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Research Article

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Genotype Dependence of Hepatitis C Virus Antibodies Detectable by the First-Generation Enzyme-linked Immunosorbent Assay with C100-3 Protein

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Abstract

Hepatitis C virus (HCV) samples in 155 sera, from patients with chronic non-A, non-B liver disease and blood donors, were grouped into four genotypes (I, II, III, and IV) by amplification of core-gene sequences by polymerase chain reaction with type-specific primers. HCV genotypes were compared with various HCV-associated antibodies detectable by the first-generation ELISA (ELISA-1) with C100-3 protein and a second-generation immunoblot assay with four recombinant HCV proteins. Antibodies to C100-3 protein and those to its subsequence (5-1-1) were detected in 13 (93%) and 12 (86%), respectively, of 14 sera with genotype I HCV; 56 (79%) and 58 (82%) of 71 sera with genotype II; 13 (34%) and 6 (16%) of 38 sera with genotype III; and 11 (34%) and 4 (13%) of 32 sera with genotype IV. Amino acid sequences of C100-3 of genotype I HCV are conserved by ~ 90% in genotype II, but only by ~ 75% in genotypes III and IV. The sensitivity of ELISA-1, therefore, would be influenced by heterogeneity in C100-3 sequences of different genotypes. (J. Clin. Invest. 1993. 92:1529–1533.) Key words: hepatitis C virus • genotype • hepatitis antibodies • enzyme-linked immunosorbent assay • polymerase chain reaction

Introduction

The discovery of hepatitis C virus (HCV)[1] by Choo et al. (1) is the major breakthrough in the research of non-A, non-B (NANB) hepatitis. HCV is a positive-stranded RNA virus of ~ 9,400 nucleotides with a 5'-noncoding region of ~ 341 nucleotides and a single open reading frame consisting of the core and envelope genes as well as nonstructural (NS) regions (2). The genomic sequence of HCV has been applied most practically to immunoassays for the detection of antibodies raised by hosts in response to HCV infection (3).

Serological diagnosis of HCV infection is a prerequisite to treatment and prevention of hepatitis C with a known preponderance for chronicity and serious sequelae ranging to hepatocellular carcinoma (4, 5). The first-generation ELISA (ELISA-1), using a recombinant fusion protein (C100-3) deduced from NS3 and NS4 regions (3), has been useful in detecting HCV infection in patients with acute and chronic NANB liver disease (3, 4) and in screening blood units for the prevention of post-transfusion NANB hepatitis (6–8).

The C100-3 protein used in ELISA-1 is deduced from the nucleotide sequence of the prototype HCV (HCV-1 [9]). During the past few years, the entire nucleotide sequence was determined on a number of HCV isolates from different regions of the world. They can be grouped, by similarity in nucleotide sequence, into four genotypes provisionally designated I, II, III, and IV (10, 11). The prototype HCV (HCV-1 [9]) belongs to genotype I.

HCV samples in 155 sera from patients with chronic NANB liver disease and blood donors were genotyped, and HCV-associated antibodies in them were tested for by ELISA-1 and a second-generation recombinant immunoblot assay (RIBA-2) involving four recombinant HCV proteins. Then, the sensitivity of antibodies to C100-3 and its subsequence (5-1-1), in detecting viremia, was correlated with each of four different genotypes of HCV.

Methods

Sera containing HCV RNA. Sera from patients with chronic NANB liver disease, hemophiliacs with liver function abnormalities, and blood donors were screened for antibody to a synthetic HCV core peptide (anti-CP10) by ELISA (12), and those with A_{492} values $\geq$ 1.00 were tested for HCV RNA by polymerase chain reaction (PCR). There were 155 sera with HCV RNA, including 41 from patients with chronic NANB hepatitis, 26 from patients with primary hepatocellular carcinoma, 10 from hemophiliacs, and 78 from donors of whom 18 were with alanine aminotransferase (ALT) levels > 45 IU/liter.

HCV-associated antibodies. Antibodies to C100-3 protein were determined by ELISA-1 (Ortho Diagnostic Systems, Tokyo, Japan), and sera with A_{492} values > 0.450 were considered reactive. Antibodies to four recombinant HCV proteins were determined by RIBA-2 (Ortho) with C100-3 deduced from NS3 and NS4 regions corresponding to amino acid (aa) 1569–1931, 5-1-1 representing its subsequence (aa 1694–1735), C33c (NS3 region, aa 1192–1457), and C22-3 (the putative core gene, aa 2–120); they all were deduced from the nucleotide sequence of the prototype HCV of genotype I [HCV-1 (9)].

Anti-CP10 was determined as previously described (12). Briefly, CP10 (19-mer) with a sequence of Pro-Lys-Pro-Gln-Arg-Lys-Thr-Lys-Arg-Asn-Thr-Asn-Arg-Arg-Pro-Gln-Asp-Val-Lys (aa 5–23 of the putative core protein) was synthesized chemically, which was deduced from HC-J1 (genotype I) and HC-J4 (genotype II) isolates (13). Wells of an immunoplate were coated with CP10 (250 ng/ml in 10 mM phosphate

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1. *Abbreviations used in this paper:* aa, amino acid; ALT, alanine aminotransferase; HCV, hepatitis C virus; NANB, non-A, non-B; NS, nonstructural; PCR, polymerase chain reaction.

buffer, pH 7.5), and overcoated with buffer supplemented with 40% (vol/vol) bovine serum. Wells received 10 μl of test serum and 50 μl of specimen diluent [saline supplemented with 40% (vol/vol) bovine serum and 0.05% (vol/vol) Tween 20], and the plate was incubated at room temperature for 1 h. Bound antibodies were detected with mouse monoclonal antibody against human IgG/γ labeled with horseradish peroxidase. Sera with A_{492} values > 0.30, which were inhibited by > 50% in the presence of CP10 (50 μg/ml), were considered reactive.

Detection of HCV RNA and genotyping. RNA was extracted from 1 ml of serum, reverse transcribed, and amplified by a two-stage PCR with nested primers deduced from the 5'-noncoding region of the HCV genome (14). Antisense primer in the original method (no. 36), for cDNA synthesis and the first stage of PCR, was replaced by no. 299 with a sequence of 5'-ACCCAACACTACTCGGCTAG-3' representing nucleotides 250–269 in the 5'-noncoding region (13). The modified PCR amplified a product of 225 bp with a sensitivity comparable with that of the original method.

Four genotypes of HCV, designated I, II, III, and IV (10, 11), were determined by amplification of HCV RNA with a universal primer (sense) and the mixture of four type-specific primers that were deduced from comparison of the putative core-gene sequences of 44 HCV isolates (10). The primer specific for genotype I (no. 132) was replaced by no. 296 with a sequence of 5'-GGATAGGCTGACGTCTACCT-3' representing nucleotides 518–539 of the putative core gene. The size of PCR product with primer no. 296 was 49 bp, compared with 57 bp in the original method. Products of PCR amplification were run on electrophoresis, stained with ethidium bromide, and observed under ultraviolet light. Genotypes were judged by the size of products: 49 bp for genotype I, 144 bp for genotype II, 174 bp for genotype III, and 123 bp for genotype IV.

Statistical analysis. Frequencies were compared using Fisher's exact probability test or the χ^2 test.

Results

Table I compares the frequency of various HCV-associated antibodies, detectable by ELISA-1 or RIBA-2, in 155 sera with HCV of four different genotypes. Antibodies to C100-3 protein were detected by ELISA-1 or RIBA-2 in 13 (93%) of 14 sera with genotype I HCV and in 56 (79%) of 71 with genotype II, more frequently than in 13 (34%) of 38 with genotype III or in 11 (34%) of 32 with genotype IV ($P < 0.001$). Similarly, antibodies to 5-1-1 protein, representing a subsequence of C100-3, were detected in 12 (86%) sera with genotype I HCV and 58 (82%) with genotype II, more often than in 6 (16%) with genotype III and 4 (13%) with genotype IV ($P < 0.001$).

By contrast, antibodies to C33c or C22-3 protein were detected in all 155 sera with HCV RNA, irrespective of genotypes. These sera had been selected by anti–CP10 ELISA with a

synthetic core peptide (12), and they all were positive with A_{492} values ≥ 1.00.

Table II compares the detection of antibodies to C100-3 and 5-1-1 in blood donors with normal or elevated transaminase levels and in patients with chronic NANB liver disease, all of whom were infected with HCV of genotypes other than I. They were divided into two groups, one with HCV of genotype II and the other with HCV of genotype III or IV. Antibodies to C100-3 and 5-1-1 were detected less frequently in blood donors with normal ALT levels than in donors with elevated ALT ($P < 0.01$) or in patients ($P < 0.001$), all of whom were infected with HCV of genotype II. Similarly, the detection of these antibodies was influenced by the clinical status of hosts infected with HCV of genotype III or IV; antibody to C100-3 was detected most hardly in donors with normal ALT levels.

Four representative HCV isolates of different genotypes were compared within amino acid sequences of the four antigen probes used in ELISA-1 and RIBA-2 (Table III). The sequence of C100-3 protein, deduced from genotype I HCV (HCV-1 [9]), is conserved by almost 90% in genotype II (HCV-BK [15]), but only by ~ 75% in genotype III (HC-J6 [16]) or IV (HC-J8 [11]). Variations dependent on genotypes are much greater in the sequence of 5-1-1; it is conserved by 83% in genotype II HCV, but only by ~ 50% in genotype III or IV.

In Fig. 1, the maximal number of discordance within every 10 amino acids, among HCV isolates of four different genotypes, is plotted along the sequence of C100-3 protein. Considerable variations dependent on genotypes are scattered over the C100-3 sequence, and 5-1-1 protein falls on an area of the highest variation.

The sequence of C33c is well conserved among HCV isolates of four different genotypes, with a similarity of > 83% (Table III). The sequence of C22-3 is the most preserved of the four antigen probes, with a similarity of > 89% among them. The sequence of CP10 is highly conserved also, 17 (89%) of 19 amino acids being identical in HCV isolates of four different genotypes (9, 11, 14, 15).

Discussion

Although ELISA-1 with the recombinant C100-3 protein has established a undisputable impact on the diagnosis and prevention of HCV infection, it is not without flaws. ELISA-1 is not very sensitive, missing viremia in some blood units that could transmit HCV infection to recipients (6–8) or in sera in which HCV RNA was detected by PCR (17). ELISA-1 is not specific,

Table I. HCV-associated Antibodies Detectable by ELISA-1 or RIBA-2 in Sera with HCV of Four Different Genotypes

			Detection by RIBA-2 of antibodies to[†]			
Genotype of HCV	n	ELISA-1* (anti–C100-3)	C100-3[†]	5-1-1	C33c	C22-3
I	14	13 (93%)	13 (93%)	12 (86%)	14 (100%)	14 (100%)
II	71	56 (79%)	55 (77%)	58 (82%)	71 (100%)	71 (100%)
III	38	13 (34%)	13 (34%)	6 (16%)	38 (100%)	38 (100%)
IV	32	11 (34%)	9 (28%)	4 (13%)	32 (100%)	32 (100%)

* Antibodies to C100-3 protein, deduced from the prototype HCV genome (HCV-1 [9]) of genotype I, was determined by the first-generation enzyme immunoassay (Ortho). [†] Antibodies to four HCV proteins, deduced from various regions of the prototype HCV genome (HCV-1 [9]) of genotype I, were detected by a second-generation recombinant immunoblot assay (Ortho).

Table II. Detection of Antibodies to C100-3 and 5-1-1 in Blood Donors and Patients with HCV of Genotype II, III, or IV*

Infection with HCV of:	Origin of sera	n	ELISA-1 (anti–C100-3)	RIBA-2	
				C100-3	5-1-1
Genotype II	Donors (ALT ≤45)	30	16 (53%)	15 (50%)	19 (63%)
	Donors (ALT >45)	12	12 (100%)	12 (90%)	11 (92%)
	Patients	29	28 (97%)	28 (97%)	29 (100%)
Genotype III or IV	Donors (ALT ≤45)	26	3 (12%)	3 (12%)	3 (12%)
	Donors (ALT >45)	6	2 (33%)	2 (33%)	0 (0%)
	Patients	38	19 (50%)	17 (45%)	7 (18%)

* Detectability of anti-C100-3 by ELISA-1 and RIBA-2, as well as antibody to 5-1-1 by RIBA-2, was compared in blood donors and patients with chronic NANB liver disease who were infected with HCV of genotypes other than I. Donors were divided into two groups with normal (≤45 IU/liter) or elevated ALT levels (>45 IU/liter).

either, testing falsely positive in some sera with high levels of IgG, typically in those from patients with autoimmune chronic active hepatitis (18). Antibodies to superoxide dismutase, making a part of the antigen probe, were implicated in false positivity in some sera (19).

As a confirmatory test for ELISA-1, the first-generation recombinant immunoblot assay (RIBA-1) has been introduced (20), which involves the binding of immunoglobulins to C100-3 protein and its subsequence (5-1-1), as well as superoxide dismutase, fixed on nitrocellulose strips. Reactivity with both C100-3 and 5-1-1 is interpreted as confirmatory for ELISA-1 and that with superoxide dismutase alone as false positive.

Both ELISA-1 and RIBA-1 use C100-3, as well as its subsequence (5-1-1), which is deduced from the prototype HCV [HCV-1 (9)]. Being an RNA virus that is evolving rapidly (21), HCV variants are expected to occur that would have a good deal of sequence divergence. This proved to be the case for HCV isolates, which have been sequenced partially or along the entire genome. To date, the entire nucleotide sequence is determined for HCV isolates of four genotypes, which differ from each other by > 21% in the entire nucleotide sequence, and they are provisionally designated genotypes I, II, III, and IV (10, 11). On the basis of sequence similarities, genotypes I and II are closely related, as are genotypes III and IV (11).

Table III. Comparison of Amino Acid Sequences of Antigen Probes Used in ELISA-1 and RIBA-2 among HCV Isolates of Different Genotypes*

Antigen probes	Amino acids in comparison n (position†)	Numbers of amino acids of an HCV isolate of genotype I shared by HCV isolates of		
		Genotype II	Genotype III	Genotype IV
C100-3	363 (1569–1931)	324 (89.3%)	278 (76.6%)	271 (74.7%)
5-1-1	42 (1694–1735)	35 (83.3%)	22 (52.4%)	21 (50.0%)
C33c	266 (1192–1457)	245 (92.1%)	223 (83.8%)	224 (84.2%)
C22-3	119 (2–120)	116 (97.5%)	106 (89.1%)	108 (90.8%)

* Amino acid sequences of antigen probes used in ELISA-1 and RIBA-2, deduced from the nucleotide sequence of an HCV isolate of genotype I (HCV-1 [9], the prototype HCV) are compared with those deduced from HCV isolates of genotype II (HCV-BK [15]), III (HC-J6 [16]), or IV (HC-J8 [11]). † Amino acid positions are numbered in accordance with those of the prototype HCV genome (HCV-1 [9]).

Genotype-dependent variations are not evenly distributed over the entire genome. The 5'-noncoding region of ~ 341 nucleotides is best conserved with a similarity of > 92% among HCV isolates of different genotypes, and therefore, would be ideal for use as primers in PCR for a sensitive detection of HCV RNA (14, 22, 23). Variations in nucleotide and deduced amino acid sequences are much greater within the coding region of HCV, which is divided into core and envelope genes and five nonstructural regions (NS1–NS5).

The sequence of C100-3, coded for by NS3 and NS4 regions, is very variable among HCV isolates of four genotypes, and 5-1-1 falls on an area of extensive variation within it (Fig. 1). Genotype-dependent sequence diversities were faithfully reflected on the sensitivity of ELISA-1 and RIBA-2 in detecting antibodies to C100-3 and 5-1-1 (Table II). Reasonably, these assays were most sensitive in detecting antibodies raised in the infection with HCV of genotype I. The sensitivity was somewhat lower for antibodies in the infection with genotype II HCV, but reasonably high at 80%; it was much lower in the infection with genotype III or IV at 34% for antibodies to C100-3 and 13–16% for antibodies to 5-1-1. Genotype dependence of antibodies to C100-3 and 5-1-1 has been pointed out by others also (2, 24), and they would be responsible for a limited performance of ELISA-1 in detecting viremia.

Among those infected with HCV of genotypes other than I, antibodies to C100-3 and 5-1-1 were detected in blood donors with normal transaminase levels less sensitively than in donors with elevated transaminase levels or in patients with chronic NANB liver disease. Detectability of these antibodies would depend on the intensity of immune responses to HCV antigens, which might be involved in the pathogenesis of NANB hepatitis.

The efficacy of ELISA-1 in decreasing the incidence of posttransfusion hepatitis C varies widely depending on countries where it was evaluated. In Spain (6), the efficacy is 56%, much lower than 84% in the United States (7). The efficacy in Japan is in between at 73% (8). HCV of genotype I prevails in the United States (2, 11), where the prototype HCV has been propagated (9), while genotype II is most common in the Far East, being determined in 131 (82%) of 159 blood donors with viremia in Japan (10). These observations are in agreement with good performance of ELISA-1 in these two countries. A poor performance in Spain may indicate that HCV of genotypes other than I or II would be more common there than in the United States and Japan.
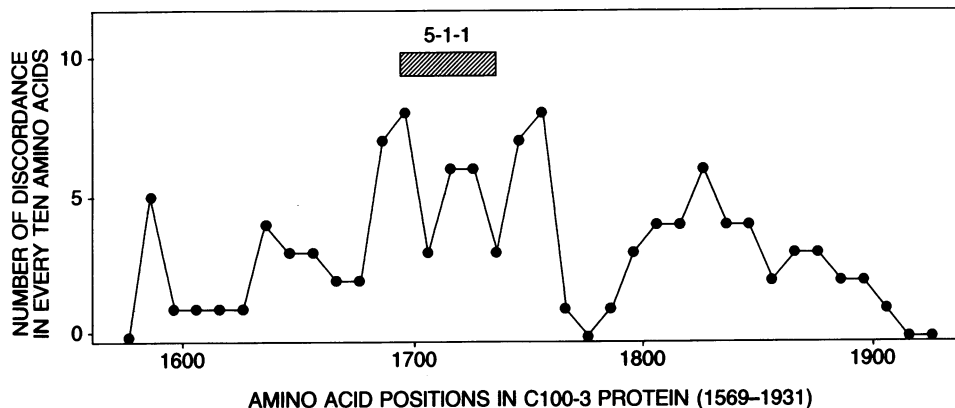
Figure 1. Variations in the amino acid sequence of C100-3 protein among four HCV isolates of different genotypes. The sequence of C100-3 protein spanning aa 1569–1931 was divided into 36 sections, each with 10 aa (the last 3 aa ignored). The number of maximal discordance among four HCV isolates of different genotypes, within every 10 aa, is plotted. HCV isolates in comparison are HCV-1 of genotype I (9), HCV-BK of genotype II (15), HC-J6 of genotype III (16), and HC-J8 of genotype IV (11). The position of 5-1-1 protein (1694–1735) is indicated by the shaded bar.

Recently, RIBA-2 was introduced, which uses, in addition to C100-3 and 5-1-1, C33c protein encoded by the NS3 region and C22-3 protein by the putative core gene (25). Superb sensitivity and specificity of RIBA-2 were extended to ELISA-2, which has gained an instantaneous popularity in the diagnosis of HCV infection in clinical and epidemiological settings.

Among HCV isolates of four different genotypes, the amino acid sequences of C33c and C22-3 are more conserved than that of C100-3 or 5-1-1 (Table III). C33c and C22-3 blots in RIBA-2 are reported to be more sensitive than C100-3 or 5-1-1 blot in identifying sera contaminated with HCV (26). Marvelously, antibodies to C33c and C22-3 were detected in all 155 sera with viremia in the present study. Hence, an advantage is obvious for employing antigen probes whose sequences are conserved by HCV of any genotypes. In actuality, ELISA-2s involving conserved sequences have expanded the power of diagnosing HCV infection; 90–98% of patients with chronic NANB liver disease possess antibodies detectable by ELISAs with the HCV core protein or its sequences (27).

Even ELISA-2 is not flawless in the detection of hepatitis C viremia (28), however. There still remains a residual risk of developing posttransfusion hepatitis C in recipients of blood units screened by ELISA-2 (28, 29). A third-generation ELISA may yet be developed, with additional conserved sequences bearing immunodominant epitopes, which will be delineated by comparison of many HCV isolates with different genotypes from all over the world. It would have to cope with infections with HCV of still other genotypes. Recently, HCV isolates are reported from Edinburgh (30) and Thailand (31), partial sequences of which differ substantially from those of genotypes I–IV, and they could be classified as genotypes V and VI, respectively.

Unfortunately, protective immunity is weak or lacking in chimpanzees with experimental HCV infection, allowing reinfection to occur even by the same HCV inoculum used in the initial transmission (32, 33). There may still be a long way before prophylactic measures are taken by hepatitis C vaccines. In the interim, we may work towards further improvements in ELISA for infallible detection of hepatitis C viremia.

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