Acta Medica Okayama

Volume 50, *Issue* 3 1996 *Article* 4

JUNE 1996

Relationship between Response to Interferon Therapy and Detection of Hepatitis C Virus RNA by Differential Flotation Centrifugation

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Relationship between Response to Interferon Therapy and Detection of Hepatitis C Virus RNA by Differential Flotation Centrifugation*

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Abstract

We purified an apurinic/apyrimidinic (AP) endonuclease from mouse ascites sarcoma (SR-C3H/He) cells. The enzyme showed nicking activity on acid-depurinated DNA but not on untreated, intact DNA. It also showed priming activity for DNA polymerase on both acid-depurinated and bleomycin-damaged DNA. The priming activity on bleomycin-damaged DNA was two times higher than that on an acid-depurinated DNA. The enzymatic properties indicate that the enzyme is a class II AP endonuclease having DNA 3' repair diesterase activity. The purified enzyme has a molecular weight of 39,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimal pH for AP endonuclease activity was 8.0 in 50 mM Tris-HCl buffer. The AP endonuclease activity depended on divalent cation such as Mg2+ and Co2+ ions, and was inhibited by 2 mM EDTA with no addition of the divalent cation. An appropriate concentration of sodium or potassium salt stimulated the activity. Partial digestion of the AP endonuclease with Staphylococcus aureus V8 protease produced 4 major peptide fragments which may be used for protein sequencing.

KEYWORDS: hepatitis C, ultracentrifugation, immune complex, interferon

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*PMID: 8805852 [PubMed - indexed for MEDLINE]

ACTA MED OKAYAMA 1996; 50(3); 139-144

Relationship between Response to Interferon Therapy and Detection of Hepatitis C Virus RNA by Differential Flotation Centrifugation

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Circulating hepatitis C virus (HCV) particles can be fractionated by means of differential flotation centrifugation. It is reported that in the bottom fraction HCV is in the form immune complexes, whereas in the top, it is free of antibodies. We evaluated the significance of circulating complex and free HCV in chronic hepatitis C, and assessed the relationship in terms of the response to interferon (IFN) therapy. We examined sera before, just after, and 1 year after administering IFN to 18 patients with chronic hepatitis C, 10 of whom responded (group CR), and 8 did not (group NR). The amounts of virus were similar between both groups before therapy. After differential flotation centrifugation with 1.063 g/ml of NaCl, the top and bottom fractions were assayed for HCV RNA. Before therapy, HCV RNA was detected in the top fraction in 1 of 10 in group CR, and in 6 of 8 in group NR ($P < 0.05$, chi-square test). HCV RNA was positive in the bottom fraction of all samples. In a follow-up study of group NR, HCV RNA was detected in the top fraction in 3 of 8 just after IFN therapy, and in 7 of 8 after 1 year. This study suggests that the presence of HCV in the top fraction can predict a poor response to IFN therapy.

Key words: hepatitis C, ultracentrifugation, immune complex, interferon

Hepatitis C virus (HCV), which was first identified by cDNA cloning in 1989 (1), is a major causative agent of non-A, non-B (NANB) hepatitis. Even though this virus has been studied extensively, the size and morphological structure of HCV remains elusive. Recently, Hijikata *et al.* (2) reported that HCV particles can be fractionated by differential flotation centrifugation into immune-complexed forms in the bottom and free particles in the top fraction. Also, an association of the infectivity to chimpanzees or the pathophysiology of liver diseases with the distribution pattern of HCV particles has been suggested. Kanto *et al.* (3, 4) have also noted that HCV was a free intact virion at a buoyant density of 1.08–1.11 g/ml, and HCV formed immune complexes at a buoyant density of 1.22–1.25 g/ml.

Interferon (IFN) has been widely used in Japan to treat chronic hepatitis C. However, more than half of those treated respond poorly to the therapy or relapse soon after discontinuation. The response to this therapy depends on several factors including the amount of HCV in serum or liver tissue prior to therapy, and the genotype of HCV (5–8).

In this study, we fractionated HCV particles by differential flotation centrifugation to evaluate the clinical significance of the circulating immune-complexed and free HCV fractions in patients with chronic hepatitis C, and assessed the relationship in terms of response of IFN therapy.

Patients and Methods

Patients. We retrospectively studied 18 patients with chronic hepatitis C, all of whom had been treated with IFN. Ten of 18 responded to the IFN therapy, becoming negative for serum HCV RNA, and were classified as group CR. The remaining 8 patients did not respond, and were classified as group NR. These patients were selected for similar pre-treatment amounts of HCV RNA and age (mean ± SD; age, 60.0 ± 9.5 in

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group CR *vs.* 54.6 ± 6.8 years old in group NR; $P = 0.195$ by Student's *t*-test; HCV RNA, 7.5 ± 0.7 in group CR *vs.* 7.8 ± 0.5 log copies/ml in group NR; $P = 0.305$ by Student's *t*-test). The genotypes of HCV were type 1b in 7, 2a in 1, 2b in 2 of group CR, and were type 1b in all cases in NR (Table 1). Patients were histologically diagnosed by means of liver biopsy under laparoscopy. The types and total administered doses of IFN are shown in Table 1. Sera taken just before the IFN administration from all patients, just after (range 0–23 days, 12.0 ± 8.8 days after the last day of administration) and 1 year after the cessation of IFN therapy from group NR were stored at −20°C until use. Written informed consent was obtained from all patients before the laparoscopy and liver biopsy. Interferon was administered only when patients gave informed consent.

Differential flotation centrifugation. Differential flotation centrifugation (DFC) was performed according to the method of Hijikata *et al.* (2). This method was described originally by Havel *et al.* for fractionation of plasma low-density lipoprotein (9). Fifty microliters of serum was overlaid on 8 ml of NaCl with a density of 1.063 g/ml and centrifuged in a Hitachi 65T rotor at 139,500 × *g* for 22 h at 14°C in a Hitachi SCP85H centrifuge. After centrifugation, 1-ml aliquots from the top and the bottom fractions were collected. Total RNA was extracted from 200 μl of each fraction by the acid-guanidinium-phenol-chloroform method (10).

Protein G Sepharose affinity chromatography. One milliliter of serum was applied to a protein G Sepharose column (HiTrap affinity columns, Pharmacia Biotech, Tokyo, Japan) at room temperature. We collected the pass-through fractions and those eluted with 0.1 M glycine-HCl, pH 2.7, then neutralized them with 1/10 volumes of 1 M Tris-HCl, pH 9.0. One-milliliter aliquots from each were fractionated by DFC. HCV RNA in both the top and bottom fractions were determined by the reverse transcriptase-polymerase chain reaction (RT-PCR).

Detection and quantitation of serum HCV RNA. The level of HCV RNA in the serum was determined by means of nested RT-PCR using the sequence of the 5' non-coding region as described before (11, 12). By this assay, as few as 100 copies of HCV

Table I Clinical data of patients

	Patient	Sex	Age	Genotype	HCV RNA[a]	Histology	IFN[b]	Total dose[c]
Group CR								
	1	M	64	1b	6	CPH	IFNα	324
	2	M	63	1b	7	CAH2B	IFNα	249
	3	M	53	1b	7	CAH2A	IFNα	504
	4	F	58	1b	8	CPH	IFNβ	192
	5	M	68	1b	8	CAH2B	IFNα	300
	6	M	72	1b	8	CAH2B	IFNα	285
	7	M	38	1b	8	CAH2A	r-IFNα2a	418
	8	M	64	2a	8	CAH2B	r-IFNα2a	126
	9	F	57	2b	7	CAH2B	IFNα	498
	10	M	63	2b	8	CAH2B	IFNα	534
Group NR								
	11	F	53	1b	7	CAH2A	IFNα	480
	12	M	43	1b	7	CAH2A	r-IFNα2b	800
	13	M	48	1b	8	CAH2A	IFNα	516
	14	M	62	1b	8	CAH2B	IFNα	123
	15	F	56	1b	8	CPH	r-IFNα2b	790
	16	F	54	1b	8	CAH2B	IFNβ	246
	17	F	63	1b	8	CAH2B	IFNα	237
	18	F	58	1b	8	CAH2A	IFNβ	480

a: Quantitation of hepatitis C virus (HCV) in the serum (log copies/0.5 ml); *b*: Type of interferon (IFN); *c*: Total dose of IFN (MU)
Abbreviations used in this table: M, male; F, female; CPH, chronic persistent hepatitis; CAH2A, chronic active hepatitis with moderate activity; CAH2B, chronic active hepatitis with severe activity; Group CR: Hepatitis patients who responded to IFN therapy; Group NR: Hepatitis patients who did not respond to IFN therapy.

RNA can be detected. Competitive RT-PCR using mutant RNA in which a *Bam*HI site was introduced by *in vitro* mutagenesis was performed to quantify the level of HCV RNA in serum as reported previously (13, 14).

Genotyping of HCV. The genotype of HCV was determined by RT-PCR using mixed type-specific primers against the NS5 region according to Enomoto *et al.* (15) as modified by Chayama *et al.* (16). The HCV genotypes were described according to the system for nomenclature of HCV genotypes proposed by Simmonds *et al.* (17).

Statistical analysis. The results were analyzed by means of Student's unpaired, two-tailed t-test and the chi-square test, and $P < 0.05$ was taken as the level of significance.

Results

Protein G Sepharose affinity chromatography. The serum sample before passing protein G Sepharose affinity chromatography was positive for HCV RNA in both DFC fractions, at a titer ratio of 1:1000 (top:bottom). In the pass-through fraction from protein G Sepharose column, the ratio became to 1:1. On the other hand, in the eluate from the column, HCV RNA was detected only in the bottom fraction.

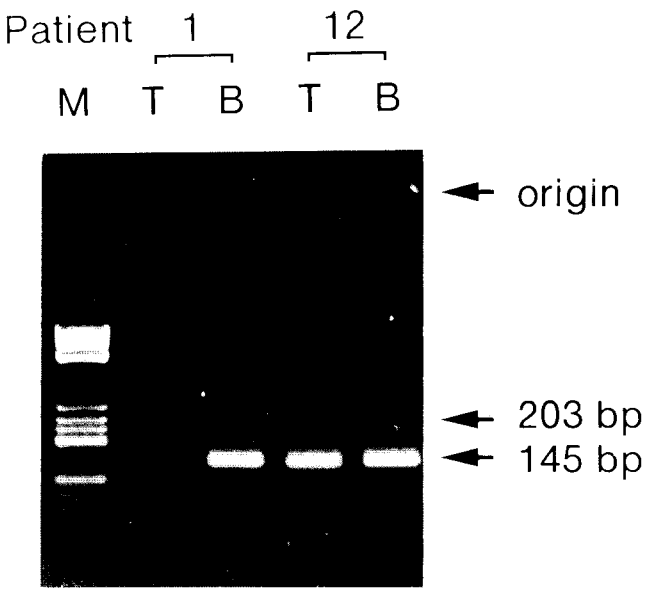

Fig. 1 Detection of hepatitis C virus (HCV) RNA by nested RT-PCR from top and bottom fraction of patients 1 and 12. **T**: top fraction. **B**: bottom fraction. **M**: molecular marker (pBR322 *Hae* III). Arrows indicate the HCV specific bands amplified by first PCR (203 bp) and amplified by second PCR (145 bp).

Table 2 HCV RNA in the top and bottom fractions before treatment with IFN

	Patient	HCV RNA	
		Top	Bottom
Group CR			
	1	−	+
	2	−	+
	3	+	+
	4	−	+
	5	−	+
	6	−	+
	7	−	+
	8	−	+
	9	−	+
	10	−	+
Group NR			
	11	−	+
	12	+	+
	13	+	+
	14	+	+
	15	−	+
	16	+	+
	17	+	+
	18	−	−

HCV, IFN, Group CR and Group NR: See Table 1.

HCV fraction and IFN therapy. Before IFN therapy, HCV RNA was detected in the bottom DFC fraction of all tested sera. In the top fraction, HCV RNA was detected in 1 of 10 (10 %) patients in group CR and in 6 of 8 (75 %) of group NR (Table 2, Fig. 1). The positive rate of HCV RNA in the top fraction was significantly higher in group NR than in group CR ($P < 0.01$, chi-square test).

To follow-up group NR, we analyzed the transition of HCV RNA in the top and bottom fractions. HCV RNA was detected in the top fraction in 6 of 8 (75 %) before therapy, in 3 of 8 (37.5 %) just after the cessation of the therapy, and in 7 of 8 (87.5 %) after 1 year. HCV RNA was positive in the bottom fraction of all samples both before therapy and after 1 year (Table 3). In only one patient (case 18) HCV RNA was not detected in both the

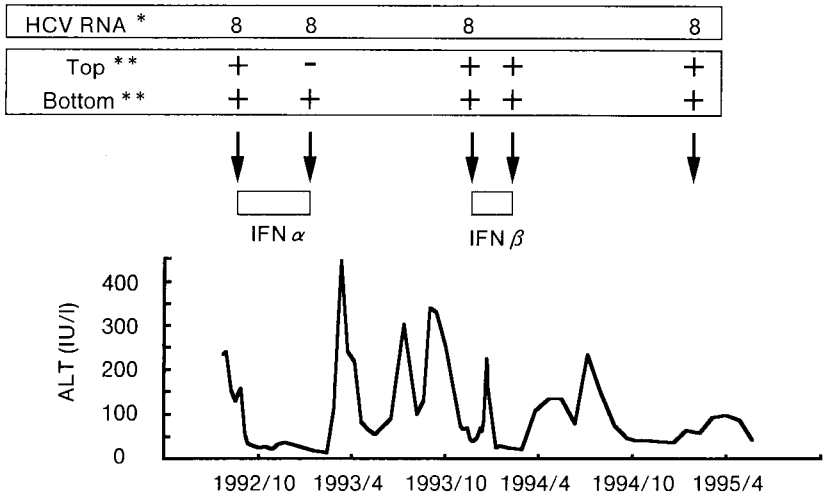

Fig. 2 Transition of HCV RNA in patient 17.
* The HCV RNA titer is indicated as a logarithm of the copy numbers of HCV RNA per 0.5 ml of serum. ** Presence of HCV RNA in the top and bottom fractions after differential flotation centrifugation. IFN: interferon, and HCV: See Fig. 1.

Table 3 Transition of HCV RNA in group NR

Patient	Fraction	HCV RNA		
		Before IFN[a]	After IFN[b]	After 1 year[c]
11				
	Top	−	+	+
	Bottom	+	+	+
12				
	Top	+	+	+
	Bottom	+	+	+
13				
	Top	+	−	+
	Bottom	+	+	+
14				
	Top	+	−	+
	Bottom	+	+	+
15				
	Top	−	−	+
	Bottom	+	+	+
16				
	Top	+	+	−
	Bottom	+	+	+
17				
	Top	+	−	+
	Bottom	+	+	+
18				
	Top	+	−	+
	Bottom	+	−	+

a: Just before IFN therapy; *b*: Just after cessation of IFN therapy; *c*: 1 year after IFN therapy. IFN, HCV: See Table 1.

top and bottom fractions just after the cessation of the therapy, but HCV RNA became positive in both fractions after 1 year.

The clinical course of case 17 in group NR, who was treated by two courses of IFN administration with unsuccessful results is presented in Fig. 2. HCV RNA was detected in the top DFC fraction before the first and the second administrations. Although the serum titers were equal at baseline, after the first, before the second, and 1 year after the second IFN administrations, HCV RNA was detected in the top fraction at all times except for immediately after the first IFN administration.

Discussion

Since part of the HCV genome has been identified (1), several characteristics of this virus have been discovered. Hijikata *et al.* (2) have separated two fractions of HCV particles in sera from patients and chimpanzees with hepatitis C by means of DFC. In their study, the results of immunoprecipitation indicated that the HCV population in the bottom fraction after centrifugation was mainly antibody-bound particles and that in the top was mostly free particles. Kanto *et al.* have also reported that the HCV fraction with a buoyant density of 1.08–1.11 g/ml in sucrose consisted of free intact virions, whereas that with

a density of 1.22-1.25 g/ml was composed primarily of immune-complexed or nucleocapsid particles (3, 4). To confirm the distribution of immune-complexed particles in our DFC, we compared the titers of HCV RNA before and after protein G Sepharose affinity chromatography. In our study, depletion of IgG and IgG-associated fractions through protein G Sepharose affinity chromatography from serum with an HCV RNA titer ratio of 1:1000 (top:bottom) resulted in a decrease in the relative amount of HCV RNA in the bottom to 1:1. This result indicated that HCV particles bound to IgG mainly went to the bottom fraction, and this finding is consistent with published reports.

Before HCV was identified, there were several descriptions of circulating immune complexes (CIC) in NANB hepatitis. Among them, Araki *et al.* detected CIC in patients with NANB hepatitis as well as hepatitis B by means of the C1q binding test (18). They found that CIC increased before and after the exacerbation of transaminases in a patient with chronic NANB hepatitis. Dienstag *et al.* (19) and Poralla *et al.* (20) also detected CIC in patients with chronic NANB hepatitis. Poralla identified IgG and C3 in CIC fractions. HCV RNA has been detected in CIC by immunoprecipitation with an anti-human immunoglobulin antibody (2, 21). Morita has detected HCV RNA in immunoprecipitates from the sera of 91% of tested patients (21), but the clinical significance of HCV RNA in CIC in the pathophysiology of hepatitis C has remained to be elucidated. Hijikata has reported that serum rich in low-density HCV was highly infectious to chimpanzees, whereas that with high density was less infectious (2). This suggests that free virions have the potential to spread widely to uninfected liver cells during the course of chronic hepatitis, while virus in the immune complex would be blocked from infecting by neutralizing antibody bound to the viral particles.

Interferon therapy for patients with chronic hepatitis C has been developed, but its efficiency is not satisfactory. More than half of treated individuals respond poorly. Several predictive factors for the response to IFN have been reported, including viral genotypes (5), the titer of viremia (6), and in liver tissue (8) before therapy. Besides these factors, it may be postulated that the infectivity of HCV particle could be related to the IFN response. Therefore we examined the fractionation of HCV particles in sera and assessed the relationship to the IFN response.

Sera taken from 18 patients with chronic hepatitis C were fractionated by DFC and analyzed for HCV RNA. Because the pre-treatment titer of HCV RNA has been considered as a predictive factor for the response to IFN therapy (6), the patients in two groups in this study were selected so as to have similar titers. HCV RNA was detected in all patients in both groups in the bottom fraction before IFN therapy. On the other hand, it was detected in the top fraction in 6 of 8 (75%) in group NR, but only 1 of 10 (10%) of group CR. This suggests that the presence of free particles could be a useful marker to predict a poor response to IFN therapy.

We studied the serial transition of HCV RNA in the top fraction in group NR (Table 3). In the top fraction, HCV RNA was detected in 75% of the patients before therapy, in 37.5% after the cessation of the therapy, but in 87.5% after 1 year. On the other hand, in the bottom fraction, HCV RNA was negative in only one patient just after IFN administration. This result raises the question as to why HCV emerged as high density particles immediately after IFN therapy. Studies of chimpanzees inoculated with HCV (2) have indicated that circulating HCV first exists as free intact virions, then binds to antibodies or is broken into nucleocapsids. Apart from acute infection, neutralizing antibodies against certain strains of virus may be present in the serum after IFN therapy. When IFN administration was stopped, the remaining viruses might replicate in the liver cells and be released into the circulation. Probably most of the released virus is captured by circulating antibodies. This would explain why we found that most HCV particles had been detected in the bottom just after IFN therapy. Eventually, virus may mutate and escape from the antibodies as reported by Kato (22), which would explain why we detected HCV RNA in the top fraction later. Kanto also examined the sera of patients with chronic hepatitis C at just before, and 24 weeks after therapy (23). They reported that patients who have high-density HCV dominance after therapy show persistent normalization of alanine aminotransferase despite the presence of viremia. Further examination of the serial changes in HCV particles for sequencing the envelope region of the HCV genome and the relationship to the pathophysiology of liver injuries are necessary to resolve this issue.

In conclusion, the present results suggest that the presence of HCV in the top fraction after differential flotation ultracentrifugation could be a useful marker to predict a poor response to IFN therapy, and that the physical and immunological characteristics of HCV particles must be related to the pathophysiology of chronic

hepatitis C.

Acknowledgments. We thank Miss Hiroko Kajitani for her excellent technical assistance. This work was partly supported by a grant for Non-A, Non-B Hepatitis Research from the Ministry of Health and Welfare, and the Viral Hepatitis Research Foundation.

This work was presented in part at the 46th Annual Meeting of the American Association for the Study of Liver Diseases, November 3-7, 1995, Chicago, IL. USA.

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Received February 7, 1996; accepted March 8, 1996.