Hepatic and Extrahepatic HCV RNA Strands in Chronic Hepatitis C: Different Patterns of Response to Interferon Treatment

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We investigated the presence of positive (genomic) and negative (replicative intermediate) hepatitis C virus RNA strands in liver, peripheral mononuclear cells and serum from patients with chronic hepatitis C using a selective and semiquantitative polymerase chain reaction procedure. Negative and positive hepatitis C virus RNA strands were present in liver, serum and lymphoid cells in all untreated patients and in all those who did not respond to interferon therapy. In the latter group of patients, the titers of RNA strands in the liver and peripheral mononuclear cells at the end of the treatment were similar to those encountered in untreated patients, but the serum titers were about 100 times lower than pretreatment values. In patients who responded to interferon with normalization of serum aminotransferase levels (n = 10), the rate of detection and the titer of the two viral strands in liver, serum and mononuclear cells were markedly decreased at the end of the therapy. In the six responders who did not relapse after interferon withdrawal, both hepatitis C virus RNA strands were absent from the liver, serum and lymphoid cells. By contrast, the positive RNA strand was present in liver cells, mononuclear cells or both at the end of therapy in all patients who experienced posttherapy relapse. In conclusion, our results indicate that interferon can clear hepatitis C virus from hepatic and extrahepatic sites only in responder patients. Disappearance of genomic hepatitis C virus RNA from the liver and from mononuclear cells may predict complete response without posttherapy relapse. (HEPATOLOGY 1993;18:1050-1054.)

Hepatitis C virus (HCV) is a positive-stranded RNA virus (1). On the basis of its putative amino acid sequence similarity this virus is now considered to be part of the *Flaviviridae* family (2). The replication of these viruses has been thought to occur through the production of a complementary negative RNA strand with the positive RNA strand as a template (3). The positive and negative HCV RNA strands have been reported to be present in the livers of patients with chronic hepatitis C, indicating active viral replication in this organ (4-6). Recently both strands were also found in peripheral-blood mononuclear cells (PBMCs) (7-10). Although the presence of the replicative intermediate of HCV in lymphoid cells might merely represent the uptake through phagocytosis of the negative strand from the serum (4), some recent evidence suggests that HCV also replicates in PBMCs (10).

Interferon has been used as an effective treatment in chronic hepatitis C, although only approximately 50% of treated patients respond to this therapy and half of those whose serum aminotransferase levels become normal with interferon treatment relapse after cessation of therapy (11, 12). The mechanisms responsible for relapse of hepatitis after cessation of interferon have not been clarified. In particular, it has been shown that the disappearance of hepatitis C virus RNA from serum is not predictive of long-term response to interferon (13-17).

We evaluated the effect of interferon on the replicative activity of HCV using a selective reverse-transcription–polymerase chain reaction (RT-PCR) procedure to estimate the positive and negative hepatitis C virus RNA strands in liver, PBMCs and serum of patients with chronic hepatitis C treated with interferon. Our findings indicate that interferon appears to eliminate HCV from the liver and from extrahepatic sites only in those patients who respond to this therapy with normalization of serum aminotransferase levels. Our results show that sustained biochemical response after interferon withdrawal is associated with complete clearance of both hepatitis C virus RNA strands from the liver, serum and PBMCs.

MATERIALS AND METHODS

Twenty patients with histologically proven CAH (6 males and 14 females; mean age, 46.5 yr; range, 17 to 72 yr) were included in this study. Antibodies to HCV (anti-HCV) and

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TABLE 1. Serum titers of positive and negative strands of HCV RNA and ALT in untreated patients with chronic hepatitis C and in interferon-treated patients before and after 12 mo of therapy

Patient no.	Treatment	HCV RNA strands (PCR units/ml)				ALT (U/L)[a]
		Positive (before treatment)	Negative (before treatment)	Positive (after treatment)	Negative (after treatment)	
1	None	2×10^4	2×10^3			210
2	None	2×10^5	2×10^3			165
3	None	2×10^5	2×10^3			266
4	None	2×10^2	2×10			26
5	None	2×10^4	2×10^2			128
6	None	2×10^4	2×10			98
7	NR	2×10^6	2×10^4	2×10^3	2×10	114
8	NR	2×10^5	2×10^4	2×10^4	2×10^2	46
9	NR	2×10^5	2×10^4	2×10^2	2×10	152
10	NR	2×10^4	2×10^2	2×10^3	2×10	177
11	RR	2×10^4	2×10^4	–	–	74
12	RR	2×10^5	2×10^4	2×10	–	172
13	RR	2×10^4	2×10^4	–	–	139
14	RR	2×10^5	2×10^4	2×10^3	2×10	48
15	R	2×10^6	2×10^4	–	–	143
16	R	2×10^3	2×10	–	–	67
17	R	2×10^4	2×10^4	–	–	92
18	R	2×10^3	2×10^3	–	–	145
19	R	2×10^4	2×10^4	–	–	154
20	R	2×10^3	2×10^2	–	–	54

NR, nonresponders to interferon therapy; *RR,* responders to interferon who relapsed after discontinuation of treatment; *R,* responders to interferon without posttherapy relapse.

Dashes indicate negative results.

[a]Normal value, 0 to 30 U/L.

serum HCV RNA were detected in all patients. Six patients had not received treatment and 14 had completed a 12-mo course of therapy with interferon (lymphoblastoid alpha interferon, Wellferon; Wellcome Research Laboratories, Beckenham, UK). Treated patients received 3 MU subcutaneously/day for 2 mo, 3 MU three times/week for 3 mo and 1.5 MU three times/week for 7 mo. Patients with normal serum aminotransferase activities at the end of the therapy (mo 12) were considered responders (n = 10), whereas those with increased values were considered nonresponders (n = 4). Among the 10 responders, 4 relapsed after therapy, with increased serum aminotransferase levels within 3 mo of completion of therapy, whereas serum aminotransferase levels were persistently normal after interferon withdrawal in 6 (mean follow-up, 10.3 mo; range, 6.5 to 15 mo). In all patients treated with interferon both positive and negative HCV RNA strands were analyzed in the liver, serum and PBMCs at the end of the therapy (mo 12) and in serum before the initiation of treatment (month 0). All patients gave written informed consent, and the study was approved by the local ethics committee.

Liver specimens, PBMCs and serum samples were obtained on the same day (PBMCs from cases 12 and 13 were not available). Liver samples were immediately frozen in liquid nitrogen and stored at −80° C. PBMCs were isolated with Lymphoprep (Nycomed Pharma AS, Oslo, Norway), washed five times, dissolved in 4M guanidinium solution and stored at −80° C. Serum was stored at −40° C. Total cellular or serum RNA was isolated by means of a single-step method with extraction in guanidinium thiocyanate and phenol/chloroform (18).

RT-PCR for both the positive and negative strands of HCV RNA was performed as described previously (7). Briefly, 250 ng of cellular RNA or RNA from 50 μl of serum was reverse transcribed with outer antisense primer (for positive strands) or the outer sense primer (for negative strands). The cDNA produced was used for the first PCR amplification (35 cycles), and 2 μl of the first PCR product was used for the second PCR amplification (30 cycles). After amplification, 15-μl aliquots of the final PCR reaction were subjected to electrophoresis in a 2% agarose gel, and the bands were visualized on ethidium bromide staining. The primers for HCV cDNA synthesis and PCR amplification were: outer sense, GTATCTCGAGGCGACACTCCACCATAGAT, and outer antisense, ATACTCGAGGTGCACGGTCTACGAGACCT; and inner sense, CCACCATAGATCTCTCCCCTGT, and inner antisense, CACTCTCGAGCACCCTATCAGGCAGT (Garson JA et al., Lancet 1990;336: 878-879, Correspondence). Titers of HCV RNA were estimated in a semiquantitative fashion by means of serial 10-fold dilution of 250 ng of cellular RNA from liver and PBMCs or RNA from 50 μl of serum. The highest dilution giving a positive second-round PCR signal was taken as the viral titer. The viral titer was expressed as PCR units per microgram of RNA (liver tissue, PBMCs) or PCR units per milliliter of serum by multiplying the highest positive dilution value (considered to contain 1 PCR unit) by 4 or 20, respectively.

The procedures recommended by Kwok and Higuchi (19) to reduce the risk of contamination were strictly applied. An aliquot from the last washing of PBMCs was also included, and PCR was always negative in these samples.

RESULTS

As shown in Table 1, both the positive and negative HCV strands were detected in serum in all untreated

TABLE 2. Titers of positive and negative strands of HCV RNA in the liver and in PBMCs and serum ALT levels in untreated patients with chronic hepatitis C and in interferon-treated patients after 12 mo of therapy

Patient no.	Treatment	HCV RNA strands (PCR units/μg RNA)				ALT (U/L)[a]
		Positive (liver)	Negative (liver)	Positive (PBMCs)	Negative (PBMCs)	
1	None	4×10^3	4×10^3	4×10	4	210
2	None	4×10^3	4×10^3	4×10	4	165
3	None	4×10^3	4×10^3	4×10	4	266
4	None	4	4	4	–	26
5	None	4×10^3	4×10^2	4	–	128
6	None	4×10^3	4×10^3	4×10	4×10	98
7	NR	4×10^3	4×10^3	4×10	4	62
8	NR	4×10^3	4×10^3	4×10	4	70
9	NR	4×10^2	4×10^2	4	4	120
10	NR	4×10^3	4×10^2	4×10^2	4×10	206
11	RR	–	–	4	–	18
12	RR	4×10^2	4	ND	ND	24
13	RR	4	–	ND	ND	19
14	RR	4×10	4	4	–	25
15	R	–	–	–	–	14
16	R	–	–	–	–	8
17	R	–	–	–	–	15
18	R	–	–	–	–	23
19	R	–	–	–	–	16
20	R	–	–	–	–	15

NR = nonresponders to interferon therapy; *RR* = responders to interferon who relapsed after discontinuation of treatment; *ND* = not determined; *R* = responders to interferon without posttherapy relapse.

[a]Normal value, 0 to 30 U/L.

cases and in the pretreatment sample of all the 14 patients who subsequently underwent interferon therapy. Both HCV RNA strands were also found in serum at the end of treatment in the four patients who were not responsive to interferon. At the completion of the therapy the positive HCV RNA strand was detected in the serum of two of the four patients who responded to interferon but relapsed after interruption of the therapy, and the negative HCV RNA strand was found in one of these cases. In patients with persistently normal aminotransferase levels after cessation of the therapy neither of the two HCV RNA strands could be detected in serum at the end of treatment (Table 1). Semiquantitative analysis showed that the titers of positive and negative strands were much lower in the posttherapy serum samples from patients who did not respond to interferon (6,050 ± 4,669 PCR units/ml and 65 ± 45 PCR units/ml, respectively) than in pretreatment samples of these subjects (605,000 ± 466,931 PCR units/ml and 15,050 ± 4,950 PCR units/ml, respectively). No differences in serum pretherapy viral titers or ALT values were found among groups (Table 1).

Both HCV RNA strands were detected in the liver in all untreated patients with chronic hepatitis C (n = 6) and also in those with no response to interferon (n = 4). At the end of therapy, the positive HCV RNA strand was present in the livers of three of the four patients who responded to interferon but then relapsed; the negative strand was found in two of these cases. Interestingly, both RNA strands were absent from the liver in all six patients with sustained responses (Table 2). Semiquantitative analysis showed that titers of positive and negative RNA strands of HCV in the liver were similar in untreated patients and in patients who failed to respond to interferon (3,334 ± 660 PCR units/μg RNA and 2,734 ± 802 PCR units/μg RNA vs. 3,100 ± 900 PCR units/μg RNA and 2,200 ± 1,039 PCR units/μg RNA, respectively), but titers of both strands in the liver were markedly decreased in the responder patients in whom these HCV sequences were found (44 ± 39 PCR units/μg RNA and 0.8 ± 0.5 PCR units/μg RNA for positive and negative strands, respectively) (Table 2). In the six untreated patients serum ALT values were above 90 U/liter, except in one patient in whom ALT was normal (26 U/liter). Interestingly, in this case the liver titers of the positive and negative HCV RNA strands were both 4 PCR units/μg RNA, whereas in the rest of untreated cases the titer of the positive HCV RNA strand was 4,000 PCR units/μg RNA and that of the negative strand was at least 400 PCR units/μg RNA.

As shown in Table 2, the positive strand of HCV RNA was found in PBMCs in all six untreated patients and, at the end of the treatment, in all patients who did not respond to therapy and in the two patients who appeared to respond but then relapsed. In one of the patients who relapsed after treatment (case 11; Table 2) HCV RNA was not detectable in the liver, but the positive strand of HCV RNA was present in PBMCs. The negative strand was found in PBMCs of five of six untreated patients, in all four cases who did not respond to interferon and in

none of the patients who responded to the therapy. In all six cases who displayed sustained responses to the therapy HCV RNA strands were undetectable in PBMCs at the end of treatment (Table 2). Semiquantitative analysis showed that titers of positive and negative strands in lymphoid cells (Table 2) were similar in untreated patients and in patients who did not respond to interferon (28 ± 7 PCR units/μg RNA and 9.3 ± 6 PCR units/μg RNA vs. 121 ± 93 PCR units/μg RNA and 13 ± 9 PCR units/μg RNA, respectively). The titers of the positive and negative HCV RNA strands were found to be, respectively, 100 and 500 times higher in the liver than in PBMCs (Table 2).

DISCUSSION

Our results show that the two HCV RNA strands can be found in the liver, PBMCs and serum of patients with chronic hepatitis C, confirming previous reports (4-10). We semiquantitatively assessed levels of HCV RNA in hepatic and extrahepatic sites simultaneously and found that the liver has a higher viral load (per microgram of RNA) and is seemingly the preferred site for HCV replication. Thus, in untreated patients with chronic hepatitis C, the positive and negative HCV RNA strands are 100 and 500 times, respectively, more abundant (per microgram of RNA) in the liver than in PBMCs. It is also worth noting that the negative strand—in the liver, mononuclear cells or serum—is detected only when the positive strand is also present, and it is found at lower levels than the positive strand. The positive strand, by contrast, may be present without accompanying negative strand in the liver or lymphoid cells, probably reflecting a state of nonreplicative infection.

The presence of the negative strand of HCV RNA in serum deserves some comment. In the serum the negative strand might be associated with the virion particle; alternatively, it might be membrane bound, in this case probably being released during hepatocyte lysis (4). Our results show that the detection of the negative HCV RNA strand in serum is always associated with the existence of negative strand in the liver, peripheral mononuclear cells or both and that, on the other hand, the positive strand may be detected in hepatic or extrahepatic sites in the absence of serum HCV RNA viremia (cases 11 and 13 posttreatment; Tables 1 and 2).

Although we were not able to sample the liver and PBMCs before therapy, our findings support the concept that the effect of interferon in reducing hepatocellular damage (as estimated by ALT levels) is related to the ability of this agent to diminish the viral load in the liver. Interferon appears to display its antiviral activity in hepatic and extrahepatic sites (Tables 1 and 2). Thus those patients with normal serum aminotransferase levels after interferon treatment have very low titers of or no viral RNA in liver, serum or PBMCs. In contrast, those patients with persistently increased ALT levels despite interferon therapy have levels of both HCV RNA strands in the liver and lymphoid cells, at the end of the treatment period, comparable to those found in untreated patients. In patients who did not respond to interferon, however, serum titers of HCV RNA after therapy were lower than those of pretreatment samples. These findings are in agreement with results from Magrin et al. (17) suggesting that in nonresponders interferon—although ineffective in reducing viral replication—may hamper viral assembly (20), thus reducing the number of circulating virions.

Both viral RNA strands were found to be absent from liver, PBMCs and serum of all patients with persistently normal serum aminotransferase values after interferon therapy. This indicates that sustained response without posttherapy relapse is associated with complete hepatic and extrahepatic clearance of the virus or at least with reduction of the viral load to levels below those detectable with the sensitive nested PCR technique. Interestingly, in patients whose serum aminotransferases levels became normal after interferon treatment but relapsed after withdrawal of therapy, the positive strand of HCV RNA was found at the end of the treatment, either in the liver or in PBMCs, in all cases. In these patients the liver titers of viral RNA were very low (Table 1); this probably accounts for the normal serum aminotransferase values. However, the persistence of the virus in hepatic or extrahepatic sites at the end of the therapy accounts for the occurrence of reactivation after cessation of the treatment. It must be noted that two of the patients with posttherapy relapse were serum HCV RNA negative, indicating that the absence of viremia cannot be used to predict a sustained therapeutic response. Determination of HCV RNA in the liver and in lymphoid cells at the end of the therapy appears to be the most sensitive method to anticipate whether the disease will reactivate after interferon discontinuation.

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