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Research Article

Hepatitis C virus (HCV) RNA was detected in the sera of patients with non-A, non-B chronic liver disease by polymerase chain reaction (PCR). RNA was extracted from the serum, reverse transcribed to cDNA, and amplified by PCR. With this method, 30 patients with non-A, non-B chronic liver disease and 10 healthy subjects were tested. HCV RNA was detected in 13 of 16 (81%) anti-HCV-positive patients and also in 7 of 14 (50%) anti-HCV-negative patients, but in none of 10 anti-HCV-negative healthy subjects. Specificity of this method was confirmed by direct sequencing of amplified cDNA segment. The nucleotide sequences (37 nucleotides) obtained from 15 patients showed only 68-78% homology compared with the prototype HCV nucleotide sequence. In addition, of 15 nucleotide sequences, there were 12 different types. But the translated amino acid sequences (12 amino acids) showed 83-100% homology compared with the prototype HCV amino acid sequence. These data suggest the majority of anti-HCV-positive patients are carriers of HCV. But to detect all the viremic patients, the anti-HCV antibody testing may be insufficient. Direct detection of HCV RNA may be useful in the study of virus replication and its association with various liver diseases.

Detection of Hepatitis C Virus Ribonucleic Acid in the Serum by Amplification with Polymerase Chain Reaction

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Abstract

Hepatitis C virus (HCV) RNA was detected in the sera of patients with non-A, non-B chronic liver disease by polymerase chain reaction (PCR). RNA was extracted from the serum, reverse transcribed to cDNA, and amplified by PCR. With this method, 30 patients with non-A, non-B chronic liver disease and 10 healthy subjects were tested. HCV RNA was detected in 13 of 16 (81%) anti-HCV-positive patients and also in 7 of 14 (50%) anti-HCV-negative patients, but in none of 10 anti-HCV-negative healthy subjects. Specificity of this method was confirmed by direct sequencing of amplified cDNA segment. The nucleotide sequences (37 nucleotides) obtained from 15 patients showed only 68–78% homology compared with the prototype HCV nucleotide sequence. In addition, of 15 nucleotide sequences, there were 12 different types. But the translated amino acid sequences (12 amino acids) showed 83–100% homology compared with the prototype HCV amino acid sequence.

These data suggest the majority of anti-HCV-positive patients are carriers of HCV. But to detect all the viremic patients, the anti-HCV antibody testing may be insufficient. Direct detection of HCV RNA may be useful in the study of virus replication and its association with various liver diseases. (*J. Clin. Invest.* 1990. 86:1764–1767.) Key words: anti-HCV antibody • non-A, non-B chronic hepatitis • direct sequencing • cDNA • hepatocellular carcinoma

Introduction

Serologic evidence of hepatitis B virus infection is present in only 20 to 30% of our patients with nonalcoholic chronic liver disease (1). Alcoholic liver disease is far less common in our country than in the U.S.A. (2). Thus, presumed non-A, non-B disease probably accounts for more than 50% of our chronic liver disease. Recent success in cloning hepatitis C virus (HCV)[1] RNA has made an assay system to detect antibody against HCV available (3, 4). However, an assay system to detect HCV, namely viral antigen or viral nucleic acid, is not widely available. It is estimated that HCV contains a positive-strand RNA molecule of ~ 10 kb (3, 4) and ~ 70% of the nucleotide sequence was disclosed (5). In this study, we developed an assay system to detect HCV RNA using polymerase chain reaction (PCR), and analyzed the serum samples obtained from patients with non-A, non-B chronic liver disease.

Methods

Extraction of RNA from the serum. RNA was extracted from the serum as previously described (6). In short, 1 ml of serum was diluted with three times volume of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 100 mM NaCl, and centrifuged at 100,000 *g* for 3 h. The pellet was digested with 1 mg/ml proteinase K and 0.1% SDS, extracted with phenol/chloroform, and precipitated with ethanol.

Preparation and amplification of HCV cDNA. With an antisense primer (5′-TTTGGTGACTGGGTGCGTCA-3′) corresponding to the position 3477-3496 of prototype HCV cDNA nucleotide sequence, HCV RNA was transcribed to cDNA by reverse transcriptase according to the manufacturer's instruction (cDNA Synthesis System Plus; Amersham International, plc, Amersham, UK). By adding a sense primer (5′-AAGTGTTTGATTCGCCTCAA-3′) corresponding to the position 3562-3581, the cDNA was amplified by PCR.

Amplification of HCV cDNA was performed by the method described by Saiki et al. (7). Briefly, 100-μl reaction mixtures containing 20 μl of specimen cDNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 1 μM each of primers, 200 μM dNTP, 200 μg/ml of gelatin, and 4 U of *Thermus aquaticus* DNA polymerase (New England Biolabs, Beverly, MA) were overlaid with 100 μl of mineral oil. Samples were heated at 95°C for 2 min (denatured), cooled to 42°C for 2 min (annealed), and heated to 70°C for 2 min (elongated). These steps were repeated for 35 cycles.

Analysis of amplified samples. After the final step of amplification, each sample (10 μl) was applied to an 8% acrylamide gel in Tris-borate buffer (pH 8.0) and electrophoresed alongside of pBR 322 DNA digested with Alu I as molecular size markers. After electrophoresis the gel was stained with ethidium bromide and observed under an ultraviolet lamp.

Direct sequencing of amplified samples. For direct sequencing of HCV cDNA; the PCR products were centrifuged in a microconcentrator (Centricon 30; Amicon Corp., Scientific Systems Div., Danvers, MA). Then one-tenth of this was directly sequenced by the modified method of Sanger et al. (8) using Sequenase (United States Biochemical Corp., Cleveland, OH) as previously described (9). A third primer (5′-AAGCCCACCCTCCATGGGCC-3′) corresponding to the position 3495-3514 of the prototype HCV cDNA nucleotide sequence was radiolabeled with γ-[^{32}P]ATP and T4 polynucleotide kinase and used for direct sequencing. Electrophoresis was performed on an 8% polyacrylamide/ 8 M urea sequencing gel and autoradiography was performed by standard techniques.

Patients. 30 serum samples from 30 patients with non-A, non-B chronic liver disease (26 chronic hepatitis, 2 cirrhosis, 2 cirrhosis with

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1. *Abbreviations used in this paper:* HCV, hepatitis C virus; PCR, polymerase chain reaction.

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Table I. Demographic Data of 30 Patients with non-A, non-B Chronic Liver Diseases

Patients	Age	Sex	ALT	Histology of liver	Serum anti-HCV antibody	HCV RNA
1	23	M	87	CPH	+	+
2	35	M	71	CPH	+	+
3	40	M	83	CPH	+	+
4	34	M	242	CPH	+	+
5	63	F	74	CAH	+	+
6	61	F	78	CAH	+	+
7	49	F	93	CAH	+	+
8	41	M	156	CAH	+	+
9	49	F	178	CAH	+	+
10	42	M	213	CAH	+	+
11	38	F	460	CAH	+	+
12	68	F	42	Cir & HCC	+	+
13	65	M	105	Cir & HCC	+	+
14	43	F	164	CAH	+	−
15	61	M	98	CAH	+	−
16	66	F	108	CAH	+	−
17	48	F	37	CPH	−	+
18	14	F	80	CPH	−	+
19	32	M	149	CAH	−	+
20	54	F	82	CAH	−	+
21	60	F	201	CAH	−	+
22	63	F	176	CAH	−	+
23	55	F	178	Cir	−	+
24	56	M	101	CAH	−	−
25	30	F	24	CAH	−	−
26	53	M	33	CAH	−	−
27	30	M	58	CAH	−	−
28	46	M	62	CAH	−	−
29	38	F	89	CAH	−	−
30	60	M	45	Cir	−	−

ALT, alanine amino transferase; CPH, chronic persistent hepatitis; CAH, chronic active hepatitis; Cir, cirrhosis; HCC, hepatocellular carcinoma.

hepatocellular carcinoma) were tested for the presence of HCV RNA by this method. Of these 30 patients, 16 were seropositive and 14 were seronegative for anti-HCV antibody by enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan). Details of clinical data are shown in Table I. In addition to these 30 patients with non-A, non-B chronic liver disease, anti-HCV-negative serum samples from 10 healthy subjects with normal liver function tests were also tested.

Results

Detection of HCV cDNA. The observed size of the PCR products was ~ 105 bp in the acrylamide gel (Fig. 1). This size was consistent with the expected length of 105 bp between the two primers used for PCR. With this method, HCV RNA was detected in 13 of 16 (81%) anti-HCV-positive patients and also in 7 of 14 (50%) anti-HCV-negative patients. This was not detected in any of 10 healthy subjects who were seronegative for anti-HCV antibody.

Direct sequencing of HCV cDNA after PCR. To confirm that these amplified segments contain the nucleotide sequences homologous to that of the prototype HCV, we performed direct sequencing of the PCR products from 15 patients using the radiolabeled sequencing primer. A portion of cDNA segment corresponding to the position 3525-3561 (37 nucleotides) was analyzed in 15 patients who were positive for HCV RNA (Table II). Of these 15 patients, 8 were seropositive and 7 were seronegative for anti-HCV antibody. The nucleotide sequences (37 nucleotides) obtained from 15 patients showed only 68–78% homology compared with those of the prototype HCV. In fact, none of 15 nucleotide sequences completely matched the prototype HCV nucleotide sequence. These variations were also observed among our Japanese patients. Of 15 patients analyzed, 12 different types of nucleotide sequences were observed.

Then the translated amino acid sequences (12 amino acids) obtained from 15 patients were analyzed. The amino acid sequences showed higher homology (83–100%) than the nucleotide sequence. The nucleotide substitutions frequently occurred at the third position of the codons: the amino acid substitutions rarely occurred. Of these 15 patients, no amino acids were substituted (100% homology) in two patients (20 and 23 in Table I), only one amino acid was substituted (92% homology) in 11 patients, and two amino acids were substituted (83% homology) in two patients (3 and 18 in Table I). Furthermore, the amino acid substitution occurred at the same position in 13 patients.

Discussion

Recent success in isolation of a cDNA clone from a bloodborne non-A, non-B viral hepatitis genome made the development of an assay system for determination of anti-HCV antibody available (3, 4) and subsequently the nucleotide sequence of HCV cDNA was disclosed (5). However, an assay system for detection of the virus itself is not widely available. Conventional immunological methods may not be useful to identify insufficient concentration of this viral antigen. An alternative approach is to detect viral nucleic acids by the hybridization method. However, the sensitivity of conventional spot hybridization tests is thought to be 0.1–1.0 pg from the previous studies of hepatitis B virus (10, 11). Thus, at least 10^{3-5} virions have to be present in the specimen to be detected by the conventional spot hybridization method.

Recently PCR, an efficient method to amplify DNA sequence, was described by Saiki et al. (7). HCV RNA was re-

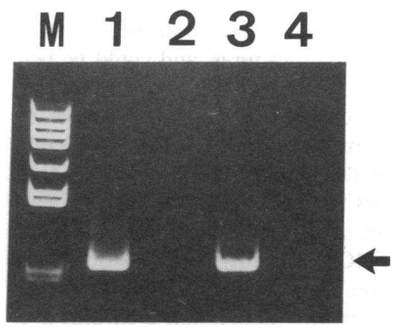

Figure 1. Ethidium bromide staining of amplified DNA after 35 cycles of PCR in acrylamide gel electrophoresis. The amplified DNA was ~ 105 bp in length (*arrow*) and observed in lanes *1* and *3*, but not in lanes *2* and *4*. Lane *M*; molecular size markers (pBR 322 DNA digested with Alu I); lane *1*, anti-HCV-antibody seropositive patient (5 in Table I) lanes *2* and *3*, anti-HCV-antibody seronegative patients (25 and 22 in Table I, respectively); lane *4*, anti-HCV-antibody seronegative healthy subject.

Table II. The Nucleotide Sequences of the Prototype HCV cDNA and Our 15 Patients Determined by PCR and Direct Sequencing Method

Prototype HCV	(3525) CTA	TAC	AGA	CTG	GGC	GCT	GTT	CAG	AAT	GAA	ATC	ACC	(3561) C	Homology
1	--G	--T	--G	--A	--A	--C	---	--A	---	--G	G--	---	-	76%
2	T-G	---	--G	--A	--A	--C	--C	---	---	--G	G--	---	-	76%
3	--G	--T	--G	--A	--A	--C	--C	--A	---	---	G--	-A-	-	73%
4	--G	--T	--G	--A	--A	--C	--C	--A	---	--G	G--	--T	-	70%
5	T-G	---	--G	--A	--A	--C	--C	---	---	--G	G--	---	-	76%
6	--G	--T	--G	--A	--A	--C	--C	--A	--C	--G	G--	--T	-	68%
12	--G	--T	--G	--A	--A	--C	--C	---	---	--G	G--	---	-	76%
13	--G	--T	--G	--A	--A	--C	---	--A	---	---	G--	---	-	78%
17	--G	--T	--G	--A	--A	--C	--C	--A	---	--G	G--	---	-	73%
18	T-G	--T	--G	--A	--A	--C	--C	--A	---	--G	G--	G--	-	68%
19	--G	--T	--G	--A	--A	--C	--C	--A	---	--G	G--	--T	-	70%
20	--G	--T	--G	--A	--A	--C	---	--A	---	--G	---	---	-	78%
21	--G	--T	--G	--A	--A	--C	--C	--A	---	--G	G--	--T	-	70%
22	T-G	--T	--G	--A	--A	--C	--C	--A	--C	--G	G--	---	-	68%
23	--G	--T	--G	--A	--A	--C	---	--A	--C	--G	---	---	-	76%

Numbers in parentheses represent the nucleotide positions of the prototype HCV cDNA (5). Dashes represent nucleotides that are identical to the prototype HCV cDNA.

verse transcribed to cDNA and PCR was used to amplify the cDNA.

Regarding the specificity of the PCR products, we analyzed the size of the PCR products by an acrylamide gel and found them to have the expected length between the primers. To confirm that the PCR products have homology with HCV cDNA, we determined the nucleotide sequence by the direct sequencing method. We analyzed 15 amplified cDNA segments from 15 different patients, and found that a portion of viral cDNA nucleotide sequences showed 68–78% homology with those of the prototype HCV reported by Houghton (5). This result was consistent with the previous report that the nucleotide sequence obtained from one Japanese anti-HCV antibody-positive serum showed comparatively low homology with that of the prototype HCV (12). However, the amino acid sequences varied less and 83–100% homology was observed between our Japanese amino acid sequences and the American prototype amino acid sequence. This indicates that the nucleotide variations frequently occur in the third nucleotide of the codons. In fact, of 150 mismatches with the American prototype HCV nucleotide sequence, 131 (87%) were in the third, 1 was in the second, and 18 were in the first nucleotide of codons. The direct sequencing method using amplified products of PCR could omit many steps of complicated conventional cloning and sequencing procedures, and could be performed in a few days.

In this study, we could compare the results obtained by anti-HCV-antibody determination and HCV RNA detection by PCR. HCV RNA was detected in the majority of anti-HCV-positive sera. Anti-HCV antibody is supposed to react with nonstructural protein of HCV (5). This study revealed that the presence of anti-HCV antibody in our patients was associated with the viremia. However, the viremia was also found in some of the patients with non-A, non-B chronic liver disease who were seronegative for anti-HCV antibody. It was previously reported that HCV RNA was detected in the liver of anti-HCV seronegative patients (13). The finding of HCV RNA in the sera that were negative for anti-HCV antibody strongly suggests that some of the viremic patients may not produce a sufficient amount of antibody against the peptide of the nonstructural portion of the virus genome. In fact, a recent transfusion study revealed that anti-HCV-negative sera still transmitted HCV infection (14). The anti-HCV antibody assay kit is made to detect antibody against peptide (C100-3) synthesized by American HCV prototype (5), that some of our Japanese HCV carriers may have the antibody that does not react with that peptide, but does with our own.

Detection of HCV RNA in human liver and in chimpanzee sera of acute phase was previously reported (13). We have now reported that HCV RNA could be detected in human sera of 1 ml or less. In addition, the direct sequencing method using amplified products of PCR revealed heterogeneity of the nucleotide sequences derived from Japanese patients with various non-A, non-B chronic liver disease, including hepatocellular carcinoma. This detection method of viral RNA may have wide applications in the diagnosis, the prevention, and the treatment of hepatitis C virus infection. However, for the application of this method in routine laboratories, it is necessary to take several precautions. Since PCR is highly sensitive, the contamination of samples should be avoided. Each nucleotide sequence obtained from our Japanese patients showed variations from the prototype HCV nucleotide sequence or even from our own, so that the analysis of the nucleotide sequence variations may help to check contamination to some extent.

For universal adaptation of this method, whole procedures could be automated, and the possibility of contamination might be eliminated in the future. But until that time, quality control by taking positive and negative controls is needed in every laboratory that conducts PCR.

References

1. Yokosuka, O., M. Omata, F. Imazeki, Y. Ito, and K. Okuda. 1986. Hepatitis B virus RNA transcripts and DNA in chronic liver disease. *N. Engl. J. Med.* 315:1187–1192.

2. Omata, M., Y. Ito, O. Yokosuka, F. Imazeki, K. Uchiumi, S. Takano, K. Hosoda, and M. Ohto. 1989. Histological changes of the liver by treatment of chronic non-A, non-B hepatitis with recombinant interferon alpha. Comparison with histological changes in chronic hepatitis B. *Dig. Dis. Sci.* 34:330–337.

3. Choo, Q.-L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradeley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science (Wash. DC).* 244:359–362.

4. Kuo, G., Q.-L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, et al. 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science (Wash. DC).* 244:362–364.

5. Houghton, M. 1989. International application published under the patent cooperation treaty (PCT). 1–242.

6. Tagawa, M., M. Omata, and K. Okuda. 1986. Appearance of viral RNA in the early stage of duck hepatitis B virus infection. *Virology.* 152:477–482.

7. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC).* 239:487–491.

8. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

9. Tada, M., M. Omata, and M. Ohto. 1990. Analysis of ras gene mutations in human hepatic malignant tumors by polymerase chain reaction and direct sequencing. *Cancer Res.* 50:1121–1124.

10. Bonino, F., B. Hoyer, J. Nelson, R. Engle, G. Verme, and J. Gerin. 1981. Hepatitis B virus replication in the liver. *Hepatology (Baltimore).* 1:386–391.

11. Matsuyama, Y., M. Omata, O. Yokosuka, F. Imazeki, Y. Ito, and K. Okuda. 1985. Discordance of hepatitis B e antigen/antibody and hepatitis B virus deoxyribonucleic acid in serum: analysis of 1063 specimens. *Gastroenterology.* 89:1104–1108.

12. Kubo, Y., K. Takeuchi, S. Boonmar, T. Katayama, Q. -L. Choo, G. Kuo, A. J. Weiner, D. W. Bradley, M. Houghton, I. Saito, and T. Miyamura. 1989. A cDNA fragment of hepatitis C virus isolated from an implicated donor of post-transfusion non-A, non-B hepatitis in Japan. *Nucleic Acids Res.* 17:10367–10372.

13. Weiner, A. J., G. Kuo, D. W. Bradley, F. Bonino, G. Saracco, C. Lee, J. Ronsenblatt, Q.-L. Choo, and M. Houghton. 1990. Detection of hepatitis C viral sequences in non-A, non-B hepatitis. *Lancet.* i:1–3.

14. Alter, H. J., R. H. Purcell, J. W. Shih, J. C. Melpolder, M. Houghton, Q. -L. Choo, and G. Kuo. 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N. Engl. J. Med.* 321:1494–1500.