MAJOR ARTICLE

Equal Amounts of Intracellular and Virion-Enclosed Hepatitis C Virus RNA Are Associated with Peripheral-Blood Mononuclear Cells In Vivo

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Background. Hepatitis C virus (HCV) replicating in peripheral-blood mononuclear cells (PBMCs) may represent an extrahepatic viral reservoir. Quantitation of HCV RNA with regard to its subcellular distribution and longitudinal course is needed for better understanding of the largely unexplored in vivo dynamics and potential pathogenetic significance of HCV in PBMCs.

Methods. Plasma and PBMCs from 30 patients coinfected with HCV and human immunodeficiency virus were evaluated in cross-sectional and longitudinal analyses, for up to 40 months. Differential extraction of virion-enclosed HCV RNA associated with cells was performed in parallel with extraction of total cellular HCV RNA. HCV RNA of either orientation was quantified by real-time polymerase chain reaction.

Results. HCV RNA was detected only in PBMCs from patients with viremia and at relatively stable quantities over time. Intracellular HCV RNA corresponding to ~60% of total cellular HCV RNA was strongly correlated with virion-enclosed HCV RNA but was only weakly associated with viral loads in plasma. In contrast, the ratio of HCV RNA load in PBMCs versus that in plasma was patient specific and stable over time.

Conclusions. The substantial and patient-specific amounts of intracellular HCV RNA found by the present study support a concept of low-level replication in PBMCs. There was no evidence for persistent HCV infection in PBMCs after clearance of viremia in plasma.

Hepatitis C Virus (HCV) is a small positive-strand RNA virus responsible for a heavy worldwide burden of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Although primarily hepatotropic, HCV is also assumed to replicate, to some degree, at extrahepatic sites [1–4], with peripheral-blood mononuclear cells (PBMCs) being the site most investigated. Evidence for low-level replication at this site has been drawn mainly from detection of either negative-strand replicative intermediates [5, 6] or cell-bound HCV antigens, including nonstructural proteins [7, 8]. Moreover, HCV quasi species or genotypes may be compartmentalized between plasma and PBMCs [9–11]. Finally, transient low-level HCV propagation can occasionally be observed in ex vivo leukocyte cultures [12, 13].

The concept of HCV lymphotropism is further supported by overrepresentation of certain lymphoproliferative disorders in HCV-infected persons [14] and may affect the pathogenesis and course of HCV infection. Reports of both recurrence of HCV viremia in immunocompromised hosts after years of sustained viral response [15] and posttransplantation recurrence after successful pretransplantation antiviral treatment [16, 17] indicate that HCV may persist for years in a reservoir of long-lived lymphoid cells [18], precisely because they do not support vigorous replication and therefore may offer shelter from the immune system. Indeed, HCV RNA of either orientation could be detected occasionally in PBMCs of persons who had achieved sustained clearance of HCV viremia in plasma for up to 9 years beforehand [19, 20]. In support of the potential implications that HCV infection of PBMCs has for horizontal as well as vertical spread of disease, the risk of mother-to-child transmission has

Received 24 May 2006; accepted 14 July 2006; electronically published 13 November 2006.

Financial support: Kamillo Eisner Stiftung (grant 20 to M.F.).

Potential conflicts of interest: none reported.

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The Journal of Infectious Diseases 2006; 194:1713–23

0022-1899/2006/19412-0014$15.00

Table 1. Characteristics of and basic polymerase-chain-reaction data on patients with hepatitis C virus (HCV) viremia.

Patient (sex; age at first visit, years)	HCV genotype[a]	Sample (time point, months[b])	CD4 cell count, no./mL	Viral load in plasma, copies/mL		PBMC-associated HCV RNA, $\log_{10}$ copies/5×10^6 cells[c]		
				HIV	HCV, $\log_{10}$[d]	Total[d] Positive strand	Total[d] Negative strand	Virion enclosed[d]
1 (M; 46)	3A	1 (0)	483	1234[e]	7.9	5.3	+	5.0
2 (M; 37)	4C/D	1 (0)	381[f]	1208[e]	7.9	5.2	+	5.0
3 (M; 41)	3A	1 (0)	211	<5[e]	6.6	5.6	+	5.6
4 (M; 29)	NA	1 (0)	189	7702[e]	6.1	2.4	–	2.6
5 (M; 32)	NA	1 (0)	1024	1220[e]	6.3	3.3	–	3.5
6 (M; 44)	1A/B	1 (0)	321	<7[e]	5.6	4.3	–	3.9
7 (M; 36)	1a	1 (0)	1135[f]	<7[e]	7.6	5.1	+	5.0
8 (M; 27)	1B	1 (0)	597	<50[e]	6.4	3.1	–	2.5
9 (M; 39)	4	1 (0)	392	<6[e]	6.5	2.9	–	2.4
		2 (5)	427	11[e]	7.0	3.4	–	2.9
10 (M; 30)	1A	1 (0)	251	<4[e]	6.7	4.7	–	4.6
		2 (6)	309	<6[e]	7.4	5.0	–	4.1
11 (M; 42)	1B	1 (0)	314	<3[e]	7.2	4.6	–	4.6
		2 (4)	387[f]	<4[e]	6.5	4.5	–	4.7
12 (M; 33)	NA	1 (0)	599[f]	19[e]	6.8	4.3	–	4.7
		2 (1)	609	<5[e]	7.5	4.2	–	4.2
13 (M; 33)	4C/D	1 (0)	302	3,900,000	8.3	3.4	–	3.3
		2 (0.4)	519	1,950,655	7.8	3.0	–	2.7
		3 (1.6)	735	1310[e]	7.0	2.4	–	2.3
		4 (3)	737	57[e]	–	–	ND	ND
14 (M; 35)	1A	1 (0)	308	273[e]	6.8	3.9	–	3.4
		2 (6)	193	217[e]	7.0	4.7	–	4.5
		3 (11)	131	117[e]	7.6	4.0	–	3.5
15 (F; 38)	NA	1 (0)	598	<5[e]	7.6	4.6	–	3.8
		2 (4)	671[f]	<6[e]	7.9	5.1	–	4.9
		3 (11)	877	<5[e]	6.7	5.4	–	4.9
16 (F; 32)	3A	1 (0)	872	<6[e]	6.6	0.9	–	–
		2 (15)	775	<7[e]	6.8	1.4	–	–
		3 (18)	872	<6[e]	7.1	1.7	–	1.3
		4 (22)	398	<9[e]	1.8	–	–	–
		5 (24)	301	<9[e]	–	–	ND	ND
17 (M; 45)	NA	1 (0)	494	<24[e]	3.9	2.6	–	1.9
		2 (4)	970[f]	16[e]	5.0	2.7	–	2.7
		3 (7)	994	23,341	4.9	2.5	–	1.8
		4 (10)	617[f]	30,000	4.4	2.6	–	1.9
		5 (11)	509	3780	3.5	2.1	–	1.3
		6 (14)	440	8130	5.0	2.8	–	2.5
		7 (17)	474	4120	5.1	2.1	–	1.7

(continued)

Table 1. ***(Continued.)***

Patient (sex; age at first visit, years)	HCV genotype[a]	Sample (time point, months[b])	CD4 cell count, no./mL	Viral load in plasma, copies/mL		PBMC-associated HCV RNA, $\log_{10}$ copies/5×10^6 cells[c]		
						Total[d]		
				HIV	HCV, $\log_{10}$[d]	Positive strand	Negative strand	Virion enclosed[d]
18 (F; 40)	3A	1 (0)	1335	<7[e]	4.7	2.4	–	2.2
		2 (5)	789	1233	2.8	–	–	–
		3 (13)	529	112	4.2	2.0	–	1.6
		4 (18)	727	1034	5.1	2.7	–	2.3
		5 (24)	741	191	5.5	2.8	–	2.5
		6 (30)	496	1960	5.6	2.9	–	2.5
		7 (35)	402	4610	5.8	2.5	–	1.8
		8 (36)	483	19	4.2	–	–	1.0
		9 (38)	414	327[e]	–	–	ND	ND
		10 (40)	474	396[e]	–	–	ND	ND
19 (M; 51)	1A	1 (0)	350	<5[e]	7.7	5.4	–	4.7
		2 (5)	369	23[e]	7.8	5.6	–	5.1
		3 (8)	255[f]	58,000[e]	7.4	5.1	–	4.8
		4 (13)	250	336[e]	7.7	5.2	+	4.8
		5 (14)	259	27[e]	7.8	5.4	–	4.9
		6 (20)	323	<6[e]	7.3	5.0	–	4.2
		7 (24)	365	20[e]	7.7	5.2	–	4.7
		8 (27)	360	<5[e]	7.6	5.2	+	4.7
		9 (33)	465	<15[e]	7.1	5.1	–	4.4
20 (M; 43)	1B	1 (0)	715	26[e]	7.6	2.3	–	2.2
		2 (3)	715	<5[e]	7.1	2.0	–	1.8
		3 (7)	640	33	6.9	2.3	–	2.1
		4 (10)	613	6	6.7	2.7	–	2.4
		5 (14)	752	<4	7.0	2.9	–	2.6
		6 (19)	716	7323	6.5	2.4	–	2.0
		7 (23)	626	5149	6.8	2.0	–	1.5
		8 (28)	564	7774	6.7	2.6	–	2.0
		9 (31)	408	3084	6.5	2.6	–	1.6
		10 (33)	410	16[e]	6.6	3.1	–	2.5
21 (F; 41)	4	1 (0)	749	<5[e]	6.4	4.8	–	4.4
		2 (5)	873	10	6.1	4.9	–	4.3
		3 (8)	900[f]	2509	6.0	4.3	–	3.8
		4 (13)	949	17,466	5.7	4.1	–	3.6
		5 (19)	633	9466	6.4	4.3	–	3.3
		6 (24)	494	3123	6.2	3.6	–	3.1
		7 (26)	427	11,195	6.8	3.4	+	3.4
		8 (29)	392	7310	6.4	4.1	–	4.0
		9 (34)	513	8370	6.4	4.3	–	3.8
		10 (39)	357	6190	6.9	4.4	–	3.7

NOTE. Except for patient 13, who experienced primary infection and spontaneous clearance, all patients showed chronic seropositivity for HCV. Patients 16 and 18 underwent treatment with peginterferon/ribavirin at the end of the observational period. PBMC, peripheral-blood mononuclear cell.

[a] NA, not available.

[b] Nos. indicate months after the collection of the earliest sample; underlined nos. denote the sample/time points randomly chosen for cross-sectional analysis.

[c] Mean of 2 independent samples.

[d] A plus sign (+) denotes that the sample tested positive for HCV RNA but was below the level of reliable quantification; a minus sign (−) denotes that the sample was negative for HCV RNA; "ND" denotes that analysis was not performed, because sample was limited.

[e] Patient was undergoing antiretroviral therapy.Interpolated from adjacent time points (≤2 months after RNA determination).

[f] Patient was undergoing antiretroviral therapy.

been reported to be associated with the presence of HCV in maternal PBMCs [21].

Despite considerable evidence for extrahepatic replication, PBMC-associated HCV RNA does not necessarily reflect solely viral replication. Because of HCV's propensity to bind to a wide range of cells possibly nonpermissive for HCV replication [22, 23], an as-yet-uncharacterized proportion of viral RNA may represent virions nonspecifically attached to blood cells. Hence, viral dynamics in PBMCs remain largely unexplored, despite considerable evidence for extrahepatic replication. To address these issues, we quantified HCV RNA, of positive and negative polarity, with respect to its long-term course and relation to PBMCs, using novel techniques allowing for selective assessment of viral RNA enclosed within cell-attached virions.

PATIENTS, MATERIALS, AND METHODS

Patients and specimens. A total of 87 samples of plasma and 87 corresponding samples of PBMCs were obtained from 30 HIV/HCV-coinfected persons monitored at the University Hospital Zürich center who were enrolled in either the Early Antiretroviral Treatment Study [24], the Swiss Spanish Intermittent Treatment Trial [25–27], or the Simplified Maintenance Therapy Trial [28]. Selection of patients for the present study was based on positive serological results for HCV. The trials had been approved by the Ethics Review Committee, and all subjects had provided written informed consent to provide frozen blood samples for further virological examinations. Basic demographic and virological characteristics of the patients are listed in table 1. PBMCs were isolated from anticoagulated blood by Ficoll-gradient purification and were stored at −80°C.

Differential extraction of HCV RNA. RNA from 0.14 mL of plasma was extracted by use of a QIAamp Viral RNA kit (QIAGEN). Total RNA from "dry" pellets of PBMCs was extracted, in the presence of synthetic internal-standard RNA coding for murine prion protein (mPrP), by use of the RNeasy mini kit (QIAGEN) [29].

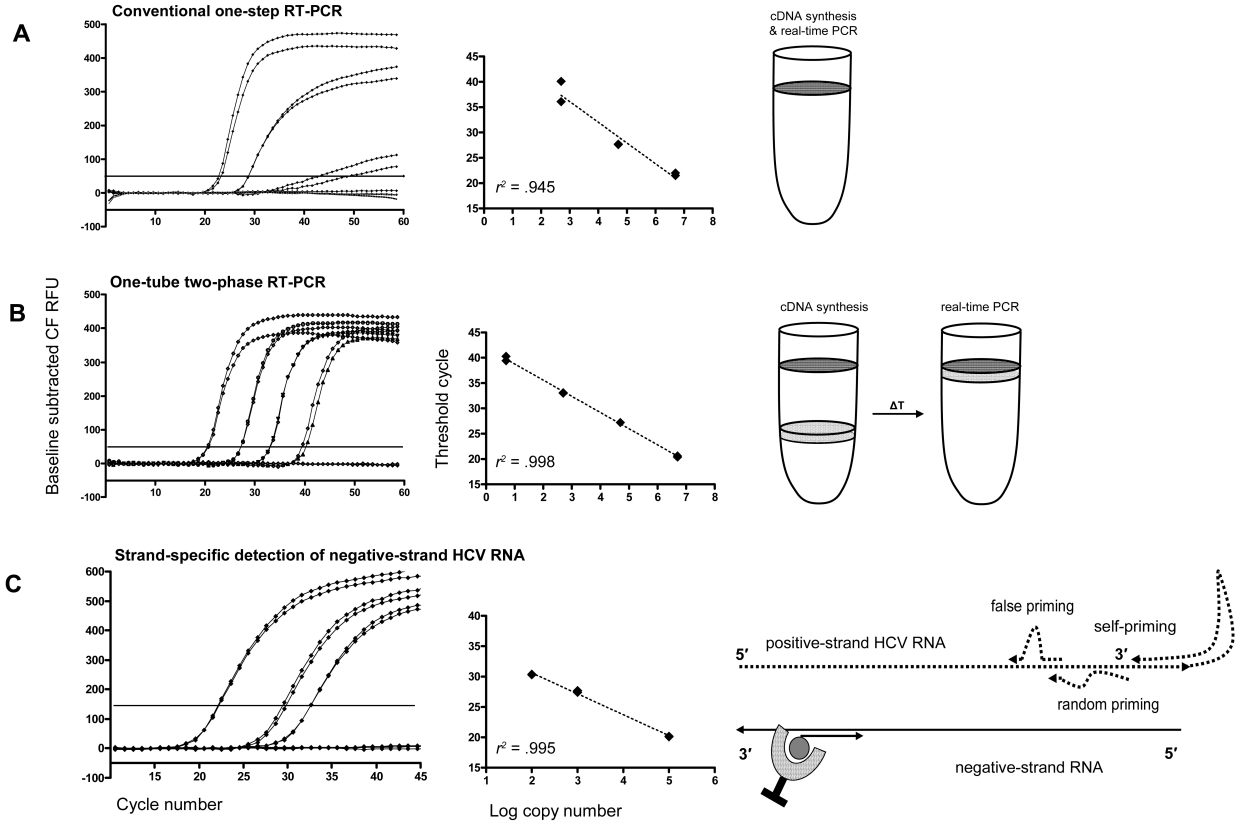

Figure 1. Quantitation of positive- and negative-strand hepatitis C virus (HCV) RNA. *A,* Results of conventional 1-step reverse transcription (RT) polymerase chain reaction (PCR) procedure. The presence of high concentrations of multiple oligonucleotides during RT favors synthesis of by-products of unspecific amplification. Premature substrate consumption may entail loss of specific amplification signals at late cycles. *B,* Separation of cDNA synthesis and PCR. Sequestration of PCR primers by means of a paraffin layer releasing reactants only during "hot-start" activation of the thermostable *Taq* polymerase results in improvement of both sensitivity and accuracy of quantitation. *C,* Strand-specific quantitative RT-PCR assay for negative-strand HCV RNA. Reactions with an input of up to 2.5×10^5 copies of positive-strand HCV RNA remained negative, representing a differentiation of 10^3–10^4 between HCV positive- and negative-strand RNA.

HCV RNA in virions attached to PBMCs was extracted from "dry" pellets by freeze-thaw nuclease (FTN) digestion [29, 30]. In brief, pellets were thawed and resuspended in 130 μL of PBS containing 10 mmol/L Tris HCl, 1 mmol/L $MgCl_2$, 1 mg/mL DNAse I (Roche), and 1.8 mg/mL RNAse A (QIAGEN). Samples were snap-frozen on dry ice, were thawed twice, and then were incubated, with constant agitation, at 37°C for 60 min. To inactivate nucleases, protease (10 μL, 50 mg/mL; QIAGEN Protease) was added, and incubation was continued for 45 min. The lysates were mixed with Buffer RLT (QIAGEN) supplemented with 1% β-mercaptoethanol, 10 μg/mL carrier RNA, and internal-standard mPrP RNA [29]. Extraction efficiency was controlled by quantitation of internal-standard RNA, and samples with low (<10%) extraction efficiency were excluded from analysis. Samples of RNA from healthy donor PBMCs were extracted in parallel and were used as negative controls (6 per PCR plate).

One-tube, 2-phase reverse-transcription polymerase chain reaction (RT-PCR) assays. Positive-strand HCV RNA was quantified by duplicate real-time RT-PCR using sense primer ME81 [31] (5′-CTGCGGAACCGGTGAGTACACC-3′), reverse/cDNA primer Ky78 [32] (5′-CTCGCAAGCACCCTATCAGGCAGT-3′), and dual-labeled fluorescent probe mf190 (5′-FAM-TAGCCGAGTAGTGTTGGGTCGCGAA-3′-TAMRA).

RT-PCR was performed in 96-well PCR plates by a single-tube system (QIAGEN 1-step RT-PCR kit) modified with an additional "hot start" using 15 μL of paraffin wax (Fluka) to separate reverse transcription from the amplification steps (figure 1*A* and 1*B*), under conditions described elsewhere [33, 34].

For specific detection of HCV negative-strand RNA (figure 1), 10 μL of template RNA was reverse transcribed as follows: RNA was incubated, at 65°C for 5 min, in a volume of 21 μL in the presence of 0.2 μmol/L biotinylated primer mf209 (BIO-5′-AAAGCGTCTAGCCATGGCGTTAGTA-3′) and 0.5 mmol/L dNTP. Subsequently, 7 μL of 4× reaction buffer containing 56 U of ribonuclease inhibitor RNaseOUT (Invitrogen), 200 U of SuperScript III reverse transcriptase (Invitrogen), and 0.14 μmol of dithiothreitol was added, and the mixture was incubated at 50°C for 45 min and at 70°C for 15 min. Recovery of specific cDNA was performed by use of streptavidin magnetic particles (Roche) (figure 1*C*): 2.2 μg of equilibrated streptavidin magnetic beads was incubated with cDNA, under constant agitation at 25°C for 60 min, in 0.1 mL of TEN100 (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 100 mmol/L NaCl, pH 7.5, 0.1% Triton X-100). Beads were washed 3 times each with TEN1000 (with 1 mol/L NaCl) and TN50 (50 mmol/L NaCl). Beads were resuspended in 30 μL of Hotmaster PCR mix. For additional specificity, PCR was preceded by linear multiplication of specific cDNA, by use of Hotmaster *Taq* polymerase (Vaudaux-Eppendorf) and 0.2 μmol/L primer mf202 (5′-ACGAGACCTCCCGGGGCACTCGCAA-3′), in a 30-μL volume, at 95°C for 2 min; PCR comprising 15 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 60 s was performed. A 2-μL portion of preamplified cDNA was subsequently PCR-quantified in real time during 40 cycles of denaturation at 95°C for 10 s, annealing and extension at 60°C for 60 s, by use of 30 μL of HotStar *Taq* master mix supplemented with 1.5 mmol/L $MgCl_2$, 1 μmol/L ME81, 1 μmol/L Ky78, and 0.3 μmol/L mf190tq. Calibration was performed by use of i-cycler software (Bio-Rad).

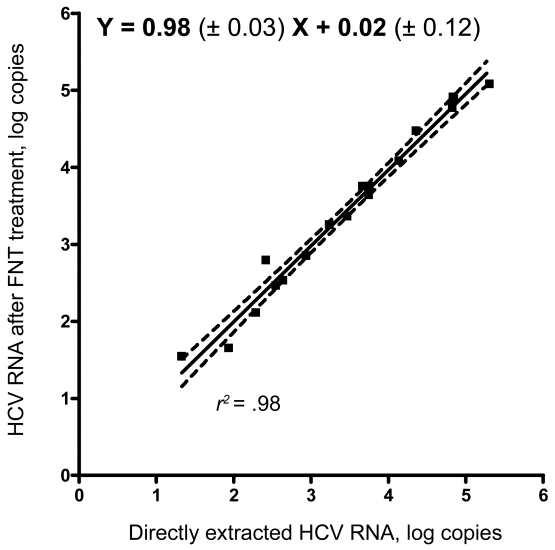

Figure 2. Evaluation of freeze-thaw nuclease (FTN)–digestion protocol for use with hepatitis C virus (HCV). Viremic plasma was spiked, on pellets, in dilution series of 5×10^6 HCV-negative peripheral-blood mononuclear cells and was extracted either directly or after FTN digestion. HCV RNA copies were quantified by reverse-transcription polymerase chain reaction. The amounts of HCV RNA were highly correlated: the slope of the linear regression line was not significantly different from 1 ($P = .124$), and the *y* intercept was not significantly different from 0 ($P = .96$).

Quantitation of mPrP mRNA and glyceraldehyde-3-phosphate dehydrogenase mRNA was performed as reported elsewhere [33].

Synthetic standard RNA of either positive- or negative-strand polarity was produced by T7 polymerase–mediated in vitro transcription of PCR products [35]: The 5′ untranslated region of patient 20 (nt 1–395, numbered according to the H77 sequence in the HCV-sequence database of the National Institute of Allergies and Infectious Diseases (http://hcv.lanl.gov/content/hcv-db/index) was amplified by RT-PCR using T7 promotor-tailed amplification primers, and RNA was transcribed by use of an Ampliscribe T7 flash-transcription kit (Epicentre). RNA was treated with DNAse I repeatedly and was purified by use of RNeasy columns, and concentrations were measured by photometry at 260 nm. External standards were prepared by serial

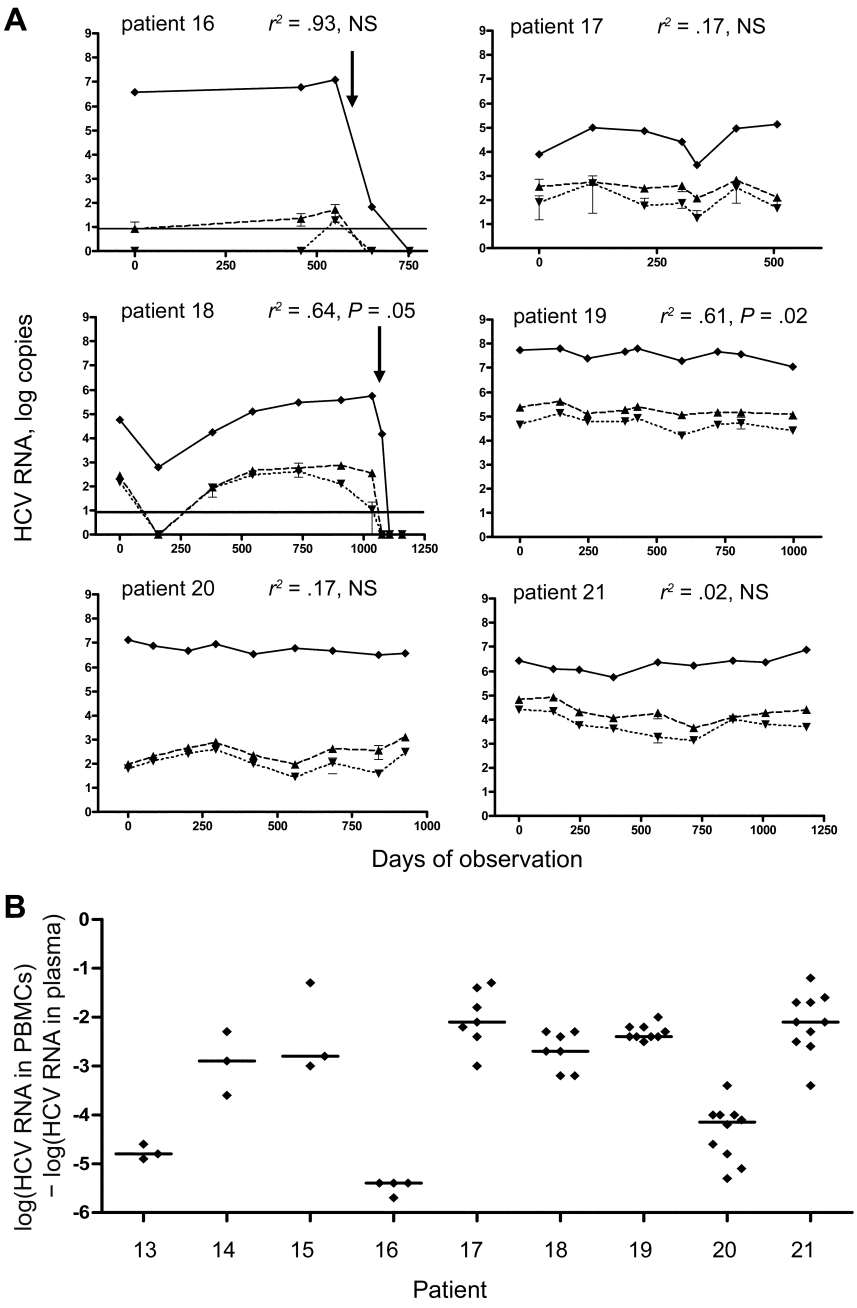

Figure 3. Course of plasma and peripheral-blood mononuclear cell (PBMC)–associated hepatitis C virus (HCV) RNA over time. *A,* Longitudinal course of HCV RNA load in plasma (◆) and of total (▲) and virion-enclosed (▼) PBMC-associated HCV RNA in patients 16–21. Correlation coefficients and *P* values derived from linear regression analyses of the correlation between total PBMC-associated and plasma HCV RNA are indicated within the graphs. Time points at which RNA levels were below the limit of detection (as indicated in the graphs for patients 16 and 18) were excluded from analyses. Patients 16 and 18 achieved sustained viral response by the end of the observational period; downward-pointing arrows (↓) indicate initiation of treatment. Note the pronounced fluctuations in viremia in plasma from patients 17 and 18, as well as the parallel kinetics of cellular HCV RNA. Plasma HCV RNA load was normalized per 1 mL, and PBMC-associated HCV RNA load was normalized per 5×10^6 cells. NS, not significant. *B,* Ratios between total PBMC-associated HCV RNA and HCV RNA loads in plasma, in patients 13–21. Plasma HCV RNA load was normalized per 1 mL, and PBMC-associated HCV RNA load was normalized per 5×10^6 cells.

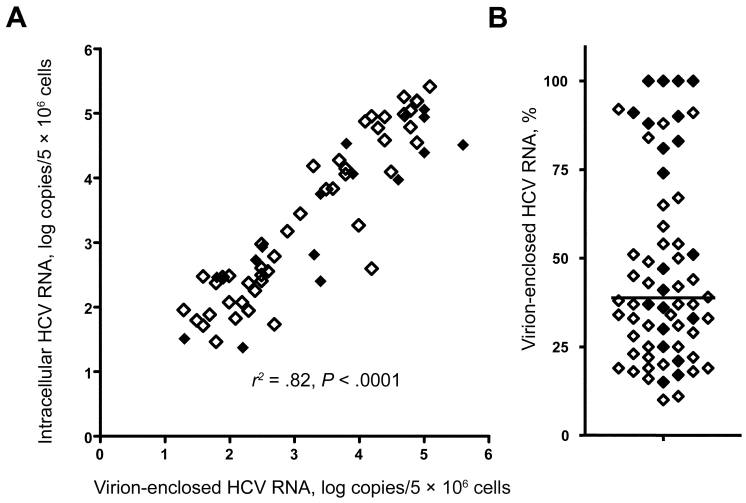

Figure 4. Distribution of peripheral-blood mononuclear cell (PBMC)–associated hepatitis C virus (HCV) RNA. Data points both for samples included in the initial cross-sectional analysis (◇) and from longitudinal analyses (◆) are shown. *A,* Correlation between virion-enclosed and intracellular HCV RNA. The level of intracellular HCV RNA was calculated by subtraction of virion-enclosed from total cell-associated HCV RNA. *B,* Virion-enclosed HCV RNA as a percentage of total cellular HCV RNA. The horizontal bar indicates the geometric mean (40%).

dilution of synthetic RNA, and assay sensitivities were assessed by limiting-dilution analysis.

Statistics. HCV RNA measurements were normalized according to 1 mL of plasma or an input cell count of 5×10^6, and statistical analyses were performed on $\log_{10}$-transformed values. Intracellular HCV RNA was calculated as the difference between total and virion-enclosed PBMC-associated HCV RNA. Parametric procedures (paired *t* test, linear regression) were used for the logarithmic data set; otherwise, either the Mann-Whitney test, Spearman's signed-rank test, or the Kruskal-Wallis test was used. Data points at which measurements of intracellular or extracellular PBMC-associated HCV RNA loads fell below the limits of detection were excluded from correlation analyses.

Correlation within the combined data set was assessed by robust linear regression accounting for multiple and unbalanced sampling. Statistic analyses were performed by use of GraphPad Prism4.0 software (AMPL Software) and Stata (version 9.2; StataCorp).

RESULTS

Differential extraction and quantitation of HCV RNA. HCV RNA was quantified by a novel real-time RT-PCR assay with enhanced sensitivity and accuracy (figure 1); this assay minimizes amplification of by-products by using a layer of paraffin wax to prevent cDNA synthesis of amplification primers. Specific and reproducible amplification (figure 1*B*) was detected over a linear range from ≥ 6 $\log_{10}$ units down to a detection limit of <5 copies per reaction.

To detect negative-strand HCV RNA, we developed an assay that used biotinylated primers to ensure specificity by affinity purification of properly primed cDNA; this assay (figure 1*C*) revealed precise reproducibility of quantitation within a linear range from ≥ 3 $\log_{10}$ down to a detection limit of 50–100 copies per reaction. An input of 2.5×10^5 copies of complementary positive-strand RNA gave rise to an occasional positive signal, because of either truly false priming events or amplification of residual template DNA within the RNA standard; however, because the inputs from samples from patients invariably were $<5 \times 10^4$ HCV positive-strand RNA copies per PCR, the specificity of the assay can be assumed to be quite sufficient to ensure the validity of the results.

To assess the distribution of HCV RNA within different compartments of peripheral blood, we quantified positive-strand HCV RNA in (1) plasma; (2) direct, "total" PBMC extracts (total PBMC-associated HCV RNA); and (3) extracts derived exclusively from virions attached to PBMCs (virion-enclosed PBMC-associated HCV RNA). The intracellular fraction of PBMC-associated HCV RNA was calculated as the difference between items (2) and (3) from replicate samples; negative-strand HCV RNA, not being incorporated within viral particles, was quantified only in (2).

Selective isolation of virion-enclosed PBMC-associated HCV RNA included disintegration of cells by repeated FTN digestion [29, 30] and relied on the virions' resistance to such treatment. To validate the procedure, we performed model experiments using HCV particles from high-HCV-titer- plasma from the patients. We found that HCV RNA copy numbers in FTN-digested samples correlated highly with the amounts recovered by direct RNA extraction ($r^2 = 0.976$; $P < .0001$) (figure 2), without a significant difference in yield ($P = .96$, by paired *t* test), thereby confirming that the HCV particles were completely resistant to

FTN digestion; conversely, intracellular nucleic acids, as quantified by use of glyceraldehyde-3-phosphate dehydrogenase RNA, were reduced to 2.1% (95% confidence interval [CI], 0.7%–3.6%) of those in directly extracted samples.

PBMC-associated HCV RNA: partial intracellular location. In a cross-sectional analysis of plasma samples from 30 HIV/HCV-seropositive patients (6 women and 24 men; median age, 38 years), 21 specimens tested positive for HCV RNA, with viral loads of 3.9–8.3 $\log_{10}$ copies (median, 6.8 $\log_{10}$ copies) (table 1). No HCV RNA was detected in any of the PBMC extracts from the 9 patients with plasma in which no HCV was detectable; conversely, all PBMC samples from patients with HCV-positive plasma tested positive for HCV RNA, which was 1.7–5.6 $\log_{10}$ copies (normalized to an input of 5×10^6 cells) in directly extracted samples and 1.3–5.6 $\log_{10}$ copies in FTN-digested samples (table 1). Thus, we observed widespread presence of viral RNA in PBMCs from HCV-viremic patients but did not find evidence for persistence of HCV in PBMCs in the absence of HCV viremia in plasma. Moreover, the amounts of virion-enclosed HCV RNA were consistently lower than those of total PBMC-associated HCV RNA ($P = .0025$, by paired t test); thus, a significant proportion of cell-associated HCV RNA was found to reside intracellularly, indicating HCV infection of PBMCs.

As a marker of ongoing replicative activity of HCV in PBMCs, we determined the occurrence of negative-strand HCV RNA in total PBMC RNA extracts (table 1). HCV negative-strand replicative intermediates could be detected in 9.6% of all PBMC samples that tested positive for positive-strand HCV RNA, accounting for sporadic detection in 28.5% of all patients with HCV viremia. Exact quantitation of positive samples was generally precluded by the low copy numbers (<50 RNA copies per reaction) of negative-strand RNA samples, which indicates that the prevalence of negative-strand RNA might have been underestimated because of limitations in sensitivity.

Furthermore, in terms of both CD4 cell count (median, 494 [range, 131–1336] vs. 508 [26–842] cells/mL; $P = .93$] and HIV load in plasma (median, 50 [range, <3–3.9×10^6] vs. 25 [<5–54,200]/mL; $P = .53$), the HCV RNA–positive patients did not differ from the HCV RNA–negative patients (table 1). Although some patients started or stopped highly active antiretroviral treatment during the observation period, no significant association with any of the above-mentioned markers was observed ($P \geq .34$).

Course of PBMC-associated HCV RNA over time. Sequential measurements of plasma, total, and virion-enclosed PBMC-associated HCV RNA were performed in a subset of patients (table 1). Patients 16–21 underwent repeat sampling (4–10 times) over time periods of 17–40 months, which allowed detailed longitudinal analyses (figure 3*A*). Except for patients 16 and 18 (who, by the end of the observation period, experienced treatment-induced clearance of viremia in plasma), HCV RNA loads in plasma showed no overall increase or decrease over time ($P = .56$). Patients 17 and 18 had patterns of pronounced fluctuations: >10-fold changes in plasma levels of HCV within 1 year. The course of total as well as virion-enclosed PBMC-associated HCV RNA levels revealed similar characteristics: no overall increase or decrease over time ($P = .27$ and $P = .28$, respectively) and, albeit less pronounced than those for viremia in plasma, fluctuations of 0.5–1.5 $\log_{10}$. Again, intracellular HCV RNA was consistently present.

Table 2. Correlation between plasma and peripheral-blood mononuclear cell (PBMC)–associated levels of hepatitis C virus (HCV) RNA.

	PBMC-associated HCV RNA		
Sample	Total	Virion enclosed	Intracellular
Cross-section of patients 1–21			
r^2	0.167	0.191	0.089
P	.066	.047	.246
Combined data sets[a]			
r^2	0.263	0.358	0.220
P	.044	.018	.068

[a] Robust linear regression accounting for multiple sampling was used.

Correlation between virion-enclosed and intracellular HCV RNA in PBMCs. Virion-enclosed and intracellular HCV RNA were strongly correlated ($r^2 = 0.801$; $P < .0001$) in cross-sectional analysis (figure 4*A*), a result that reflects that a consistent proportion—approximately half (geometric mean, 51% [95% CI, 39%–68%])—of total PBMC-associated HCV RNA was enclosed within virions. Longitudinal data confirmed this balanced distribution (geometric mean, 40% [95% CI, 35%–46%) (figure 4). To determine whether an apparently bimodal distribution of this proportion among patients could be assigned to host or viral factors, interdependencies with virological (plasma or cellular HCV RNA loads, HCV genotype, HIV load, and antiretroviral treatment), immunological (lymphocyte, monocyte, and CD4 cell counts), or potentially confounding (cell pellet size, clinical trial, and age of sample) factors were assessed by correlation analyses and rank tests of association ($P > .16$ in all cases). Moreover, the proportion of virion-enclosed HCV RNA did not significantly differ between patients ($P > .3$, by Kruskal-Wallis test).

Limited correlation between plasma and PBMC-associated HCV RNA. HCV RNA was observed solely in the PBMCs from patients with detectable HCV viremia; notably, HCV RNA became undetectable in the PBMCs of patients who experienced spontaneous (in the case of patient 13) or treatment-induced (in the case of patients 16 and 18) resolution of HCV viremia (table 1). Thus, it is conceivable that, in PBMCs, a significant contribution to the measured HCV RNA loads might come from free virus binding on leukocyte surfaces. However, the

association between viral loads in plasma and PBMC-associated HCV RNA was weak in cross-sectional analysis and, at most, of only borderline significance (table 2). To further elucidate this possibly weak interdependency, which might have been obscured by patient- and virus-specific factors, intrapatient correlation analysis of sequential samples was performed. In 2 of 6 patients, a significant association between plasma and total PBMC HCV RNA could be detected (figure 3*A*). Again, interdependency may have been concealed by the typically narrow range of virological fluctuation and by the limitations of the data set. Conversely, in patients with more-pronounced changes in plasma HCV RNA loads (patients 17 and 18), the course of PBMC-associated HCV RNA appeared to mirror the course of viremia; adjusted analysis of the combined data set revealed comparably weak but significant interdependence (table 2). Thus, the level of viremia in plasma might partly, but not entirely, account for the observed variation in PBMC-associated HCV RNA. Furthermore, the ratio of total cell-associated HCV RNA to plasma HCV RNA differed between patients but was relatively stable, over time, for a given patient ($P < .001$) (figure 3*B*). This finding suggests the importance of additional, host- or virus-specific determinants that affect systemic HCV replication, both in plasma and in PBMCs.

DISCUSSION

The mounting evidence for extrahepatic replication of HCV in vivo [5–12] relies mainly on findings of a qualitative nature, whereas little is known about viral dynamics in the PBMC compartment. Moreover, the relative contributions of HCV RNA within either the cells or virions attached to the cells have not been examined so far.

The presence of approximately equal amounts of intracellular and virion-enclosed HCV RNA associated with PBMCs, as clearly demonstrated in our analysis during chronic infection but also in a single primary case (patient 13), confirms the evidence for HCV infection of PBMCs in vivo. However, it remains unresolved whether there was active ongoing replication in the analyzed PBMC specimens; the fact that intracellular HCV RNA was strongly correlated with virion-enclosed HCV RNA, whereas levels of viremia in plasma were only loosely associated with cellular viral load, may be interpreted as being supportive of virus production in PBMCs, by the argument that the cell-associated virions attached to PBMCs may originate directly from intracellular production, rather than reflect viral particles captured from the plasma surrounding these cells.

Conversely, replicative intermediates were detected in only a minority (9.5%) of the samples tested. This result may be due to the limitations of the sensitivity of the assay of HIV negative-strand RNA, or it may indicate low replicative activity in PBMCs. Hence, intracellular HCV RNA might represent viral particles that have fused solely with the cell membrane and are not going to replicate themselves there. Such virions, which are engaged in the early entry process, are expected to be sensitive to FTN digestion and would qualify as "intracellular" in our analysis. In preliminary experiments, we have observed that nuclease sensitivity of otherwise completely FTN digestion–resistant plasma-derived HCV RNA may be induced in vitro by binding of HCV to primary human leukocytes but not to some immortalized human T and hepatoma cell lines (M.F. and P.K., unpublished data). These in vitro observations, which are reminiscent of ongoing viral entry, suggest that intracellular PBMC-associated HCV RNA in vivo might to some extent reflect ongoing fusion of virions in plasma. Thus, at least some of the infected cells may not actively transcribe the viral genome at a given time point but, rather, may passively carry intracellular viral-RNA genomes.

Although the present study found that plasma and cell-associated HCV RNA are subject to some common constraints, the relatively low strength of their correlations suggests, in accordance with the results of previous reports [36–38], differential regulation of PBMC-associated HCV RNA, by as-yet-unknown host- or virus-specific factors. In contrast, proportions of cell-associated HCV RNA load versus plasma HCV RNA load were observed to be patient specific and relatively stable over time. Thus, the ratio of plasma and cellular HCV burden may be characteristic of host-virus interaction and might mirror the infectivity that the predominant viral quasi species in plasma has for the patient's PBMCs.

To our knowledge, the present study is the first in which PBMC-associated HCV RNA has been both quantified with regard to its intra- versus extracellular localization and compared longitudinally to viral loads in plasma over a time period of up to several years (figure 3*A*). HCV RNA concentrations in plasma were found to have relatively stable courses without progressive changes. These findings are in concordance with reports that monitor viral loads in plasma over comparable periods [39–41], although other studies have indicated increases in HCV viremia [42, 43]. These discrepancies may be explained by the longer periods of observation and the differences in the patient populations (e.g., HIV coinfection) and assays used. The course of PBMC-associated HCV RNA, both in the total and in the virion-enclosed fraction, was found to be equally steady over time and appeared to mirror the course of virus concentrations in plasma. Notably, typical major fluctuation [44, 45] of the virus in the plasma of patients 17 and 18 corresponded to changes in the cellular compartment (figure 3*A*). Furthermore, successful antiviral treatment of patients 16 and 18 reduced PBMC-associated HCV RNA to undetectable levels before completely clearing it from plasma, a finding that is consistent with the lack of detection of HCV RNA in the PBMCs of patients whom the cross-sectional analysis had shown to

have undetectable viral loads in plasma (table 1). This latter finding contradicts reports of prolonged HCV persistence in the PBMC compartment when viremia is absent [19, 46]. More precisely, Radkowski et al. [19] have detected HCV RNA of both polarities in the leukocytes of patients experiencing years of sustained viral response. Occasionally, HCV was also detectable in serum and liver samples, suggesting the presence of more-widespread low-level persistence. Conversely, Castillo et al. [46] have reported HCV RNA in the PBMCs of patients with "occult" HCV infection, which implies undetectable serum HCV RNA loads but does not exclude its presence in the liver. Given the almost single-copy sensitivity of our PCR assay for positive-strand HCV RNA, methodological flaws are unlikely to have obviated detection of persistent HCV RNA, whereas the restricted sample size in our study represents a potential limitation that should be considered. On all accounts, our findings reveal that HCV-infected long-lived leukocytes are, at most, extremely rare in the peripheral blood.

In conclusion, our experiments reveal a substantial contribution of intracellular to total PBMC-associated HCV RNA. Although our findings describe a setting of HIV/HCV coinfection and remain to be confirmed for monoinfection, they support a concept of low-level replication in PBMCs. Indisputably, our data suggest that infection and expression of HCV in PBMCs is of minor quantitative importance for systemic replication and persistence of HCV; nevertheless, HCV infection of PBMCs may influence pathogenesis, not only as an independent reservoir but also because infected PBMCs might carry and deliver virions into other potential HCV reservoirs. Particularly in view of the notable patient specificity of HCV RNA loads in PBMCs, future studies should investigate whether this readily accessible cellular compartment of HCV infection provides any independent predictive value for either the early prognosis of the course of disease or its response to treatment.

Acknowledgments

We thank our patients, for their commitment to the study; R. Hafner, D. Baumann, C. Grube, and C. Schneider, for excellent patient care; C. Vögtli and I. Nievergelt, for administrative assistance; F. Burgener, for laboratory assistance; B. Ledergerber, for statistical advice; A. Zinkernagel, for helpful discussions; and J. Wong, for support and critical review of the manuscript.

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