S. Occult hepatitis C: how convincing are the current data? Hepatology 2009;49:665-675.
6. Pearlman BL, Traub N. Sustained virologic response to antiviral therapy for chronic hepatitis C virus infection: a cure and so much more. Clin Infect Dis 2011;52:889-900.
7. Pham TN, MacParland SA, Mulrooney PM, Cooksley H, Naoumov NV, Michalak TI. Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. J Virol 2004;78: 5867-5874.
8. Radkowski M, Gallegos-Orozco JF, Jablonska J, Colby TV, Walewska-Zielecka B, Kubicka J, et al. Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. Hepatology 2005;41: 106-114.
9. Hui CK, Cheung WW, Zhang HY, Au WY, Yueng YH, Leung AY, et al. Kinetics and risk of *de novo* hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. Gastroenterology 2006;131:59-68.
10. Lee WM, Polson JE, Carney DS, Sahin B, Gale M,Jr. Reemergence of hepatitis C virus after 8.5 years in a patient with hypogammaglobulinemia: evidence for an occult viral reservoir. J Infect Dis 2005;192: 1088-1092.
11. Lin A, Thadareddy A, Goldstein MJ, Lake-Bakaar G. Immune suppression leading to hepatitis C virus re-emergence after sustained virological response. J Med Virol 2008;80:1720-1722.
12. Fujii H, Itoh Y, Ohnishi N, Sakamoto M, Ohkawara T, Sawa Y, et al. Relapse of hepatitis C in a pegylated-interferon-alpha-2b plus ribavirin-treated sustained virological responder. Hepatol Res 2010;40:654-660.
13. Conry-Cantilena C, VanRaden M, Gibble J, Melpoler J, Shakil AO, Viladomiu L, et al. Routes of infection, viremia, and liver disease in blood donors found to have hepatitis C virus infection. N Engl J Med 1996;334:1691-1696.
14. Bare P, Massud I, Parodi C, Belmonte L, Garcia G, Nebel MC, et al. Continuous release of hepatitis C virus (HCV) by peripheral blood mononuclear cells and B-lymphoblastoid cell-line cultures derived from HCV-infected patients. J Gen Virol 2005;86:1717-1727.
15. Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636-642.
16. Bernardin F, Tobler L, Walsh I, Williams JD, Busch M, Delwart E. Clearance of hepatitis C virus RNA from the peripheral blood mononuclear cells of blood donors who spontaneously or therapeutically control their plasma viremia. Hepatology 2008;47:1446-1452.
17. Nicot F, Kamar N, Mariamé B, Rostaing L, Pasquier C, Izopet J. No evidence of occult hepatitis C virus (HCV) infection in serum of HCV antibody-positive HCV RNA-negative kidney-transplant patients. Transpl Int 2010;23:594-601.
18. Lanford RE, Chavez D. Strand-specific rTth RT-PCR for the analysis of HCV replication. In: Lau JYN, ed. Hepatitis C Protocols. Totowa, NJ: Humana Press; 1998:471-481.
19. MacParland SA, Pham TNQ, Guy CS, Michalak TI. Hepatitis C virus persisting after clinically apparent sustained virological response to antiviral therapy retains infectivity *in vitro*. Hepatology 2009;49: 1431-1441.
20. Pham TN, Michalak TI. Occult persistence and lymphotropism of hepatitis C virus infection. World J Gastroenterol 2008;14:2789-2793.
21. Alter MJ, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, et al. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N Engl J Med 1999;341:556-562.
22. Maylin S, Martinot-Peignoux M, Moucari R, Boyer N, Ripault MP, Cazals-Hatem D, et al. Eradication of hepatitis C virus in patients successfully treated for chronic hepatitis C. Gastroenterology 2008;135: 821-829.
23. Page EE, Cox A, Atkins M, Nelson MR. Clearance of hepatitis C virus RNA from serum in HIV/hepatitis C virus coinfection indicates eradication from peripheral blood mononuclear cells. AIDS 2010;24: 1267-1271.
24. Veerapu NS, Raghuraman S, Liang TJ, Heller T, Rehermann B. Sporadic reappearance of minute amounts of hepatitis C virus RNA after successful therapy stimulates cellular immune responses. Gastroenterology 2011;140:676-685.
25. George SL, Bacon BR, Brunt EM, Mihindukulasuriya KL, Hoffmann J, DiBisceglie AM. Clinical, virologic, histologic, and biochemical outcomes after successful HCV therapy: a 5-year follow-up of 150 patients. Hepatology 2009;49:729-738.
26. Morishima C, Morgan TR, Everhart JE, Wright EC, Apodaca MC, Gretch DR, et al. Interpretation of positive transcription-mediated amplification test results from polymerase chain reaction-negative samples obtained after treatment of chronic hepatitis C. Hepatology 2008; 48:1412-1419.
27. Blackard JT, Kemmer N, Sherman KE. Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences. Hepatology 2006;44:15-22.
28. Parodi C, Culasso A, Aloisi N, García G, Bastón M, Corti M, et al. Evidence of occult HCV genotypes in haemophilic individuals with unapparent mixed infections. Haemophilia 2008;14:816-822.
29. Boisvert J, He XS, Cheung R, Keeffe EB, Wright T, Greenberg HB. Quantitative analysis of hepatitis C virus in peripheral blood and liver: replication detected only in liver. J Infect Dis 2001;184:827-835.
30. Riva E, Maggi F, Abbruzzese F, Bellomi F, Giannelli G, Picardi A, et al. Immune complexed (IC) hepatitis C virus (HCV) in chronically and acutely HCV-infected patients. Med Microbiol Immunol 2009; 198:13-18.
31. Marukian S, Jones CT, Andrus L, Evans MJ, Ritola KD, Charles ED, et al. Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. Hepatology 2008;48:1843-1850.
32. Operskalski EA, Mosley JW, Tobler LH, Fiebig EW, Nowicki MJ, Mimms LT, et al. HCV viral load in anti-HCV-reactive donors and infectivity for their recipients. Transfusion 2003;43:1433-1441.
33. McHutchison JG, Poynard T, Esteban-Mur R, Davis GL, Goodman ZD, Harvey J, et al. Hepatic HCV RNA before and after treatment with interferon alone or combined with ribavirin. Hepatology 2002; 35:688-693.