MAJOR ARTICLE

Occult Hepatitis C Virus Infection in Patients in Whom the Etiology of Persistently Abnormal Results of Liver-Function Tests Is Unknown

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(See the editorial commentary by Lerat and Hollinger, on pages 3–6.)

Background. There are patients in whom the etiology of long-standing abnormal results of liver-function tests is unknown (ALF-EU) after exclusion of all known causes of liver diseases. We analyzed the presence of hepatitis C virus (HCV) RNA in liver-biopsy specimens from 100 patients who were negative for anti-HCV antibodies and for serum HCV RNA and who had ALF-EU.

Methods. HCV RNA status was tested by reverse-transcription polymerase chain reaction (RT-PCR) and by in situ hybridization, in liver and peripheral-blood mononuclear cells (PBMCs).

Results. HCV RNA was detected in liver-biopsy specimens from 57 of 100 patients negative for anti-HCV antibodies and for serum HCV RNA (i.e., who had occult HCV infection). HCV RNA of negative polarity was found in the liver of 48 (84.2%) of these 57 patients with occult HCV infection. Nucleotide-sequence analysis confirmed the specificity of detection of HCV RNA and that patients were infected with the HCV 1b genotype. Of these 57 patients with intrahepatic HCV RNA, 40 (70%) had viral RNA in their PBMCs. With regard to liver histology, patients with occult HCV infection were more likely to have necroinflammatory activity ($P = .017$) and fibrosis ($P = .022$) than were patients without intrahepatic HCV RNA.

Conclusion. Patients with ALF-EU may have intrahepatic HCV RNA in the absence of anti-HCV antibodies and of serum HCV RNA.

Abnormal results on liver-function tests have various causes, and the etiology of the liver damage can be established in most cases. However, there are patients in whom the etiology of long-standing abnormal results on liver-function tests is unknown (ALF-EU) after rigorous exclusion of all known causes of liver diseases and for whom no final diagnosis can be made even after liver biopsy.

In an attempt to determine the etiology of the elevated liver-enzyme levels in these patients, the presence of hepatitis C virus (HCV) RNA in serum, in the absence of detectable anti-HCV antibodies, has been analyzed. Although some investigators have found serum HCV RNA in some of these patients [1], others have not [2]. However, it may be speculated that HCV RNA might be present in the liver, in the absence of viral RNA in serum, as has been shown for the hepatitis B virus (HBV) infecting humans and woodchucks [3–5]. Thus, we have analyzed the presence of HCV RNA, by reverse-transcription polymerase chain reaction (RT-PCR) and by in situ hybridization, in liver-biopsy specimens from a cohort of patients who are negative for anti-HCV antibodies and for serum HCV RNA and who have ALF-EU.

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Table 1. Characteristics of patients negative for anti–hepatitis C Virus (HCV) antibodies and for serum HCV RNA who had abnormal liver-enzyme levels of unknown etiology.

Category	Data
No. of patients	100
Sex, male/female	76/24
Age, mean ± SD (range), years	45.2 ± 11.1 (22–66)
Body-mass index, mean ± SD (range)	26.6 ± 3.9 (18–44)
History of transfusion, no. of patients[a]	8
Estimated duration of abnormal liver-function tests, mean ± SD (range), months	71.2 ± 66.4 (12–288)
Liver-enzyme levels, mean ± SD (range), IU/L:	
Aspartate aminotransferase[b]	37.5 ± 16.2 (9–109)
Alanine aminotransferase[b]	61.1 ± 33.6 (11–207)
γ Glutamyl transpeptidase[c]	93.5 ± 9.7 (6–683)
Cholesterol level, mean ± SD (range), mg/dL	210.9 ± 43.5 (30–311)
Triglyceride level, mean ± SE (range), mg/dL	140.2 ± 17.8 (37–1796)

[a] The only risk factor, for HCV infection, reported by patients.
[b] Normal value, ⩽43 IU/L.
[c] Normal value, ⩽45 IU/L.

PATIENTS, MATERIALS, AND METHODS

This study was conducted according to the ethical guidelines of the 1975 Helsinki Declaration, and all patients gave written informed consent to participate in the study. Two institutions, attended by a total of 5000 patients with liver diseases, participated in the enrollment of patients. The enrollment period lasted 2 years, and, during that time, 100 patients were selected for the study. The selection criterion was presentation, for ⩾12 months before the liver biopsy, of sustained abnormal liver-enzyme levels that were above the normal values (tested every 3 months) for ⩾1 of the 3 liver enzymes (aspartate aminotransferase [AST], alanine aminotransferase [ALT], and γ glutamyl transpeptidase [GGTP]) and that were of unknown etiology. All known causes of liver diseases were excluded on the basis of analytical, clinical, and epidemiological data: infection by HBV (i.e., subjects were negative for hepatitis B surface antigen and for serum HBV-DNA), HCV (i.e., subjects were negative for anti-HCV antibodies and for serum HCV RNA, on the basis of in-house RT-PCR as described below), autoimmunity, metabolic and genetic disorders, alcohol intake, and drug toxicity—and all cases were negative for anti-HIV antibodies. Eight patients had abnormal levels of AST, ALT, and GGTP simultaneously, 48 had abnormal values of 2 liver enzymes (ALT and GGTP, 34 patients; ALT and AST, 14 patients), and 44 had abnormal levels of only 1 liver enzyme (GGTP, 32 patients; ALT, 12 patients). None of the patients reported a clinical or biochemical history of acute hepatitis. In addition, all patients were carefully interviewed for risk factors for HCV infection, including drug abuse, blood transfusions, tattoos, body piercings, and sexual behavior; patients were negative for all of these factors, except for 8 patients who reported having had blood transfusion(s). The characteristics of the patients included in the study are shown in table 1. As control subjects, 30 patients with histologically proven liver damage of nonviral origin (12 with primary biliary cirrhosis, 7 with alcoholic hepatitis, 10 with autoimmune chronic hepatitis, and 1 with Gilbert syndrome) also were studied.

The patients underwent a liver biopsy (using Tru-cut needles; Baxter Healthcare) for diagnostic purposes. Liver-biopsy specimens were divided into 2 portions. One portion was fixed in 10% formalin and was paraffin-embedded for routine histological diagnosis and for in situ hybridization. Histological evaluation was performed by 2 pathologists who were blinded with respect to the HCV RNA status of the liver-biopsy specimens. Necroinflammatory activity and fibrosis were scored according to the METAVIR score system [6, 7]. Another portion, a minor (4–5-mm) fragment of the specimen, was immediately snap frozen in liquid nitrogen (elapsed time from extraction of the liver-biopsy specimen to freezing, 25–30 s) and was stored until used for detection of HCV RNA by RT-PCR.

Samples of serum and of peripheral-blood mononuclear cells (PBMCs) were collected from all patients on the same day that the liver biopsy was performed and were aliquoted and stored either at −80°C (for serum) or in liquid nitrogen (for PBMCs) until used. Anti-HCV antibodies were assayed on ⩾2 occasions, by 2 different commercial EIAs: a third-generation test (Ortho HCV 3.0 ELISA; Ortho Diagnostic Systems) with estimated sensitivity and specificity of 98.9% and 100%, respectively [8], and a fourth-generation test (INNOTEST HCV Ab IV; Innogenetics) with 100% sensitivity and 100% specificity, respectively (National Serologic Reference Laboratory, Australia; http://www.nrl.gov.au).

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HCV RNA in serum, liver, and PBMCs was tested by RT-PCR using primers from the 5′ noncoding region (5′NCR) of the HCV genome [9]. Total RNA was isolated from 200 μL of serum and from liver and PBMCs, by the SV Total RNA Isolation System (Promega); and, after precipitation, the RNA pellet was dissolved in 10 μL of diethylpyrocarbonate-treated water. Reverse transcription and the first amplification were performed by the Access RT-PCR System (Promega), with either 2 μL of total RNA from serum or 0.5 μg of total RNA from either liver, PBMCs, or HepG2 cells (as the negative control). The RT-PCR was performed for 45 min at 48°C, followed by 2 min at 94°C and by the first amplification round, which consisted of 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 68°C. The second PCR was performed with 2 μL of the product of the first PCR reaction, under the same conditions that were used for the first PCR. The sensitivity of this RT-PCR was 10 IU/mL, as determined by testing of serial dilutions of a serum sample that was positive for HCV RNA (genotype 1b) and in which HCV RNA quantification had previously been assayed by an Amplicor HCV Monitor 2.0 (Roche Diagnostics).

Detection of HCV RNA was also performed in all liver samples by use of degenerated primers designed to amplify the HCV core region of HCV genotypes 1–3. The core primers were as follows: outer sense, 5′-AGGACGTYAAGTTCCCG-3′ (nt 391–407); outer antisense, 5′-GARAARGAGCAACCRGG-3′ (nt 840–856); inner sense, 5′-TSYTGCCGCGCAGGGGC-3′ (nt 439–455); and inner antisense, 5′-GTACCCCATGAGRTC-GGC-3′ (nt 723–740) (Nucleotide positions are per Takamizawa et al. [10], with Y = C or T, R = A or G, and S = C or G.) The conditions for RT-PCR were identical to those used for detection of HCV RNA by use of the 5′NC primers, except that the annealing temperature was 50°C. The sensitivity of this RT-PCR (tested as described above) was 100 IU/mL.

All PCR assays were performed according to the recommendations of Kwok and Higuchi [11]. Furthermore, several negative controls (no-RNA) controls were included in each PCR step, to assure the specificity of the results.

Genotyping of the HCV RNA amplified from liver-biopsy specimens was performed by a standard methodology (INNO-LIPA HCV II; Innogenetics). Paraffin-embedded liver sections (4 μm) and PBMCs were pretreated for in situ hybridization, as described elsewhere [12, 13]. The HCV RNA of positive polarity was detected by use of a cRNA probe obtained by in vitro transcription of the pC5′NCR, which contains the complete 5′NCR of the HCV genome. Detection of the HCV RNA of negative polarity was performed by use of a cRNA probe spanning 390 nt of the HCV core coding region, obtained by in vitro transcription of the pC core plasmid. Hybridization conditions for the in situ detection of the HCV RNA of both polarities were as described elsewhere [12]. The specificity of the in situ hybridization was assessed by (1) digestion of the sections by either RNase A (0.2 mg/mL) or DNase I (20 U/mL), for 2 h at 37°C, before hybridization; (2) hybridization with an unrelated RNA probe (a 360-base fragment of the chloramphenicolacetyltransferase gene); and (3) omission of the probe in the hybridization mixture. The percentage of infected cells was determined by visual inspection of ⩾2000 cells from each liver-biopsy specimen.

Immunohistochemical detection of the HCV core antigen in liver-biopsy specimens was performed automatically (Tech Mate 500; Dako) by use of an anti–HCV core monoclonal antibody (Hepatitis C Virus Monotope; ViroStat) at a 1:30 dilution, after antigenic-retrieval pressure cooking (3 min in a buffer of 10 mmol of citrate/L, pH 6.0), by the Dako Envision staining procedure and with aminoethylcarbazole as the chromogen, after the endogenous peroxidase had been blocked by treatment with H_2O_2. Liver-biopsy specimens from 3 patients with chronic hepatitis C (positive for anti-HCV antibodies and for serum HCV RNA) and in which the presence of HCV core antigen had previously been demonstrated were included as positive controls.

For sequence analysis of HCV RNA, PCR products corresponding to the HCV core region of the genome that were amplified from liver-biopsy specimens of 10 randomly selected patients were cloned into the pCRII-TOPO vector (Invitrogen). Five clones from each sample were sequenced by an ALF Express DNA Automated Sequencer (Amersham Pharmacia Biotech), and the sequences obtained were aligned and compared with published HCV sequences. In 26 randomly selected patients with intrahepatic HCV RNA, the total number of CD3, CD4, and CD8 cells was counted in a flow cytometer (EPICS XL; Beckman-Coulter).

The statistical analyses were performed by SPSS package release 9.0 (SPSS). Comparisons between groups were made by Student's *t* test (for continuous variables) and by either the χ^2 test or Fisher's exact test (for categorical data). Correlations were determined by Spearman's rank correlation test. Epidemiological and clinical data of the patients (gender; age; body-mass index; estimated duration of abnormal liver-function test results; previous blood transfusions; levels of AST, ALT, GGTP, cholesterol, and triglycerides; and presence or absence of intrahepatic HCV RNA) were included in a logistic regression analysis to identify independent factors associated with necroinflammatory activity and fibrosis. A 2-tailed *P* value <.05 was considered to denote statistical significance.

RESULTS

All patients were negative for anti-HCV antibodies and for serum HCV RNA when they were enrolled in the study; and, when assays for the 2 markers were subsequently repeated (by 1 of the participating institutions) in the serum sample obtained

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when the liver biopsy was performed, all patients were again found to be negative for the 2 markers. RT-PCR using primers from the 5′NCR detected intrahepatic HCV RNA in 57 (57%) of 100 patients with ALF-EU, but it did not detect it either in the liver-biopsy specimens from the 30 control patients with nonviral liver diseases or in HepG2 cells; we will refer to these 57 patients with intrahepatic HCV RNA as having "occult HCV infection." HCV RNA was also tested in the liver-biopsy specimens from all patients and control subjects, by RT-PCR using primers from the HCV core region of the genome. Of the 57 patients who were found to be positive for intrahepatic HCV RNA when primers from the 5′NCR were used for testing it, 40 (70%) also were positive when the core primers were used for testing it; the remaining patients and the control subjects were negative for intrahepatic HCV RNA. In terms of epidemiological or clinical data, no significant differences were found between the patients with occult HCV infection and the patients without it (table 2).

By in situ hybridization, the HCV RNA of positive polarity was detected in the biopsy specimens from the 57 patients with occult HCV infection, whereas no hybridization signals were observed in the liver-biopsy specimens from the remaining patients and the control subjects (figure 1); the HCV RNA of negative polarity was detected in 48 (84.2%) of the 57 patients with occult HCV infection. The specificity of in situ hybridization for HCV RNA of both polarities was confirmed by the absence of signals when an unrelated probe was used or when the probe was omitted from the hybridization mixture. In addition, predigestion of the slides with RNase abolished the hybridization signals, whereas predigestion with DNase did not (figure 1). Hepatocytes containing hybridization signals were randomly distributed throughout the liver-biopsy specimens, and the signals were detected in the cytoplasm. The mean ± SD percentage of hepatocytes containing the HCV RNA of positive polarity was 5.4% ± 4.3% (range, 0.1%–18.0%), which was significantly higher ($P = .001$; Student's t test) than that of hepatocytes with the HCV RNA of negative polarity (mean, 2.9% ± 3.4% [range, 0.1%–16.0%]). In the 57 patients with occult HCV infection, no correlation between the percentage of infected hepatocytes and the levels of AST, ALT, or GGTP was found. Finally, none of the liver-biopsy specimens from either the 100 patients with ALF-EU or the 30 control subjects were positive for HCV core antigen by immunohistochemical detection, whereas this antigen was present in the liver-biopsy specimens of the 3 patients with chronic hepatitis C who were included as positive control subjects.

RT-PCR detected viral RNA in PBMCs from 40 (70%) of the 57 patients with intrahepatic HCV RNA but not in PBMCs from any of the patients who did not have occult HCV infection or from any of the control subjects. The presence of HCV RNA in the PBMCs of these 40 patients was confirmed by in situ hybridization (figure 2). Although 35 (87.5%) of these 40 patients had in their liver cells the HCV RNA of negative polarity, no correlation between its presence in the liver and infection of the PBMCs was found. The total numbers of CD3, CD4, and CD8 cells in the 26 randomly selected patients with occult HCV infection were within the normal ranges.

Genotyping of intrahepatic HCV RNA showed that the 57 patients with occult HCV infection had genotype 1b, a genotype that accounts for >90% of chronic HCV infections in the population that we studied [14, 15]. The nucleotide sequence analysis of the HCV core region from 10 randomly selected patients

Table 2. Comparison between patients with or without intrahepatic hepatitis C virus (HCV) RNA.

Category	Results of test for intrahepatic HCV RNA		P
	Positive	Negative	
No. of patients	57	43	
Sex, male/female	44/13	32/11	.748
Age, mean ± SD, years	44.8 ± 10.9	45.8 ± 11.5	.669
Body-mass index, mean ± SD	27.1 ± 4.3	26.0 ± 3.2	.183
History of transfusion, no. of patients[a]	3	5	.284
Estimated duration of abnormal liver-function test levels, mean ± SD, months	72.5 ± 71.8	69.6 ± 59.3	.831
Liver-enzyme levels, mean ± SD (range), IU/L			
Aspartate aminotransferase [b]	38.0 ± 17.4	36.9 ±14.8	.741
Alanine aminotransferase[b]	62.5 ± 36.6	59.3 ± 29.5	.638
γ Glutamyl transpeptidase[c]	95.0 ± 14.7	91.6 ± 11.6	.863
Cholesterol level, mean ± SD, mg/dL	213.2 ± 44.0	207.9 ± 43.2	.547
Triglyceride level, mean ± SE, mg/dL	148.0 ± 30.4	130.0 ± 9.8	.619

[a] The only risk factor, for HCV infection, reported by patients.
[b] Normal value, ⩽43 IU/L.
[c] Normal value, ⩽45 IU/L.

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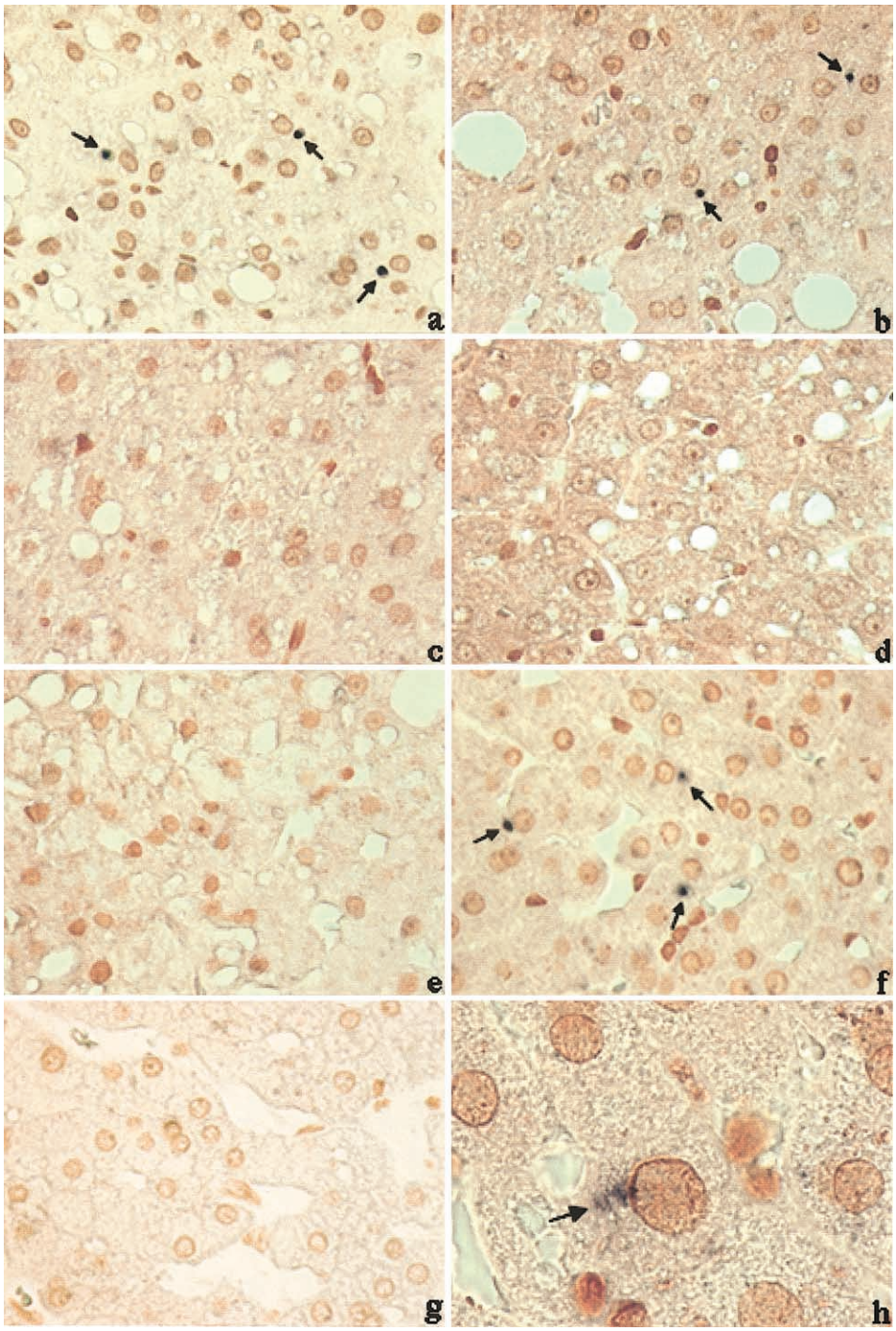

Figure 1. Detection of the hepatitis C virus (HCV) RNA of positive polarity *(a)* and of the HCV RNA of negative polarity *(b),* in a liver-biopsy specimen from a patient with occult HCV infection. Specificity controls are hybridization in the absence of the probe *(c),* hybridization with an unrelated labeled probe *(d),* RNase digestion *(e),* and DNase digestion *(f).* Absence of hybridization signals in a liver-biopsy specimen from a patient without occult HCV infection *(g)* and visualization of the infection in a single hepatocyte, showing the cytoplasmic localization of the signal *(h),* also are shown. All specimens were counterstained with safranine. Arrows in panels *a, b, f,* and *g* indicate hybridization signals. Original magnifications, ×400 for *a–g* and ×1000 for *h.*

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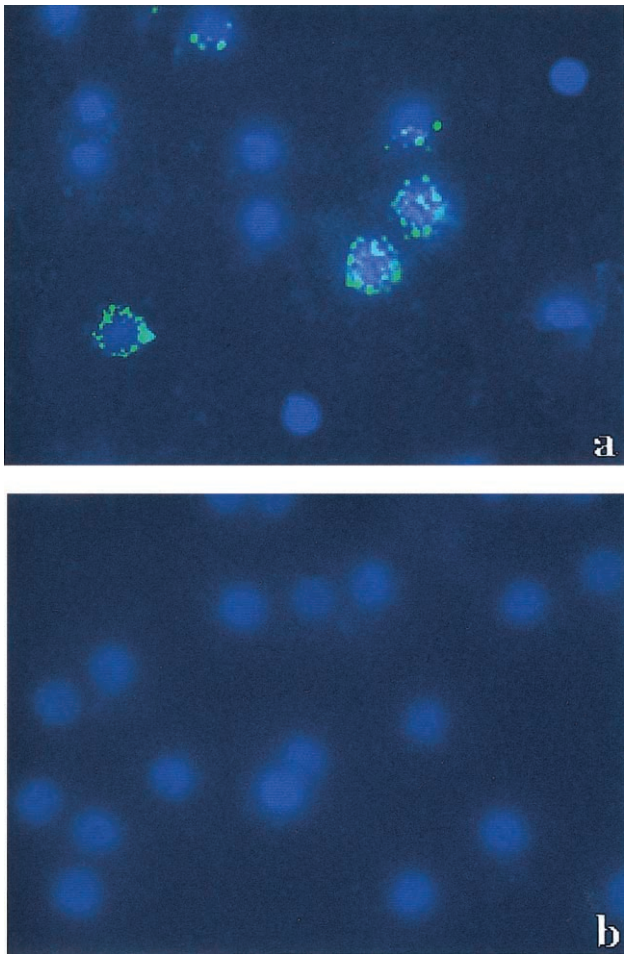

Figure 2. Fluorescent in situ hybridization of the hepatitis C virus (HCV) RNA of positive polarity, in peripheral-blood mononuclear cells from a patient with occult HCV infection *(a)* and from a patient without occult HCV RNA *(b)*. Both groups of cells were counterstained with 4–6 diamidino-2-phenylindole. Original magnification, ×1000.

confirmed that the HCV isolates belonged to genotype 1b (homology range, 93.4%–95.4%). The nucleotide identities of the C gene among the 10 patients were in the 93.5%–96.4% range, whereas the intrapatient homology among the HCV clones was in the 98.8%–99.2% range. The deduced amino acid sequence showed 100% homology among all the clones.

The histological diagnosis of the 100 liver-biopsy specimens from the patients with ALF-EU ranged from minimal nonspecific changes to liver cirrhosis (table 3). Of the 57 patients with occult HCV infection, 20 (35%) had necroinflammatory activity (level, ⩾A1 [according to METAVIR), a frequency significantly higher ($P = .017$; χ^2 test) than that found in patients without intrahepatic HCV RNA (6 of 43 [14%]). In addition, the number of cases with fibrosis (level, ⩾F1 [according to METAVIR]) was significantly higher ($P = .022$; Fisher's exact test) in patients with occult HCV infection (10 [17.5%] of 57) than in patients without intrahepatic HCV RNA (1 of 43 [2.3%]) (see table 3). In a logistic regression analysis, only the following 2 variables were found to be independently related with the existence of necroinflammatory activity (⩾A1): age ($P = .003$; odds ratio [OR], 1.08 [95% confidence interval {95%CI}, 1.027–1.14]) and the presence of intrahepatic HCV RNA ($P = .01$; OR, 4.2 [95%CI, 1.41–12.52]). Similarly, in a logistic regression analysis, these 2 variables also were independently related with the presence of fibrosis (stage, ⩾F1): age ($P = .04$; OR, 1.08 [95%CI, 1.00–1.15]); presence of intrahepatic HCV RNA ($P = .03$; OR, 10.57 [95% CI, 1.26–88.87]). Finally, the number of cases with steatosis was significantly lower ($P = .047$; χ^2 test) among patients with occult HCV infection than among patients without intrahepatic HCV RNA (10 [15.5%] of 57 vs. 15 [35%] of 43).

DISCUSSION

In the present study, 57 of 100 patients who were negative for anti-HCV antibodies and for serum HCV RNA and who had ALF-EU had occult HCV infection, as demonstrated by the detection of HCV RNA in liver-biopsy specimens by RT-PCR using primers from the 5′NCR of the HCV genome. It could be argued that the absence of anti-HCV antibodies in these patients (tested by 2 different methods in ⩾2 different samples) was due to alterations in the patients' immunological systems; however, this seems unlikely, because the numbers of CD3, CD4, and CD8 cells were within the normal ranges in 26 randomly selected patients with occult HCV infection. It also could

Table 3. Histological findings in 100 patients with abnormal liver-enzymes levels of unknown etiology.

Histological finding	Results of testing for HCV RNA in liver, no. of patients: Positive ($n = 57$)	Negative ($n = 43$)	P[a]
Minimal changes	27	22	.707
Steatosis[b]	10	15	.047
METAVIR activity score[c]			
A1	13	5	.017
A2	3	1	
A3	4	0	
METAVIR fibrosis score[d]			
F1	3	0	.022
F2	2	0	
F3	2	1	
F4 (cirrhosis)	3	0	

[a] $P < .05$ was considered to be significant.
[b] None of the patients had histological features of steatohepatitis.
[c] None of the patients had a score of A0.
[d] A total of 10 patients positive for HCV RNA and 5 patients negative for HCV RNA had a score of F0.

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be argued that the absence of serum HCV RNA in these patients (tested in ⩾2 different samples) is attributable to low sensitivity of the RT-PCR assay; however, this also seems unlikely, because the sensitivity (10 IU/mL) of the RT-PCR assay that we used is the same as that of the most sensitive methods for detection of HCV RNA that currently are available [16].

In the present study of 57 patients with occult HCV infection, the specificity of the detection of HCV RNA in the liver has been confirmed in all of them by in situ hybridization and in 40 of them by amplification of HCV RNA by primers from the HCV core region. The lower percentage of intrahepatic HCV RNA detected by use of the core primers can be attributed to the different sensitivities of the sets of primers used by RT-PCR or to the possible existence of nucleotide point mutations that preclude the annealing of the core primers. Finally, sequence analysis of the HCV core region showed nucleotide-sequence differences between the patients, as well as the existence of quasi-species in each patient, a finding that excludes the possibility of cross-contamination between samples.

To our knowledge, only 2 studies have analyzed the presence of HCV RNA in liver samples from patients with chronic hepatitis in the absence of anti-HCV antibodies and of serum HCV RNA, with negative results [17, 18]. This discrepancy probably can be explained by differences in technical conditions. For example, Geller et al. [17] used paraffin-embedded liver sections, in which HCV RNA degradation may occur [19, 20], rather than properly frozen liver-biopsy specimens (such as those in the present study), in which such degradation does not occur [21]. Bresters et al. [18] used frozen liver-biopsy specimens, but they did not report the elapsed time between extraction and freezing of the specimen—a crucial period, because degradation of viral RNA can occur if this elapsed time is too long [21]. Supporting our findings is Verslype et al.'s [22] recent report of the presence of HCV envelope 2 protein in the liver of 6 patients negative for anti-HCV antibodies and for serum HCV RNA, whereas RT-PCR found only 1 patient to be positive for intrahepatic HCV RNA. As Verslype et al. note, this discrepancy may be explained, as previously had been reported [21], by either a low viral copy number or the degradation of HCV RNA during tissue handling. Thus, because the percentage of infected hepatocytes found in occult HCV infection (and, therefore, the total amount of HCV RNA) is much lower (mean ± SD, 5.4 ± 4.3%) than that reported for patients with chronic hepatitis C (mean ± SD, 39.08% ± 28.61% in one report [12]; mean ± SD, 46.24% ± 20.22% in another report [23]), HCV RNA degradation must be strictly avoided.

Absence of serum HCV RNA in patients with occult HCV infection may reflect an absence of HCV replication. However, the HCV RNA of negative polarity was detected by in situ hybridization in the hepatocytes of 48 (84.2%) of the 57 patients with occult HCV infection, suggesting an ongoing viral replication although at a very low level. Whether the HCV infecting these patients harbors mutations affecting its translation capacity, its encapsidation capacity, or the formation and release of virions into circulation that lead to such an extremely low viremia that it is below the detection limit of our RT-PCR is currently under investigation.

On the other hand, we did not detect HCV core antigen in any of the liver-biopsy specimens from patients with occult HCV infection. It could be hypothesized that the lack of detection of HCV core antigen in the liver is due to an absence of HCV protein expression. However, this is probably not the case, because viral proteins are essential for HCV replication, and such replication was detected in 84.2% of our patients. Furthermore, we have found that occult HCV infection is associated with necroinflammatory activity in the liver; and, in chronic hepatitis C, liver damage is due to the host immune response to hepatocytes expressing HCV antigens [24]. So, these data suggest that, in occult HCV infection, HCV antigens may be expressed in the liver, but at levels so low that they are undetectable by standard immunohistochemical assays. In fact, in patients with chronic hepatitis C, immunohistochemical assays do not give an overall sensitivity >75% [25–27].

HCV RNA was also detected in the PBMCs of 40 (70%) of the 57 patients with intrahepatic HCV RNA. This finding has important diagnostic implications, because a high percentage of patients with occult HCV infection can be routinely identified by testing for HCV RNA in PBMCs rather than in liver-biopsy specimens. However, it has to be stated that a negative result in PBMCs does not exclude the existence of HCV RNA in liver cells.

With regard to the histological findings, necroinflammatory activity and fibrosis were significantly more frequent ($P = .017$ and $P = .022$, respectively) among patients with occult HCV infection than among patients without intrahepatic HCV RNA. In fact, 3 patients with occult HCV infection had liver cirrhosis, whereas this was not seen in patients negative for HCV RNA. Furthermore, in a logistic regression analysis, intrahepatic HCV RNA was independently associated with the presence of necroinflammatory activity and fibrosis ($P = .01$ and $P = .03$, respectively) These findings suggest that occult HCV infection may play a pathogenic role in some patients with ALF-EU. However, in general, occult HCV infection seems to induce a mild liver disease, because 65% of our 57 patients with occult HCV infection had either minimal nonspecific changes or steatosis. Liver steatosis was less frequent ($P = .047$) in patients with intrahepatic HCV RNA than in those without it. Although steatosis is a common liver feature in chronic hepatitis C [28], only HCV genotype 3 is directly associated with this fat accumulation [29, 30], and all 57 of our patients with occult HCV were infected with the 1b genotype.

In summary, the findings of the present study may influence

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thoughts on the epidemiological and clinical aspects of liver disease. In the present study, 70% of patients with occult HCV infection were found to have HCV RNA in their PBMCs, and the possible impact that this has on the diffusion of the disease in different circumstances (e.g., blood donations, liver or bone-marrow transplantation, and hemodialysis) should be assessed. Although neither is currently a standard diagnostic procedure in such patients, either RT-PCR or in situ hybridization should be performed to test for HCV RNA in liver-biopsy specimens from patients with ALF-EU. Finally, future studies should consider whether patients with occult HCV infection should receive antiviral therapy.

References

1. Berasain C, Betes M, Panizo A, et al. Pathological and virological findings in patients with persistent hypertransaminasaemia of unknown etiology. Gut **2000**; 47:429–35.
2. Kodali VP, Gordon SC, Silverman AL, McCray DG. Cryptogenic liver disease in the United States: further evidence for non-A, non-B, and non-C hepatitis. Am J Gastroenterol **1994**; 89:1836–9.
3. Bläckberg J, Kidd-Ljunggren K. Occult hepatitis B virus after acute self-limited infection persisting for 30 years without sequence variation. J Hepatol **2000**; 33:992–7.
4. Yuki N, Nagaoka T, Yamashiro M, et al. Long-term histologic and virologic outcomes of acute self-limited hepatitis B. Hepatology **2003**; 37:1172–9.
5. Michalak TI, Pardoe IU, Coffin CS, et al. Occult lifelong persistence of infectious hepadnavirus and residual liver inflammation in woodchucks convalescent from acute viral hepatitis. Hepatology **1999**; 29: 928–38.
6. Bedossa P. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. METAVIR Cooperative Study Group. Hepatology **1994**; 20:15–20.
7. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology **1996**; 24:289–93.
8. Colin C, Lanoir D, Touzet S, et al. Sensitivity and specificity of third-generation hepatitis C virus antibody detection assays: an analysis of the literature. J Viral Hepat **2001**; 8:87–95.
9. McOmish F, Yap PL, Dow BC, et al. Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. J Clin Microbiol **1994**; 32:884–92.
10. Takamizawa A, Mori C, Fuke I, et al. Structure and organization of the hepatitis C virus genome isolated from human carriers. J Virol **1991**; 65:1105–13.
11. Kwok S, Higuchi R. Avoiding false positives with PCR. Nature **1989**; 339:237–8.
12. Gosalvez J, Rodriguez-Iñigo E, Ramiro-Diaz, JL, et al. Relative quantification and mapping of hepatitis C virus by in situ hybridization and digital image analysis. Hepatology **1998**; 27:1428–34.
13. Rodriguez-Iñigo E, Casqueiro M, Navas S, Bartolome J, Pardo M, Carreño V. Fluorescent in situ hybridization of hepatitis C virus RNA in peripheral blood mononuclear cells from patients with chronic hepatitis C. J Med Virol **2000**; 60:269–74.
14. Quiroga JA, Martín J, Navas S, Carreño V. Induction of interleukin-12 production in chronic hepatitis C virus infection correlates with the hepatocellular damage. J Infect Dis **1998**; 178:247–51.
15. Rodríguez-Iñigo E, Bartolomé J, de Lucas S, et al. Histological damage in chronic hepatitis C is not related to the extent of infection in the liver. Am J Pathol **1999**; 154:1877–81.
16. Pawlotsky JM. Use and interpretation of virological tests for hepatitis C. Hepatology **2002**; 36 (Suppl 1):S65–73.
17. Geller SA, Nichols WS, Rojter SE, et al. Hepatitis C is not recoverable from liver tissue in cryptogenic cirrhosis: failure to identify hepatitis C virus–RNA using reverse transcription–mediated polymerase chain reaction. Hum Pathol **1996**; 27:1161–5.
18. Bresters D, Cuypers HT, Reesink HW, et al. Detection of hepatitis C viral RNA sequences in fresh and paraffin-embedded liver biopsy specimens of non-A, non-B hepatitis patients. J Hepatol **1992**; 15:391–5.
19. Akyol G, Dash S, Shieh YS, Malter JS, Gerber MA. Detection of hepatitis C virus RNA sequences by polymerase chain reaction in fixed liver tissue. Mod Pathol **1992**; 5:501–4.
20. Guerrero RB, Batts KP, Brandhagen DJ, Germer JJ, Perez RG, Persing DH. Effects of formalin fixation and prolonged block storage on detection of hepatitis C virus RNA in liver tissue. Diagn Mol Pathol **1997**; 6:277–81.
21. Madejon A, Manzano ML, Arocena C, Castillo I, Carreño V. Effects of delayed freezing of liver biopsies on the detection of hepatitis C virus RNA strands. J Hepatol **2000**; 32:1019–25.
22. Verslype C, Nevens F, Sinelli N, et al. Hepatic immunohistochemical staining with a monoclonal antibody against HCV-E2 to evaluate antiviral therapy and reinfection of liver grafts in hepatitis C viral infection. J Hepatol **2003**; 38:208–14.
23. Agnello V, Abel G, Knight GB, Muchmore E. Detection of widespread hepatocyte infection in chronic hepatitis C. Hepatology **1998**; 28:573–84.
24. Nelson DR. The immunopathogenesis of hepatitis C virus infection. Clin Liver Dis **2001**; 5:931–53.
25. Yap SH, Willems M, Van den Oord J, et al. Detection of hepatitis C virus antigen by immuno-histochemical staining: a histological marker of hepatitis C virus infection. J Hepatol **1994**; 20:275–81.
26. Sansonno D, Cornacchiulo V, Jacobelli AR, Gatti P, Di Stasi M, Dammaco F. Demonstration and distribution of HCV RNA sequences by in situ hybridization and HCV-related proteins by immunohistochemistry in the liver tissue of patients with chronic HCV infection. Pathobiology **1995**; 63:239–48.
27. Brady RI, Eng S, Melamed J, et al. Immunohistochemical detection of hepatitis C antigen by monoclonal antibody TORDJI-22 compared with PCR viral detection. Am J Clin Pathol **1998**; 110:32–7.
28. Goodman ZD, Ishak KG. Histopathology of hepatitis C virus infection. Semin Liver Dis **1995**; 15:70–81.
29. Rubbia-Brandt L, Quadri R, Abid K, et al. Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3. J Hepatol **2000**; 33: 106–15.
30. Negro F. Hepatitis C virus and liver steatosis: is it the virus? yes it is, but not always. Hepatology **2002**; 36:1050–2.

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