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Presence of Hepatitis C Virus (HCV) Genomic RNA and Viral Replicative Intermediates in Bone Marrow and Peripheral Blood Mononuclear Cells from HCV-Infected Patients

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The cellular tropism of hepatitis C virus (HCV) was studied in vivo in samples from patients with persistent HCV infection. Plasma, liver, peripheral blood mononuclear cell (PBMC), and bone marrow cell (BMC) samples from 15 subjects positive for anti-HCV antibodies were tested for the presence of HCV RNA sequences by reverse transcription PCR. Virus-specific RNA sequences were found to be present in liver samples from all subjects (100%), in plasma samples from 13 of 15 patients (86.7%), in PBMC samples from 3 patients (20%), and in BMC samples from 9 (60%) of the 15 anti-HCV-positive patients enrolled in this study. The presence of the molecular intermediate of HCV replication (the negative-stranded HCV RNA) was evident in two of the three PBMC and in five of the nine BMC HCV RNA-positive samples. Finally, we studied the nucleotide sequence of a large portion (−270 to −59) of the 5′untranslated region of HCV amplified from plasma samples of 12 of the 15 patients with and without HCV in BMCs; the degree of heterogeneity compared with the prototype HCV sequence was similar in both groups. The data principally indicate that HCV infection of PBMCs and BMCs is frequent in persistently infected patients, as shown by the occurrence of positive- and negative-stranded HCV RNA, thus suggesting the possibility of extrahepatic replication of HCV.

Hepatitis C virus (HCV) is a positive-stranded hepatotropic RNA virus, and it is considered the causative agent of a wide spectrum of liver diseases, including posttransfusion non-A non-B hepatitis (2), chronic hepatitis and cirrhosis (12), an autoimmune form of hepatitis (14, 17), and primary hepatocellular carcinoma (23). Additionally, circulating antibodies to HCV (1, 11, 19–21) and HCV RNA sequences in serum samples (16) have been detected in the vast majority of patients with mixed cryoglobulinemia, an autoimmune disorder.

In the last few years, several reports have indicated that HCV is capable of infecting cells other than hepatocytes, e.g., peripheral blood lymphocytes (3, 22, 25). In this context, a more complete evaluation of cell and tissue tropism of HCV during persistent infection may be important for both a better understanding of the pathophysiology of this infection and a more correct clinical management of HCV-infected patients.

We planned a molecular analysis of persistent HCV infection in vivo. Accordingly, in this study, several molecular features of HCV infection were investigated in samples from anti-HCV-positive patients, including (i) the presence of specific HCV sequences in liver, plasma, peripheral blood mononuclear cells (PBMCs), and bone marrow cells (BMCs); (ii) the presence of negative-stranded HCV RNA in liver, plasma, BMC, and PBMC samples; (iii) the distribution of HCV RNA in PBMC and BMC subpopulations; and (iv) the sequence of the 5′-untranslated region (5′-UTR) of the HCV RNA isolated from these patients.

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MATERIALS AND METHODS

Patients and serological assays. Fifteen patients positive for anti-HCV antibodies (six males and nine females; age, 22 to 72 years; mean, 54.6 years) were included in this study. Antibodies to HCV were detected by two different enzyme immunoassays (from United Biomedical, Inc., Hauppage, N.Y., and Sorin Biomedica S.p.A., Saluggia, Italy) and were confirmed by a second-generation immunoblotting assay (Chiron RIBA 2; Ortho Diagnostic Systems, Raritan, N.J.). Hepatitis B virus markers were assayed with commercial kits from Murex (Dartford, United Kingdom) and Sorin Biomedica. All patients included in this study were positive for anti-HCV antibodies when tested by the enzyme immunoassays and RIBA 2. The patients underwent complete clinical examinations, including liver biopsy. Liver biopsies from these patients showed histologic features compatible with chronic active hepatitis in five patients, chronic persistent hepatitis in eight patients, and cirrhosis in two patients (Table 1). Six patients were under therapy with alpha interferon at the time of this study (Table 1).

Molecular analysis of HCV infection. PBMCs from 10 ml of heparinized blood were isolated by Ficoll-Hypaque (Sigma, St. Louis, Mo.) density gradient centrifugation, resuspended in 10 ml of RPMI 1640 (Whittaker, Walkersville, Md.), and washed twice in cold phosphate-buffered saline (PBS). BMCs were collected by aspiration from the posterior iliac crest, and cells were isolated as for PBMCs. Total RNA was extracted from fresh (unfrozen) BMCs, PBMCs, liver tissues, and 100-μl plasma samples, using the guanidinium thiocyanate method (6). For HCV RNA detection, an aliquot (10 μl) of purified RNA was reverse transcribed and amplified with specific primers for the 5′-UTR of the HCV genome: HCV-01 (5′-ACC ATG AAT CAC TCC CCT GTG AGG AAC TA, recognizing a specific HCV sequence at positions −313 to −285) and HCV-02 (5′-CAC TCG CAA GCA CCC TAT CAG GCA GTA CCA, recognizing a specific HCV sequence

TABLE 1. HCV RNA sequences in clinical samples from anti-HCV-positive patients

Patient no.	Liver histology[a]	Detection of HCV RNA				Therapy (months)[b]
		Plasma	Liver	PBMCs	BMCs	
A01	CPH	+	+[c]	+[c]	+[c]	IFN-α (11)
A02	CPH	+	+[c]	+[c]	+[c]	IFN-α (15)
A03	CPH	+	+[c]	+	+[c]	IFN-α (20)
A04	CAH	+	+[c]	−	+[c]	IFN-α (10)
A05	CPH	+	+[c]	−	+[c]	None
A06	CPH	−	+[c]	−	+	Steroids (3)
A07	CAH	−	+[c]	−	+	Steroids (3)
A09	CPH	+	+[c]	−	−	IFN-α (14)
A12	CAH	+	+[c]	−	+	None
A13	CIR	+	+[c]	−	+	IFN-α (12)
A15	CPH	+	+[c]	−	−	None
A16	CIR	+	+[c]	−	−	None
A17	CAH	+	+[c]	−	−	None
A18	CPH	+	+[c]	−	−	None
A19	CPH	+	+[c]	−	−	None

[a] CPH, chronic persistent hepatitis; CAH, chronic active hepatitis; CIR, cirrhosis.

[b] IFN-α, human recombinant alpha interferon (3 MU intramuscularly three times a week). The steroids used was deflazacort (12 mg/day).

[c] Detection of negative-stranded HCV RNA also.

at positions −29 to −58), thus encompassing a 285-bp viral sequence (−313 to −29). Reverse transcription (RT) was carried out at 42°C for 30 min in a mixture (final volume, 20 μl) containing 10 μl of RNA sample, 1× PCR buffer (50 mM NaCl, 10 mM Tris HCl [pH 8.3], 1.5 mM $MgCl_2$, 0.01% gelatin), 0.2 mM each deoxynucleoside triphosphate, 10 pmol of antisense primer HCV-02, 20 U of RNasin, and 100 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, Md.). After a denaturation step at 94°C for 5 min, the amplification reaction (50 cycles) was performed in a mixture (final volume, 100 μl) containing 1× PCR buffer, 2.5 U of *Taq* DNA polymerase, and primers HCV-01 and HCV-02 (final concentration, 50 pmol each), using an automated thermal cycler (Perkin-Elmer 9600; Perkin Elmer Cetus, Norwalk, Conn.) and the following amplification profile: denaturation at 94°C for 60 s, annealing at 55°C for 45 s, and extension at 72°C for 60 s. We tested for the presence of the HCV replicative intermediate in liver, plasma, BMC, and PBMC samples by reverse transcribing RNA samples, using the consensus primer HCV-01 in independent RT assays.

Finally, PBMC and BMC samples from five patients (patients A01 to A05) were also separated into subsets: heparinized venous blood samples were loaded over Mono-Poly Resolving Medium (Flow Laboratories, Milan, Italy) and centrifuged at 300 × *g* for 30 min at room temperature, resulting in the formation of two cell bands. The first band, located at the plasma–Mono-Poly Resolving Medium interface, contained mononuclear cells which were 95 to 98% viable; the second band, located 10 mm below the first one, consisted of 99% pure polymorphonuclear leukocytes, which were 98% viable. The cells of the first fraction were recovered, washed twice in RPMI 1640 with 10% fetal calf serum, and divided into two aliquots. The first aliquot was incubated for 30 min at 37°C in 5% CO_2 in a plastic petri dish; nonadherent cells were dislodged with several washings of cold PBS, and adherent cells containing more than 95% nonspecific esterase-positive cells were employed for the molecular studies. The second aliquot was employed for positive selection of $CD2^+$ and $CD19^+$ cells, using Dynabeads (Dynal International, Oslo, Norway) according to the manufacturer's instructions. Bone marrow aspirates, anticoagulated with heparin, were processed as described for the peripheral blood samples.

Direct sequencing of amplified HCV sequences. For direct sequencing of a portion of the HCV 5′-UTR, PCR products were deproteinized and purified by phenol-chloroform extraction and centrifugation with Centricon 100 filters (Amicon Division, Beverly, Mass.). Double-stranded purified material was directly sequenced by using the T7 DNA polymerase chain termination method and ^{35}S-dATP (Pharmacia LKB Biotechnology, Uppsala, Sweden) as previously described (15); amplified cDNA samples were bidirectionally sequenced with both primers HCV-01 (consensus primer) and HCV-02 (antisense primer). To perform a comparative analysis of the viral genotypes among the patients studied, a portion of the HCV genome (the −270 to −59 sequence from the HCV 5′-UTR) was sequenced directly with cDNA amplified from plasma samples from 12 of the 15 patients.

RESULTS

All liver biopsy samples were positive for positive- and negative-stranded HCV RNA sequences (Table 1), confirming the specificity of the serological data and demonstrating that an active HCV infection occurs in all of these patients. When PBMC and BMC samples from these subjects were assayed for the presence of virus-specific sequences, 3 (20%) and 9 (60%) of the 15 patients tested positive by RT-PCR, respectively (Table 1). HCV RNA was also assayed in the last cell wash; all samples tested negative, thus excluding possible plasma contamination of cellular samples. Two patients (A06 and A07) exhibited the presence of HCV RNA sequences in BMCs, although RT-PCR testing for viral sequences in plasma gave negative results; six patients with HCV sequences in BMCs tested negative for viral sequences in PBMC samples, while all patients positive for HCV RNA in PBMCs showed infection in BMCs. Overall, 9 of the 15 anti-HCV-positive patients (60%) showed molecular signs of extrahepatic HCV infection.

The replicative form of the HCV genome (the negative-stranded HCV RNA) was assayed in liver tissues, plasma samples, BMCs, and PBMCs and detected in all liver samples, in five of the nine BMC samples, and in two of the three PBMC HCV RNA-positive samples. All plasma samples tested negative for the presence of HCV replicative intermediate (Table 1).

Molecular analysis of the cell populations obtained was carried out in 5 of the 15 patients and documented the presence of specific HCV RNA sequences only in mononuclear cells: in the $CD2^+$-enriched fraction in three patients (patients A01 and A02, in PBMCs and BMCs, and patient A05, in BMCs), in the $CD19^+$ subset in one patient (patient A03, in PBMCs and BMCs), and in the adherent cells in one patient (patient A04, in BMCs). Interestingly, in each sample investigated, only one cell subpopulation tested RT-PCR positive.

Direct sequencing was carried out on a large portion of the HCV 5′-UTR. Sequencing data of samples from patients with and without HCV infection of BMCs revealed the presence of highly conserved domains interpersed with clustered variable domains in viral isolates from both groups (data not shown). The degree of heterogeneity compared with the prototype HCV sequence (7) averaged 2.68% (0 to 5.66%). Nucleotide variations were primarily located in three major domains (−239 to −228, −151 to −139, and −128 to −119); otherwise, in most isolates, domains invariably conserved were observed between nucleotides −270 and −238, −188 and −78, and −76 and −29.

DISCUSSION

The data shown here principally indicate that a high proportion of HCV-infected patients (including patients whose plasma tested negative for HCV RNA) harbor specific viral sequences in PBMCs and BMCs; this extrahepatic HCV infection may be active, as documented by the presence of viral negative-stranded RNA (the replicative intermediate of HCV replication) in BMCs and PBMCs. Thus, the results of this study are in agreement (and extend to BMCs) with those of others (25) who found HCV RNA sequences by RT-PCR in PBMC samples from patients with chronic hepatitis and are in contrast with early data suggesting that this virus replicates in liver cells but not in lymphocytes (24). Furthermore, in this study, we did not observe the presence of negative-stranded HCV in plasma samples, as was recently reported by others (8). It will be important to clarify this aspect in the future in clinically and methodologically homogeneous researchs; however, in this study, the negative result in plasma samples underlines the specificity of data obtained from cell preparations.

More recently, both positive- and negative-stranded HCV RNA sequences were detected in PBMCs from 10 untreated patients with HCV infection and chronic hepatitis (22) and in the monocyte-macrophage subpopulation of PBMCs from persistently infected patients (3). The study presented here extends the molecular investigation to BMC samples from HCV-infected patients; direct evidence of active viral infection of these cells has been shown in samples from untreated patients and from patients under therapy with alpha interferon. Although the extrahepatic cellular tropism of HCV may play a role in the pathophysiology of this infection, the precise biological significance of the presence of HCV sequences in BMCs and PBMCs presently remains to be established. In particular, whether HCV replicates transiently or persistently in these cells and whether infection of cells other than hepatocytes plays a role in the development of chronic hepatitis and/or immunologic disorders associated with this human infection should be investigated.

The comparative sequence data obtained in this study exclude that significant differences in the HCV 5′-UTR exist between the patients with and those without PBMC and BMC infection. Recent evidence obtained by sequence analysis of the complete HCV genome or partial sequencing of HCV isolates has indicated that HCV exhibits high genetic variability (7); in fact, significant genetic heterogeneity has been reported not only among HCV isolates from different geographic areas (5), but also within isolates from a single individual (10), thus indicating that this virus is particularly prone to genetic variation. In this context, it may be hypothesized that appearance and selection of a new HCV variant could be responsible for a modified viral tissue tropism. This aspect deserves particular attention since circulation of HCV types with genomic heterogeneity may have implications for virus-host interactions, pathogenic potential, clinical evolution, and vaccine development. Published sequence data indicate that the HCV 5′-UTR region shares sequence similarity with the 5′ noncoding region of pestiviruses (18). More recently, contrary to early data indicating that this region is highly conserved (9), not only has significant evidence of sequence heterogeneity of 5′-UTR among different HCV isolates come to light (4, 13), but it has been suggested that this region may play a major role in viral replication and gene expression (4). Mainly for these reasons, we compared the 5′-UTR sequences of most viral isolates from the infected patients studied; although our data cannot exclude that differences in other HCV regions may exist, we did not observe any significant differential feature in sequences from the viral isolates, thus ruling out the possibility that genetic variability of the 5′-UTR may be the basis of the differential molecular aspects of infection observed in these HCV-infected subjects.

Finally, our findings may have some implications clinically. In fact, in the 6 months preceding this investigation, 6 of the 15 patients were treated with recombinant alpha interferon, but HCV RNA was still present in the BMCs of 5 of these subjects (Table 1). Although longitudinal research is clearly necessary to evaluate this point precisely, thus clarifying the temporal relationship between therapy and data reported herein, this indicates that complete molecular evaluation of the response to any treatment employed in HCV-infected patients should consider not only the presence of HCV RNA in plasma and liver but also viral infection of PBMCs and BMCs.

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