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Detection of Hepatitis C Virus RNA in Patients with Chronic Hepatitis C Virus Infections during and after Therapy with Alpha Interferon

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In 24 patients with hepatitic C virus (HCV) infection who participated in a randomized trial with alpha 2B interferon, HCV RNA analysis by the polymerase chain reaction with two separate primer sets was performed at weeks 0, 4, and 24 and during a follow-up period of 6 to 9 months. Prior to therapy all patients were HCV RNA positive. During therapy, HCV RNA decreased to an undetectable level (<1 chimpanzee infectious dose per ml) in nine patients at week 4. After week 4, no additional cases of HCV RNA disappearance (<1 chimpanzee infectious dose per ml) were observed. During follow-up, HCV RNA could not be detected in four of the six patients with a sustained alanine aminotransferase response. These results suggest the probable predictive value of HCV RNA levels for detecting the failure of therapy in an early stage of HCV infection.

In recent years, several randomized controlled studies were performed with alpha interferon (IFN) for the treatment of non-A, non-B hepatitis (NANBH) (9, 10, 13, 16, 19, 20). The effect of IFN was evaluated by measuring the alanine aminotransferase (ALT) level in serum, which reflects the activity of liver disease.

Recently, the etiological agent for NANBH has been identified (6, 12). The causative agent is now known as hepatitis C virus (HCV). The HCV genome is a positive-stranded RNA molecule of about 9,400 nucleotides. Sequence homology among the known HCV strains is about 80% (3, 7, 14, 17, 21).

In the study described here, 24 patients were investigated for the presence of HCV RNA during and after IFN therapy, to determine directly the effect of IFN treatment on viremia. HCV RNA analysis by the polymerase chain reaction was performed by using primer sets from the highly conserved noncoding (NC) region and a conserved sequence from nonstructural region 5 (NS5).

Patients between 18 and 70 years of age with elevated ALT levels (≥2 times the upper limit of normal), a biopsy-proven chronic NANBH, antibodies to HCV determined by a second-generation enzyme immunosorbent assay (Abbott, North Chicago, Ill.) and a confirmatory assay RIBA IV (Ortho, Raritan, N.J.), and no recent history of hepatitis B virus, hepatitis A virus, cytomegalovirus, or Epstein-Barr virus infection were included in the study. All patients gave informed consent prior to participation in the study. Patients were treated in a randomized controlled trial with either a standard scheme (12 patients) consisting of 3 MU of recombinant IFN-α2B (Intron A; Schering Plough, Kenilworth, N.J.) three times a week for 24 weeks or an experimental scheme (12 patients). In the experimental scheme, therapy was started with 6 MU of recombinant IFN-α2B three times a week for at least 8 weeks. Therapy was stopped at week 12 if ALT levels remained elevated. If the ALT level normalized, therapy was continued with 3 MU of recombinant IFN-α2B three times a week for 8 weeks; this was followed by treatment with 1 MU of recombinant IFN-α2B three times a week until a normal ALT level was accompanied by an undetectable HCV RNA level for a period of 1 month. Blood samples were taken prior to treatment and at least every fourth week during treatment and follow-up. For HCV RNA detection, EDTA-blood was collected and plasma was prepared within 2 h after sampling; aliquots were quickly frozen in liquid nitrogen and were stored at −70°C.

HCV RNA was extracted from 100 μl of plasma by a modification of the guanidinium method described by Chomczynski and Sacchi (5). cDNA synthesis was performed with 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-Bethesda Research Laboratories, Gaithersburg, Md.) and 20 pmol of antisense primer in a 25-μl reaction volume. Antisense primers were chosen as follows (location was that of Okamoto et al. [17]): for the NC region, residues 323 to 304 (GTGCACGGTCTACGAGACCT); for the NS5 region, residues 7020 to 7001 (AGGAGGTTG GCCTCTATGAG). For amplification of HCV cDNA (40 cycles, 94°C for 1 min, 48°C for 2 min, 74°C for 3 min), 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.), reaction buffer, and 20 pmol of sense primer (for NC, GGCG ACACTCCACCATAGAT; residues 1 to 20; for NS5, CCCTCCCATATAACAGCAGA; residues 6857 to 6867) were added to a final volume of 100 μl. Sense primers were chosen as follows: for the NC region, residues 1 to 20 (GGCGACACTCCACCATAGAT); for the NS5 region, residues 6857 to 6877 (CCCTCCCATATAACAGCAGA). Twenty microliters of the amplification product was analyzed by Southern blot hybridization. The probe for the NC region (GAGTAGTGTTGGGTCGCGAA; residues 239 to 258) detects a product of 324 bp. For detection of the NS5 product (164 bp), two probes were used: probe A (GGGTCT CCCCCCTCCTTGGCCAGCTCTTCAGCTA; residues 6902 to 6931), which is identical to HCV-J (14) and HCV-BK (21), and probe B (CATGACTCCCCTGATGCTGA; residues 6980 to 6999), which is identical to HCV-1 (7). In all cases, HCV RNA was sought with both primer sets. Known HCV cDNA sequences (7, 14, 15, 18, 21) were used for selection of the primers and probes. The detection limit of our assay was 1 chimpanzee infectious dose per ml, as estimated with

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TABLE 1. Biochemical response and HCV RNA results in 24 patients during and after IFN therapy

ALT response	No. of patients	No. (%) of patients negative for HCV RNA at:			
		0 wk	4 wk	24 wk	Follow-up[a]
Sustained[b]	6	0	5 (83)	5 (83)	4 (67)
Transient[c]	2	0	2 (100)	1 (50)	0
None	16	0	2 (13)	1 (6)	0
Total	24	0	9 (37)	7 (29)	4 (16)

[a] Follow-up was at 6 to 9 months after the end of therapy.
[b] Decrease in ALT levels during treatment and normal ALT levels during follow-up.
[c] Normalization in ALT levels during treatment and elevation in ALT levels during follow-up.

challenge plasma from the Eurohep HCV RNA panel (provided by P. N. Lelie, CLB, Amsterdam, The Netherlands). Both primer sets showed the same sensitivity. Distilled water and anti-HCV-negative and -positive plasma as negative and positive controls, respectively, were used in each experiment. All results were confirmed by repeat testing.

Prior to IFN treatment, all patients were HCV RNA positive as determined with the NC primer set. In one patient, HCV RNA was not detected by the NS5 primer set.

Four patients, which were treated by the experimental scheme, stopped therapy before week 24. Two of them stopped therapy after 12 weeks and one stopped therapy after 20 weeks because their ALT levels remained elevated. One patient stopped therapy at week 20 because of noncompliance.

After 4 weeks of treatment, 9 of the 24 patients (5 in the standard scheme, 4 in the experimental scheme) showed a decrease in HCV RNA and HCV RNA became undetectable by both primer sets. At the end of therapy (week 24), HCV RNA reappeared in two patients (standard scheme) and after 6 to 9 months of follow-up in another three patients (one of the standard scheme, two on the experimental scheme). A normal ALT level (<30 U/liter) was observed at week 4, week 24, and the follow-up period in 8 (33%), 8 (33%), and 6 (25%) patients, respectively. A simultaneous absence of HCV RNA and a normal ALT level in the follow-up study was limited to four patients (two on the standard scheme, two on the experimental scheme) (Table 1). The rapid decline in the amount of HCV RNA in responders between weeks 0 and 4 and the lack of an increase in the number of patients with HCV RNA present at a level of less than 1 chimpanzee infectious dose per ml after 4 weeks of treatment are of considerable interest; if confirmed, these findings may have implications for the duration of IFN therapy.

Similar results on the transient nature of HCV RNA disappearance, discrepancies between normalization of ALT levels and the presence of HCV RNA (1, 2), and relapse within 6 months after termination of therapy were also observed by other investigators who used primers from the NC or NS4 region (4, 20).

The use of two primer sets, NC and NS5, resulted in differences between the outcomes in plasma samples from five patients in our study. The HCV RNA was consistently undetectable with the NS5 primer set in one patient (nonresponder; standard scheme). In three other patients (nonresponders; two on the standard scheme, one on the experimental scheme), HCV RNA was not detected with the NS5 primer set at week 4 but was detected at week 24 or 48. During therapy, a change in HCV RNA positivity depending on the primer set was observed in one patient (nonresponder; experimental scheme).

The NC primer set was very suitable for monitoring HCV RNA levels during IFN therapy (7, 11, 14, 17, 21, 22). The discongruent results for the two sets of primers at the beginning of and during therapy could not be the result of a reduced sensitivity of the assay (8). In fact, these results are suggestive of the presence of HCV variants in these patients, and the individual sensitivities of these variants for IFN therapy are under investigation.

In conclusion, results of the present study suggest that monitoring of HCV RNA levels in the first months of IFN therapy may be of prognostic value for failure of therapy aimed at eradicating HCV RNA (in the present study, four patients [16%]). However, the normalization of ALT levels alone, as observed in an additional two patients, could be beneficial if they were sustained for long periods of time.

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