J Clin Pathol 1994;47:148–151

Chronic liver disease and active hepatitis C virus infection in patients with antibodies to this virus

E Petrelli, A Manzin, S Paolucci, A Cioppi, M Brugia, P Muretto, M Clementi

Abstract

***Aims*—To assess the association between active hepatitis C virus (HCV) infection and liver damage in randomly selected patients with antibodies to the virus.**

***Methods*—Thirty three consecutive subjects with serologically confirmed positivity for antibodies to HCV were studied for the presence of liver and circulating viral sequences by using the reverse transcription polymerase chain reaction (RT-PCR) and specific primers for the 5′-untranslated region (5′-UTR) of the HCV genome. Parallel clinical, biochemical, and histological investigations were carried out in all cases.**

***Results*—A comparative virological and histological investigation showed the presence of molecular signs of active viral replication and different degrees of liver damage in all cases. Baseline values of liver and plasma samples from all the patients showed (with one exception) the presence of detectable HCV RNA sequences, despite alanine amino transferase activities being within normal values or within 1·5 times the upper limit of normal in 13 of them. Examination of percutaneous liver biopsy specimens showed the presence of confirmed liver damage (ranging from chronic persistent hepatitis to cirrhosis) in all 33 patients.**

***Conclusions*—Circulating HCV RNA sequences (a direct sign of active HCV infection) are associated with liver damage, even in the absence of clinical or biochemical signs of overt liver disease. Parallel molecular, histological, and clinical follow up of these patients is needed to understand precisely the natural history of HCV infection and for correct clinical management.**

(*J Clin Pathol* 1994;47:148–151)

Division of Infectious Diseases, S Salvatore Hospital, Pesaro
E Petrelli
A Cioppi

Division of Pathology
P Muretto

Institute of Microbiology, University of Ancona Medical School Via P Ranieri, 1-60100 Ancona, Italy
A Manzin
S Paolucci
M Brugia
M Clementi

Correspondence to:
Dr A Manzin

Accepted for publication
7 October 1993

Hepatitis C virus (HCV) is a major cause of parenterally transmitted, acute, and chronic non A, non B hepatitis.[1] Furthermore, this virus has recently been associated with the pathogenesis of autoimmune chronic hepatitis[2] cirrhosis, primary hepatocellular carcinoma,[3] and with a variety of extrahepatic disorders, such as glomerulonephritis,[4] polyarteritis, and cryoglobulinemia.[5]

Infection with HCV is routinely diagnosed by testing serum specific antibodies to cloned or synthetic viral expressed proteins, but the appearance of HCV antibodies after the onset of hepatitis can be delayed from three to 52 weeks.[6] The presence of HCV antibodies in serum can indicate (especially in the absence of signs of overt liver disease) either that complete recovery after an acute infection has occurred or (more frequently) that HCV infection persists. In these circumstances individual virological and histological characterisation of HCV infected patients is needed for correct clinical management. Even though improved second generation and, more recently, third generation immunoenzymatic (EIA) and immunoblotting assays have provided more sensitive and specific tests for detecting HCV antibodies, only molecular testing for circulating HCV RNA sequences using the reverse transcription polymerase chain reaction (RT-PCR) confirms active HCV infection.[7] HCV RNA can be detected as early as two or three days after experimental inoculation of susceptible chimpanzees[8]; in man viral nucleic acid is generally detectable before the appearance of virus specific antibodies.[9]

Although it has recently been proposed that HCV is directly cytopathic,[10] as yet the pathogenesis of liver damage caused by HCV and the mechanism(s) leading to chronic infection are largely unknown. In fact, it has been observed that HCV associated chronic liver disease has two distinctive features[11]: (*i*) chronically infected patients may have intermittent flare-ups of overt disease, characterised by alternating periods of high alanine aminotransferase (ALT) activity and quiescent periods in which activities are normal; (*ii*) infected patients have raised ALT values throughout the course of chronic hepatitis. Recently, conflicting data concerning the presence of histologically confirmed liver damage (chronic persistent hepatitis (CPH), chronic active hepatitis (CAH), or liver cirrhosis) in infected patients have been reported,[12–14] indicating either that an invariable association between the presence of detectable HCV viraemia and liver injury (histological evidence of chronic liver disease even in the absence of raised ALT values) exists,[12] or that detection of persistently high levels of viraemia is possible even in the complete absence of overt liver damage.[13,14]

Methods

Thirty three consecutive patients enrolled in this study were recruited on the basis of serological data (positivity for HCV antibodies).

In all of them baseline plasma and liver HCV-RNA, ALT values, and presence of autoantibodies (anti-nuclear (anti-ANA); anti-actin smooth muscle (anti-SMA); anti-liver-kidney microsomal (anti-LKMA) were assessed. Percutaneous liver biopsy specimens were also evaluated at this point. Serum and plasma samples were collected at the time of the first clinical and histological examination and three months later. The diagnosis of chronic hepatitis was based on the presence of HCV antibodies and a history of clinically overt acute non A, non B hepatitis (consequent to blood transfusion), or previous (at least six months) episodes of a transient or persistent increase in ALT. Seven patients started IFNα treatment (three million units three times a week) two months after the first clinical and virological examination.

Control subjects included 13 HCV antibody negative patients with chronic liver disease (10 HBV chronic carriers and three with a diagnosis of non B, non C chronic hepatitis).

Antibodies to HCV were detected using two commercial enzyme immunoassays containing peptides corresponding to segments of both structural and non-structural regions of HCV (from United Biomedical Inc., Hauppage, New York, USA, and Sorin Biomedica SpA, Saluggia, Italy). Results were all confirmed by second generation immunoblotting assay (Chiron RIBA 2, Ortho Diagnostic Systems, Raritan, New Jersey USA). Serological markers for hepatitis B virus (HBV) (HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc IgG and IgM) and hepatitis D virus (HDV) (HDAg, anti-HDV IgG, and IgM) infection were assayed using commercial kits from Murex (Dartford, England). All patients were negative for HBV and HDV markers. Anti-ANA, anti-SMA, and anti-LKMA autoantibodies were tested by immunofluorescence on sections of rat organ and human Hep-2 cells at a screening dilution of 1 in 40.

Percutaneous liver biopsy specimens (at least 20 mm in length) were obtained from all patients. The evaluation of liver histology was based on the following criteria[15]:

(i) chronic persistent hepatitis (CPH): expansion of the portal zone by mononuclear cells and some fibrosis:

(ii) chronic lobular hepatitis (CLH): intralobular inflammation and necrosis, without piecemeal necrosis;

(iii) chronic active hepatitis (CAH): presence of portal inflammation with lymphocytic infiltrate and piecemeal necrosis;

(iv) cirrhosis: widespread fibrosis with nodule formation;

(v) steatosis: presence of macrovesicular or microvesicular fatty degeneration.

Total RNA was extracted from liver and plasma samples using the guanidinium thiocyanate/phenol/chloroform method.[16] In particular, 100 μl of fresh plasma or homogenised liver samples were added to 300 μl of denaturing solution (4·0M guanidinium thiocyanate, 25 mM sodium citrate (pH 7·0), 0·5% sarcosyl, 0·1M 2-mercaptoethanol), 30 μl of 3M sodium acetate (pH 4·7), 400 μl of water-saturated phenol, and 80 μl of chloroform-isoamyl alcohol (24·1), with thorough mixing of each new reagent. After cooling in ice for 20 minutes the samples were centrifuged at 14 000 rpm for 20 minutes, and the aqueous phase precipitated with absolute ethanol. The samples were then pelleted by centrifuging at 15 000 rpm for 45 minutes, washed with 70% ethanol, and vacuum dried.

For HCV RNA detection, water-resuspended purified RNA (10 μl) was reverse transcribed and amplified by using specific primers for the 5'-untranslated region (5'-UTR) of the HCV genome (HCV-01: 5'-ACCATGGAATCACTCCCCTGTGAGG-AACTA, positions –313 to—285; (HCV-02: 5'-ACTCGCAAGCACCCTATCAGG-CAGTACCA, positions –29 to –58). Reverse transcription was carried out at 42°C for 30 minutes in a mixture (final volume 20 μl) containing 10 μl 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 of dNTP, 10 pmol of antisense primer HCV-02, 20 U RNase inhibitor, and 100 U reverse transcriptase from Moloney murine leukaemia virus (MoMuLV) reverse transcriptase (BRL, Bethesda, Maryland, USA). After denaturing the cDNA at 94°C for 5 minutes the amplification reaction was carried out for 50 cycles in a mixture (final volume 100 μl) containing 1 × PCR buffer, 2·5 U *Taq* DNA polymerase and primers HCV-01 and HCV-02 (final concentration 50 pmol each), using an automated thermal cycler (Perkin Elmer Cetus, Norwalk, Connecticut, USA). The amplification profile was as follows: denaturation at 94°C for 60 seconds; annealing at 55°C for 45 seconds; extension at 72°C for 60 seconds. Human sera negative for HCV antibodies and dilutions of a sample from a posttransfusion patient with hepatitis, tested positive for HCV RNA, were included as negative and positive controls, respectively. Special care was taken to avoid any cross-contamination, including physical separation of pre- and post-amplification aliquots. Reagent controls (reagents and primers without target sequences) were tested in each RT-PCR assay. Sensitivity and specificity of the amplification results were confirmed by liquid oligomer hybridisation using a ^{32}P-labelled probe, internal to the amplified product (HCV-03: 5' CCGGGAGAGCCATAGTG-GTCTGCGGAACCGGTGAGTACACCG-GAATTG, position −213 to −166) according to the procedure described before.[17]

Results

All the 33 patients were positive for HCV antibodies when tested using two different ELISA commercial kits, and confirmation of the results was obtained by retesting the serum samples with RIBA 2. Positive results

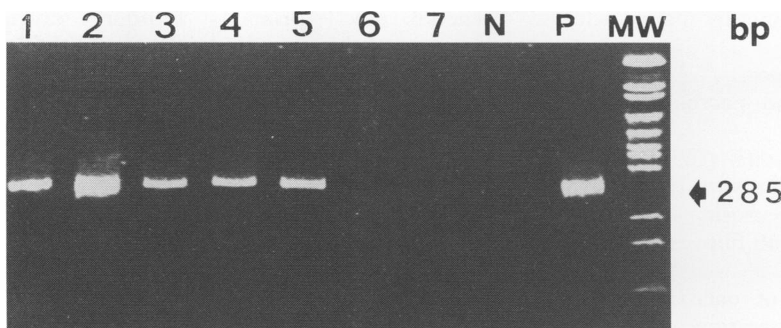
Detection of HCV RNA in plasma and liver, and liver histology HCV antibody positive patients

Case No	ALT* (U/l)	HCV-RNA			Histology‡
		Plasma 1†	Plasma 2†	Liver	
1**	16	+	+§	+	CPH
2	24	+	−	+	CLH
3	26	+	−§	+	CPH
4	30	+	+	+	CPH
5	32	+	−	+	CAH
6	35	+	−	+	CAH
7	40	+	+	+	CLH
8	41	−	+	+	CPH
9	41	+	ND	+	CPH
10	50	+	+	+	CAH
11	51	+	ND	+	CAH
12	51	+	−	+	CAH
13	53	+	+	+	CAH
14	72	+	+	+	CAH
15	75	+	−§	+	CLH
16	77	+	+	+	CAH
17	78	+	+ §	+	CPH
18	82	+	ND	+	CAH
19	83	+	+	+	CLH
20	87	+	ND	+	CPH
21	92	+	−	+	Steatosis
22	107	+	+	+	CAH
23	108	+	−§	+	CAH
24	112	+	+	+	CAH
25	113	+	−	+	CAH
26	113	+	−	+	Cirrhosis
27	120	+	+	+	CAH
28	123	+	ND	+	CAH
29	143	+	−§	+	CPH
30	159	+	−	+	CAH
31	317	+	+	+	CLH
32	338	+	−§	+	CAH
33	906	+	ND	+	CAH

*ALT values, data of HCV RNA in liver, and liver histology are related to results obtained at baseline.
†Plasma 1 = plasma sample at baseline, no treatment; plasma 2 = 3 months later.
‡CPH = Chronic persistent hepatitis; CLH = Chronic lobular hepatitis; CAH = Chronic active hepatitis.
§ IFNα treatment.
**Plasma and liver samples from 13 controls with HCV antibody negative CPH (10 hepatitis B chronic carriers, three non-B, non-C chronic hepatitis) were assayed for the presence of specific HCV RNA sequences. All these samples tested negative for HCV RNA.

were defined for both EIA assays and the RIBA 2, according to the manufacturer's instructions. Anti-ANA autoantibodies and anti-SMA were absent in all subjects. The presence of anti-LKMA autoantibodies was checked for in 12 patients: only one of them tested positive.

The baseline histological examination identified 18 (54·54%) patients with CAH, eight (24·24%) with CPH; five (15·15%) with CLH, one (3·03%) with liver cirrhosis, and one (3·03%) with steatosis (table). ALT values clustered into four classes: nine patients (five CPH, two CAH, two CLH) with values below the upper limit of normal (45 IU/l); four (cases 10–13; all CAH) with less than 1·5 times the upper limit; eight (cases 14–21; three CAH, three CPH, one CLH, and one steatosis) between 1·5 and 2·5 times the upper limit of normal values; and 12 (cases 22–33; nine CAH, one CLH, one CPH, one cirrhosis) at least 2·5 times the upper limit of normal.



Detection of HCV RNA sequences from liver and plasma samples by RT-PCR using specific primers of the HCV 5'-UTR.
Lanes 1–4: amplified specific HCV RNA sequences in liver (1–2) and plasma samples (3–4) from cases 1 and 3; lanes 5–6: liver and plasma from 8; lane 7: reagent control (without target sequences); N: negative control from an HCV antibody negative serum sample; P: positive control from an HCV-RNA positive plasma sample; MW: pGEM molecular weight marker (Promega).

Intrahepatic HCV sequences were detected in all 33 patients; HCV viraemia was detected in all but one (case 8), regardless of the ALT value and the degree of hepatic injury (table and figure). All the control subjects (HCV antibody negative with chronic hepatitis) had a histological diagnosis of CPH and tested negative for HCV RNA in plasma and liver samples (table). A second set of plasma samples were collected three months after the baseline from 27 of the 33 patients. Specific HCV RNA sequences were detected from 14 of the 27 samples; seven patients had been given IFNα treatment. At the time, two of the seven treated patients were non-responders and tested positive for plasma HCV RNA; another five had no detectable viraemia and showed normalisation of ALT values.

Discussion

Persistent HCV infection is thought to be associated with a high risk of developing chronic hepatitis and liver cirrhosis. Furthermore, hepatocellular carcinoma may be the consequence of long term, untreated HCV infection.[18] Even though it has recently been proposed that HCV is directly cytopathic,[10] our knowledge of both the mechanisms of virus persistence in infected hosts and the pathogenetic steps leading to the chronic liver disease is still incomplete. As a direct consequence, a major problem is the clinical management of patients who are persistently positive for HCV antibodies but who have no symptoms or signs of liver disease. Molecular testing for genomic HCV sequences in liver or blood by RT-PCR allows viral activity to be detected[19] and it has partially settled this issue.

In this study 33 consecutive subjects recruited on the basis of their serological positivity for HCV antibodies underwent parallel virological and histological investigations; all patients tested positive for HCV-RNA sequences in plasma and liver samples using RT-PCR. In particular, case 1 tested negative for circulating viral sequences (but positive in liver) initially and positive three months later. Interestingly, liver histology showed features of chronic liver disease in all patients studied: principally, chronic lobular hepatitis (15·15% of cases), chronic persistent hepatitis (24·24%); and chronic active hepatitis (54·54%) were detected. Two other patients had steatosis and cirrhosis, respectively. Otherwise, ALT values were not predictors of histological findings. In fact, HCV-RNA in liver and plasma samples were significantly associated with liver damage, but only 20 of the 33 patients had ALT values at least 1·5

times the upper limit of normal values; nine patients had normal ALT values. These findings agree with those of a recent report,[12] showing the invariable association between active persistent HCV infection and liver damage, and are in clear contrast with others, which detected HCV RNA in the serum[13] and liver[14] of patients with normal liver histology. In particular, Navas *et al*[14] affirmed that the absence of liver damage in patients with HCV replication in the liver might be due to HCV variant strains, or to the lack of recognition of HCV by the immune system. Experimental biological and molecular research is clearly necessary to test the hypothesis that emergence of viral genomic variants may account for differences in the viral pathogenic potential.[20]

Detection of intermittent viraemia in 13 of the 33 patients studied after three months from baseline deserves some considerations. A follow up plasma HCV RNA in five of seven patients treated with IFN who tested negative showed, from a clinical point of view, that they were responders; two HCV patients who were non-responders still tested positive. On the other hand, we detected a low and transitory replication rate in eight infected patients with intrahepatic viral sequences and, in some of these cases, raised ALT values. Even though technical problems affecting the sensitivity of HCV RNA detection[21] could not be completely ruled out, these observations suggest that persistent HCV infection may account for two, non-mutually exclusive pathogenic mechanisms: (*i*) direct liver damage (associated with persistent viral replication); and (*ii*) an HCV-induced autoimmune damage occurring in the absence of overt viral replication.[22] Furthermore, it has recently been proposed that liver recolonisation could be achieved by virus spreading from extrahepatic reservoirs.[23]

Overall, results shown here may have several pathogenic, clinical, and therapeutic implications. First, they confirm that most patients with active HCV infection have differing degrees of liver damage, even though ALT values are not raised. Second, patients with persistent viraemia should be considered for liver biopsy, in order to categorise the degree of damage and assess eligibility for antiviral treatment. Third, patients with liver injury and possible intermittent viraemia should be followed up prospectively for a more correct line of clinical management; in particular, HCV antibody positive patients who are persistently negative for HCV RNA should not have a biopsy, as infection might have resolved.

This work was partially supported by grants from the *Consiglio Nazionale delle Ricerche* (CNR), target project "Biotechnology and Bioinstrumentation", and from the *Ministero della Sanità—Istituto Superiore di Sanità*, 6th AIDS Project. SP is the recipient of a fellowship from the *Ministero della Sanità—Istituto Superiore di Sanità*.

1 Choo QL, Kuo G, Weiner AJ, *et al*. Isolation of a cDNA derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989;**244**:359–62.
2 Michel G, Ritter A, Gerken G, *et al*. Anti-Gor and hepatitis C autoimmune liver disease. *Lancet* 1992;**332**: 267–9.
3 Saito I, Miyamura T, Ohbayashi A, *et al*. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990;**87**: 2059–61.
4 Misiani R, Bellavita P, Fenili D, *et al*. Hepatitis C virus infection in patients with essential mixed cryoglobulinemia. *Ann Intern Med* 1992;**117**:573–7.
5 Pechere-Bertschi A, Perrin L, De Saussure P, *et al*. Hepatitis C: a possible etiology for cryoglobulinemia type II. *Clin Exp Immunol* 1992;**89**:419–22.
6 Farci P, Alter HJ, Wong D, *et al*. A long-term study of hepatitis C virus replication in non-A, non-B hepatitis. *N Engl J Med* 1991;**325**:98–104.
7 Kaneko S, Unoura M, Kobayashi K, *et al*. Detection of serum hepatitis C virus RNA. *Lancet* 1990;**335**:976.
8 Shimizu YK, Weiner AJ, Rosenblatt J, *et al*. Early events in hepatitis C virus infection of chimpanzees. *Proc Natl Acad Sci USA* 1990;**87**:6441–4.
9 Garson JA, Ring C, Tuke P, Tedder RS. Enhanced detection by PCR of hepatitis C virus RNA. *Lancet* 1990; **336**:1022–5.
10 Kagawa T, Saito H, Tada S, *et al*. Is hepatitis C virus cytopathic? *Lancet* 1993;**341**:316–7.
11 Weiner AJ, Geysen HM, Christopherson C, *et al*. Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: Potential role in chronic HCV infections. *Proc Natl Acad Sci USA* 1992;**89**:3468–72.
12 Alberti A, Morsica G, Chemello L, *et al*. Hepatitis C viraemia and liver disease in symptom-free individuals with anti-HCV. *Lancet* 1992;**340**:697–8.
13 Brillanti S, Foli M, Gaiani S, *et al*. Persistent hepatitis C viraemia without liver disease. *Lancet* 1993;**341**:464–5.
14 Navas S, Castillo I, Carreno V. Detection of plus and minus HCV RNA in normal liver of anti-HCV-positive patients. *Lancet* 1993;**341**:904–5.
15 Sherlock S. Chronic hepatitis. In: Sherlock S, ed. *Disease of the liver and biliary system*. 8th edn. Oxford: Blackwell Scientific Publications, 1990:339–71.
16 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-choloroform extraction. *Anal Biochem* 1987;**16**:156–9.
17 Kumar R, Goedert JJ, Huges SH. A method for the rapid screening of human blood samples for the presence of HIV-1 sequences: the probe shift assay. *AIDS Res Hum Retroviruses* 1989;**5**:345–54.
18 Colombo M, Kuo G, Choo QL, *et al*. Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. *Lancet* 1989;**ii**:1004–6.
19 Gretch D, Lee W, Corey L. Use of aminotransferase, hepatitis C antibody, and hepatitis C polymerase chain reaction RNA assay to establish the diagnosis of hepatitis C virus infection in a diagnostic virology laboratory. *J Clin Microbiol* 1992;**30**:2146–9.
20 Prince AM, Brotman B, Huima T, *et al*. Immunity in hepatitis C infection. *J Infect Dis* 1992;**165**:438–43.
21 Zaaijer HL, Cuypers HTM, Reesink HW, *et al*. Reliability of polymerase chain reaction for detection of hepatitis C virus. *Lancet* 1993;**341**:722–4.
22 Garson JA, Lenzi M, Ring C, *et al*. Hepatitis C viraemia in adults with type 2 autoimmune hepatitis. *J Med Virol* 1991;**34**:223–6.
23 Zignego AL, Macchia D, Monti M, *et al*. Infection of peripheral mononuclear blood cells by hepatitis C virus. *J Hepatol* 1992;**15**:382–6.