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Serodiagnostic Assay of Hepatitis C Virus Infection Using Viral Proteins Expressed in *Escherichia coli*

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Infection with hepatitis C virus (HCV) was analyzed by an enzyme-linked immunosorbent assay based on recombinant viral proteins encoded by regions of the putative viral core, NS3, NS4 and NS5, which were expressed in *E. coli*. Results showed that 106 of 124 cases (85.5%) of non-A, non-B chronic hepatitis and 43 of 45 cases (95.5%) of hepatocellular carcinoma, negative for HBV marker, were positive for antibodies against at least one of these viral proteins. One of 87 healthy individuals with normal alanine aminotransferase activity was positive for antibody against only the viral core, but was negative for HCV RNA. The serum of one patient with chronic hepatitis was positive for one of these proteins, but negative for HCV RNA. These findings in combination with results on detection of HCV RNA in the sera of patients with non-A, non-B chronic hepatitis indicated that 105 of 124 cases (84.6%) were positive for HCV infection. Sera that were negative for HCV antibodies against all these proteins were also negative for HCV RNA assayed by reverse transcription followed by the polymerase chain reaction. Screening of HCV infection by detecting viral antibodies in circulating blood using all these viral proteins is useful for reducing the number of ambiguous results in screening for viral infection. Thus, this assay system may be useful diagnostic purposes.

Key words: Hepatitis C virus — Serodiagnosis — Recombinant protein — Non-A, non-B hepatitis — Hepatocellular carcinoma

Infection with hepatitis C virus (HCV) is closely related to post-blood transfusion non-A, non-B chronic hepatitis (NANBCH) as well as hepatocellular carcinoma (HCC), which is negative for HBV infection. Initially, screening for HCV infection was conducted by enzyme-linked immunosorbent assay (ELISA) with C-100, which consists of HCV peptide fused with superoxide dismutase and is expressed in yeast. With this screening system, more than 60% patients with NANBCH and related HCC were found to be positive for HCV.[1-5] However, HCV RNA was detected in some sera that were negative for antibody against C-100.[6] Moreover, a protein core of HCV, expressed in *E. coli*, detected anti HCV core antibody in sera from patients with NANBCH, which were negative for the antibody against C-100.[7] The value of this protein core for detection of HCV infection was reported by us[7,8] and others.[9,10] An ELISA system revealed that 79% of patients with NANBCH were positive for antibody against the protein core while 63% of their sera were positive for antibody against C-100.[8] These results indicated the necessity of finding some viral proteins other than C-100 or the protein core for use as probes to detect viral antibodies in sera for more accurate detection of HCV infection.

In this study we expressed various peptides of viral proteins encoded by various regions spanning almost the entire virus open reading frame except the envelope region in *E. coli* as fusion proteins with β-galactosidase and used these proteins to search for antibodies against viral proteins in sera of patients with NANBCH and HCC.

MATERIALS AND METHODS

Expression of peptides of HCV in *E. coli* pUEX-1, -2 or -3 was used as a vector to express HCV peptides in *E. coli*[11] (Table I). Pieces of cDNAs of the HCV genome were inserted into the cloning site of the pUEX vector so that a fusion protein of HCV peptide with β-galactosidase would be expressed and the resultant plasmids were introduced into *E. coli*. Bacteria were grown at 30°C in LB-broth until the optical density at 540 nm reached 0.3. Then expression of the fused protein was induced by shifting the incubation temperature to 42°C for 2.5 h. Fused proteins were purified as follows: *E. coli* was lysed by sonication in lysis buffer (50 m*M* Tris (pH 8.0)-100 m*M* NaCl-1 m*M* EDTA) containing 0.6 *M* urea. The

The abbreviations used are: HCV, hepatitis C virus; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription followed by polymerase chain reaction; EIA, enzyme immunoassay; NANBCH, non-A, non-B chronic hepatitis; HCC, hepatocellular carcinoma; nt, nucleotide.

insoluble fraction was collected by centrifugation at 8,000 rpm for 20 min and the pellet was suspended in lysis buffer containing 3 *M* urea. The solution was re-centrifuged and the resulting pellet was dissolved in lysis buffer containing 6 *M* urea. As most of the fused proteins were solubilized in 6 *M* urea, the supernatant was used as a source of fused proteins. The sizes and locations of cDNA fragments on the viral genome and expression vectors used in this work are shown in Table I and Fig. 1.

Sera Sera from patients with NANBCH and HCC and from healthy persons not showing elevated transaminase activity were used. All these sera were negative for HBs antigen and anti-HBs antibody and were stored at −70°C until use.

ELISA A sample of 25 ng of fused protein was coated onto a 96-well plate and used for ELISA as described.[8] The cut-off values for ELISA were assessed statistically from the values at 490 nm obtained by ELISA of 102 sera from healthy persons with normal alanine aminotransferase activity and no indication of HCV infection and from values at 490 nm on ELISA of 50 sera from patients with NANBCH with HCV markers. In practice, the average value at 490 nm on ELISA of healthy persons plus a value of 6×(standard deviation) was used as the cut-off value. In this assay, less than 0.5% of the 300 healthy persons tested were positive.

Table I. Vectors and Locations of cDNAs Used for Expression of HCV Peptides

Proteins	Location in HCV genome (nt)	Vector used for expression
04	2466–3301	pUEX2
05	3231–3818	pUEX3
06	3657–4338	pUEX3
07	4224–4963	pUEX2
08	4864–5191	pUEX3
10	5137–5723	pUEX3
11	5658–6137	pUEX2
12	6031–6679	pUEX2
13	6573–6686	pUEX2
14	6630–7174	pUEX2
15	7071–7660	pUEX2
16	7551–8328	pUEX2
17	8228–8508	pUEX3
18	8402–8778	pUEX1
21	8618–9359	pUEX3

Reverse transcription followed by polymerase chain reaction (RT-PCR) RNA obtained from sera of patients with NANBCH or HCC was reverse-transcribed using a primer with a complementary sequence to nucleotide 349 to 329 of the HCV genome and the product was used as a template for the PCR, carried out as described elsewhere.[6] The primers used for the PCR corresponded to the 5′ conserved non-coding region of HCV, and their sequences were 5′CCATGGCGTTAGTATGAGTG3′ (nt 83 to 103, numbered according to Kato *et al.*[12]) and 5′GTGCTCATGGTGCACGGTCTA3′ (complementary sequence to nt 349 to 329). One-fiftieth of the first PCR product was then subjected to a second PCR to increase the sensitivity of detection. The primers used for the second PCR were 5′AGAGCCATAGTGGTCTGCGGA3′ (nt 134 to 154) and 5′CTTTCGCGACCCAACACTAC3′ (complementary to nt 277 to 258). After the reaction, the product was analyzed by agarose gel electrophoresis and DNA was located by staining with ethidium bromide.

Preparation of RNA fraction from sera Sera from patients with NANBCH, diagnosed according to the criteria of the Japanese Gastroenterology Society in 1979, and from patients with HCC without HBV markers were centrifuged at 60,000 rpm for 3 h. The precipitated fraction was disrupted in 4 *M* guanidine isothiocyanate-25 m*M* sodium citrate-0.5% sodium sarcosylate-0.1 *M* β-mercaptoethanol and treated with phenol and chloroform. RNA was then precipitated with 3 volumes of ethanol.[6]

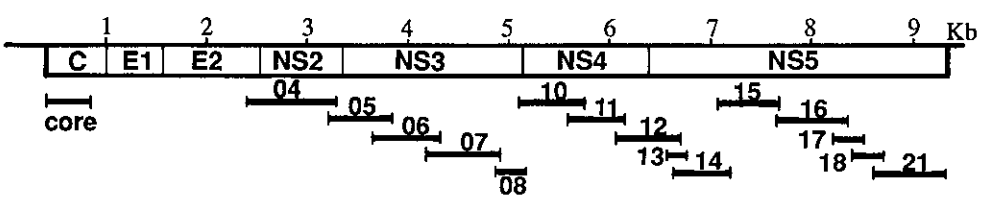

Fig. 1. Locations of cDNA used for expression of HCV peptides in the HCV genome. The box with thick bars indicates a single open reading frame of the HCV genome flanked with 5′ and 3′ noncoding sequences shown by bars. Putative structural proteins [core (C) and envelope (E)] and non-structural proteins (NS2 to NS5) of HCV are shown inside the box. Thick bars underneath the open box, except the core, indicate regions of cDNAs expressed as fusion proteins with β-galactosidase. Expression of the protein core was as described elsewhere.[7]

RESULTS AND DISCUSSION

Expression of HCV peptides as a fused form with β-galactosidase in *E. coli* Plasmids derived from the pUEX vector, which carry an insert of HCV cDNA to produce fusion proteins with β-galactosidase, were introduced into *E. coli* and expression of the fused genes was induced by shifting the temperature of the culture medium from 30°C to 42°C. Each fused protein was purified as described in "Materials and Methods." Production of each fused protein was confirmed by polyacrylamide gel electrophoresis (Fig. 2). Proteins 12 and 21 were not used for further study because their profiles indicated that degradation had occurred.

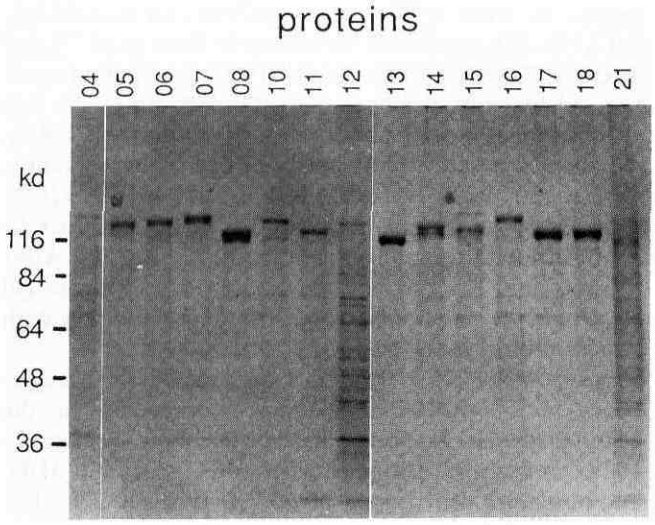

Fig. 2. SDS-polyacrylamide gel electrophoresis of fusion proteins expressed in *E. coli*. Gel was stained with Coomassie Brilliant Blue. Molecular size markers are indicated in kilodaltons. The size marker of 116 kd is β-galactosidase.

Detection of HCV infection by ELISA with proteins expressed in *E. coli* Each protein was coated onto a 96-well plate and used for ELISA to examine its ability to detect antibody against HCV protein. Fifty sera with high values (>50) of alanine aminotransferase activity were selected at random for analysis. Of four fused proteins (proteins 05, 06, 07 and 08) consisting of peptides derived from different regions of NS3 of HCV, protein 07 was found to be the most sensitive for detection of HCV antibody in these sera (data not shown). Similarly, of the NS4 region proteins protein 10 was more sensitive than protein 11 for detection of HCV antibody by ELISA and of the NS5 region proteins, protein 14 was more effective than proteins 15, 16, 17 and 18 (data not shown). Therefore, proteins 07, 10 and 14 as well as the protein core were used in further analyses.

These proteins were used to test for antibodies against HCV in 124 serum samples from patients with NANBCH and 45 sera from patients with HCC as well as 87 sera from healthy individuals with normal alanine aminotransferase activity (Table II). Positive reactions for the protein core, and proteins 07, 10 and 14, respectively, were obtained in 104 (83.8%), 100 (80.6%), 75 (60.4%) and 67 (54.0%) of the 124 sera of patients with chronic hepatitis. The rate of detection with the protein core was consistent with that reported previously.[8] The detection rate with protein 10 was similar to that with C-100 (data not shown), presumably because the amino acid sequence of HCV in protein 10 is included in the sequence of C-100.[5] Of the sera from patients with hepatocellular carcinoma, 95.5%, 93.3%, 84.4% and 66.6% were positive for antibodies against the protein core and proteins 07, 10 and 14, respectively. Of 124 cases of NANBCH and 45 cases of HCC, 106 and 43, respectively, were positive for more than one of these proteins. Only one serum sample from a healthy subject was positive for antibody against the protein core. The physiological meaning of the appearance of each anti-

Table II. Detection of HCV Antibodies in Sera by Viral Proteins Expressed in *E. coli*

	Total	Positive reaction for antibodies to proteins					Negative reaction to all proteins
		Core	07	10	14	More than two proteins	
CH	124	104 (83.8)	100 (80.6)	75 (60.4)	67 (54.0)	105 (84.6)	18 (14.5)
HCC	45	43 (95.5)	42 (93.3)	38 (84.4)	30 (66.6)	43 (95.5)	2 (4.4)
Normal	87	1 (1.1)	0	0	0	0	86 (98.9)

Numbers in parentheses are percentages. CH, non-A, non-B chronic hepatitis; HCC, hepatocellular carcinoma without HBV markers.

Table III. Classification of Cases that Were Positive for One or Two Antibodies of HCV

Disease		Antibodies against proteins				Total number	Positive for HCV RNA[a)]
		Core	07	10	14		
CH	group 1	+	−	−	+	5	5/5
	group 2	+	+	−	−	12	12/12
	group 3	−	+	+	−	1	1/1
	group 4	+	−	−	−	1	0/1
HCC	group 2	+	+	−	−	2	2/2

a) Ratio of numbers of positive cases to total number of cases examined.

body is still unknown, but it seems important to analyze all these antibodies not only for screening purposes but also for diagnostic purposes.

In all, 105 of 124 (84.6%) serum samples from patients with NANBCH were positive for antibodies against more than two proteins (Table II). This percentage was similar to that of samples positive for antibody against only the protein core. However, one serum sample that was positive for antibody against only the protein core was negative for HCV RNA (see below), while another that was positive for HCV RNA was negative for the antibody against the protein core, but positive for antibodies against other proteins (groups 3 in Table III, and unpublished result). All serum samples that were positive for antibodies against two proteins, were also positive for HCV RNA (Table III). Eighteen (14.5%) and 2 (4.4%) cases of NANBCH and HCC, respectively, were negative for HCV antibodies against all these proteins.

Detection of HCV RNA in sera by RT-PCR Sera that were positive for antibodies against one or two proteins and sera that were negative for antibodies against all these proteins were analyzed for HCV RNA by RT-PCR.

RNA fractions from 200 μl of sera were reverse-transcribed and subjected to PCR using the primers described in "Materials and Methods." Then the reaction products were analyzed by agarose gel electrophoresis. The results showed an amplified DNA fragment of the expected size in reaction mixtures with RNA from sera that were positive for antibodies against one or two proteins, except those of sera in group 4 (Table III). The sera of 17 of 18 cases (one was not available for analysis) that were all negative for HCV antibodies were also negative for HCV-RNA (data not shown). Therefore, it is likely that these 17 cases of NANBCH might not have been caused by HCV infection. Conversely, there may be cases that showed no detectable marker of HCV infection in the circulating blood.

Other groups reported improved detection systems for HCV infection, using proteins expressed by similar regions of the HCV genome to that of the protein core and proteins 07 and 10.[13, 14] As shown here, it is important to screen for virus infection using each of these proteins to obtain accurate information of virus infection. For this purpose, protein 14 is essential. Diagnosis and follow-up studies of patients using these proteins may contribute to clarification of the physiological significance, if any, of HCV in patients.

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