PREDOMINANCE OF ANTIBODIES TO HEPATITIS C VIRUS ENVELOPE PROTEINS IN VARIOUS DISEASE STATUSES OF HEPATITIS C

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Received February 23, 2000; accepted January 18, 2001

Summary. – The antibody profile to various proteins of hepatitis C virus (HCV) was studied in 113 patients positive for HCV RNA in various disease statuses of hepatitis C (HC). A single peptide (E2/NS1, aa 413–436 of HCV polyprotein) chosen from a conserved region at the C-terminus of the hypervariable region (HVR) HVR1 of HCV was found to be sufficient for reliable diagnosis of the infection, even in the acute phase. Six hundred and one suspected HC cases and 200 voluntary blood donors were tested by this peptide. The sensitivity of detection of HCV antibodies by this peptide did not increase with addition of peptides from other HCV proteins. Our results clearly demonstrate that antibodies to HCV envelope proteins occur in a higher percentage of the infected population than those to other proteins. This emphasizes the necessity of using representative sequences from HCV envelope proteins in diagnostic immunoassays of this viral infection.

Key words: hepatitis C virus; epitope masking; envelope proteins; predominant antibodies; multi-peptide analysis

Introduction

Although a decade has elapsed from the discovery of HCV as a causative agent of parenterally transmitted non-A, non-B hepatitis (Choo *et al.*, 1989) to its establishment as a heterogenic group (Bukh *et al.*, 1995; Simmonds, 1995; Clarke 1997), a confirmatory and reliable diagnostic procedure for HC is still lacking. This in part could be due to the lack of an adequate *in vitro* system for propagation of the virus. In addition, studies on the host immune response to HCV infection are hampered by the lack of an alternative animal model other than chimpanzee, restricting such studies to a few laboratories.

It has been well established clinically that infection with HCV can present both short-term disease (acute self-limiting hepatitis) (Alter 1989; Choo *et al.*, 1989; Zibert *et al.*, 1997) as well as long-term disease, namely chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (Bukh, 1995; Clarke, 1997) apart from other clinical manifestations (cryoglobulinaemia, oral carcinoma, lichen planus, etc.) (Johnson *et al.*, 1993; Nagao *et al.*, 1995; Hadziyannis, 1997) involving organs other than liver. Owing to difficulties in HCV antigen capture immunoassays, detection of HC is mostly dependent on reverse transcription–PCR (RT-PCR) procedures to detect HCV RNA and immunoassays for HCV antibodies. Presently a third-generation immunoassay is available which uses synthetic and recombinant peptides

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Abbreviations: aa = amino acid; BSA = bovine serum albumin; EIA = enzyme immunoassay; HAV = hepatitis A virus; HBV = hepatitis B virus; HC = hepatitis C; HCC = hepatocellular carcinoma; HCV = hepatitis C virus; HEV = hepatitis E virus; HVR = hypervariable region; PBEIA = peptide-based enzyme immunoassay; RT-PCR = reverse transcription–PCR; RIBA = recombinant immunoblot assay

corresponding to the core protein C (c22-3 antigen; aa 2–120), non-structural proteins NS3 to NS4 (c200 antigen; aa 1192–1931) and NS5 (aa 2054–2955) representing approximately 60% of amino acid regions of HCV (Aoki *et al.*, 1996). However, a drawback of this assay is that it does not detect HC during acute phase, early HC and latent HC (Aoki *et al.*, 1996). This could be due to HCV antibody responses mostly directed against the outermost structural proteins of the virus, namely envelope proteins, the core (nucleocapsid) protein C, and non-structural proteins.

To study the prevalence of antibodies to each of the HCV proteins, namely envelope, nucleocapsid and non-structural ones, we used a set of eight peptides (Khanna *et al.*, 1998) representing the abovementioned proteins. The presence of HCV RNA in serum is now well established as a seromarker of active HCV replication. The present analysis was carried out on patients positive for HCV RNA by RT-PCR. Based on this analysis we also report here that a single peptide (E2/NS1) selected from a conserved region at the C-terminus of the HVR (HVR1) of E2/NS1 protein of HCV is sufficient for reliable diagnosis of HC, even in the acute phase. Moreover, to check whether the sensitivity of detection of HCV antibodies using peptides could be increased, assays were carried out using E2/NS1 in combination with peptides derived from other HCV proteins.

Materials and Methods

Sera from patients with liver disorders, negative for the markers of hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis E virus (HEV) infection, referred to the Center for Liver Diseases, Hyderabad, the Rajiv Gandhi Center for Biotechnology, Trivandrum, and the Medical College Hospital, Trivandrum, were chosen for the present study. Sera from voluntary blood donors visiting the Sree Chitra Blood Bank, Trivandrum, served as controls.

HCV RNA and HCV antibodies determinations HCV RNA was detected by RT-PCR (Das *et al.*, 1993). HCV antibodies were determined by a commercial enzyme immunoassay (EIA) (Organon, United Biomedical Inc.) according to the manufacturer's protocol.

Peptides (Table 1), described in our earlier publication (Khanna *et al.*, 1998), were synthesized using the conventional F-moc chemistry. Purity of the peptides was confirmed by reverse phase HPLC on a Sephasil-C-18 column (Pharmacia).

Peptide based enzyme immunoassay (PBEIA) was carried out essentially as described earlier (Khanna *et al*, 1998). Polystyrene plates (Maxisorp 96F, Nunc) were coated with the E2/NS1 peptide or a peptide mixture (1000 ng/50 µl/well) in PBS in a standard way. To prepare peptide mixtures, the peptides were mixed with the E2/NS1 peptide in equimolar concentrations prior to coating the plates.The coated plates were blocked with 350 µl of 3% (w/v) bovine serum albumin (BSA) in PBS containing 0.1% (v/v) Tween-20, and incubated at 37°C for 2 hrs. The tested sera, diluted

Table 1. Characteristics of the peptides tested

Peptide	Amino acid region	M_r	PI	Charge at pH 7*
Peptides from envelope proteins				
PEP3	222–248	2854	8 01	+0 95
PEP4	440–464	2729	8.29	+1.0
E2/NS1	413-436	2629	7.16	+0.04
Peptide from core protein				
Core	5–27	2706	12.51	+6.99
Peptides from non-structural proteins				
NS2	857–880	2516	8.76	+1 04
NS3	1388–1410	2512	9.65	+3.08
NS4	1697–1719	2788	3.75	–5.96
NS5	2253–2276	2658	3 46	–7.0

*Calculated using the PHYCHEM Program of the PCGENE Software Package

1:20 in the blocking solution, were added (50 µl/well) and the plates were incubated at 37°C for 90 mins. Subsequently, anti-human goat IgG (γ-chain-specific), conjugated to horseradish peroxidase (Sigma) was added to the wells (50 µl/well) at recommended dilution in the blocking solution, and the plates were incubated at 37°C for 45 mins. H_2O_2 and tetramethylbenzidine (Bangalore Genei, India) were added (50 µl/well) and the color development was allowed to take place in dark for 15–20 mins. The reaction was stopped with 2N H_2SO_4 (50 µl/well) and the plates were read at 450 nm and 630 nm in a standard way. The cut-off value was set as the mean of five to ten negative control readings (taken in each assay) plus the threefold of standard deviation. Sera giving absorbancy values higher or equal to the cut-off value were considered positive.

Results

Comparison of PBEIA and commercial EIA and evaluation of the E2/NS1 peptide

Table 2 shows detection of HCV-specific antibodies in 113 HCV RNA-positive sera (divided into four groups according to the disease status) by PBEIA using different peptides and by a commercial EIA. It is evident that the E2/NS1 peptide scores over the other peptides.

Next, based on this result, we tested 601 sera from patients suspected for HCV infection and 200 sera from voluntary blood donors by PBEIA with the E2/NS1 peptide and by commercial EIA for HCV antibodies (Table 3). The obtained results show that the PBEIA with E2/NS1 scored over the commercial EIA. It should be noted that ten sera from voluntary blood donors were positive with the E2/NS1 peptide. Five of these sera were positive for HCV RNA by

Table 2. Number and percentage of HCV antibody-positive sera assayed by PBEIA with various peptides and by commercial EIA

Disease status	PBEIA with peptides								Commercial EIA
	PEP3	PEP4	E2/NS1	NS2	NS3	NS4	NS5	Core	
AH (n=28)	15 (53.57)	23 (82.14)	24 (85.71)	23 (82.14)	17 (60 71)	19 (67.85)	08 (28.57)	08 (28.57)	04 (14.28)
CH (n=21)	16 (76.20)	14 (66.67)	15 (71.43)	14 (66.67)	12 (57.14)	11 (52.38)	13 (61.90)	14 (66.67)	09 (42.85)
CIR (n=58)	48 (82.75)	43 (74.13)	47 (81.00)	42 (72.41)	40 (68.96)	46 (79.31)	48 (82.75)	45 (77.58)	30 (51.72)
HCC (n=6)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	3 (50)
Total (n=113)	85 (75.22)	86 (76.11)	92 (81.42)	85 (75 22)	75 (66.37)	82 (72.56)	75 (66.37)	73 (64.60)	46 (40.70)

Percentage of the positivity is shown in parentheses. All the 113 samples were positive for HCV-RNA.
AH = acute hepatitis; CH = chronic hepatitis; CIR = cirrhosis; HCC = hepatocellular carcinoma.

Table 3. Number of HCV antibody-positive sera in suspected HC cases assayed by PBEIA with E2/NS1 peptide and by commercial EIA

	Commercial EIA	PBEIA with E2/NS1 peptide
Suspected HC cases (n=601)		
HCV RNA-positive (n=275)	163	205
HCV RNA-negative (n=326)	34	59
Voluntary blood donors (n=200)		
HCV RNA-positive (n=5)	1	5
HCV RNA-negative (n=195)	0	5
Total tested (n=801)	198	274

RT-PCR. However, only one of these sera was positive by the commercial EIA. The distribution of sample index (signal/cut-off ratio) values of non-reactive sera (Fig. 1) and reactive sera (Fig. 2) showed a clear separation of these two groups of sera, a characteristic feature of PBEIAs using synthetic peptides.

Epitope masking

In a subset of 50 sera (Table 4), peptides were mixed in equimolar concentrations with the E2/NS1 peptide prior to coating the plates. The aim was to check whether the sensitivity of detection of HCV antibodies using the E2/NS1 peptide could be increased. It is evident from these

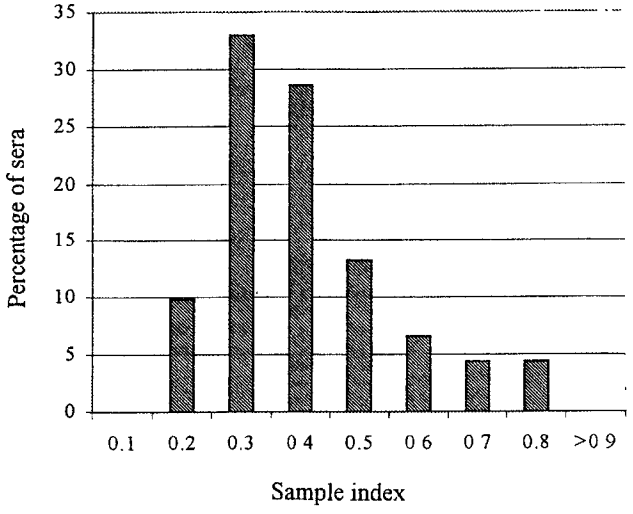

Fig. 1
Distribution of sample index values of a representative set of sera negative in PBEIA with the E2/NS1 peptide

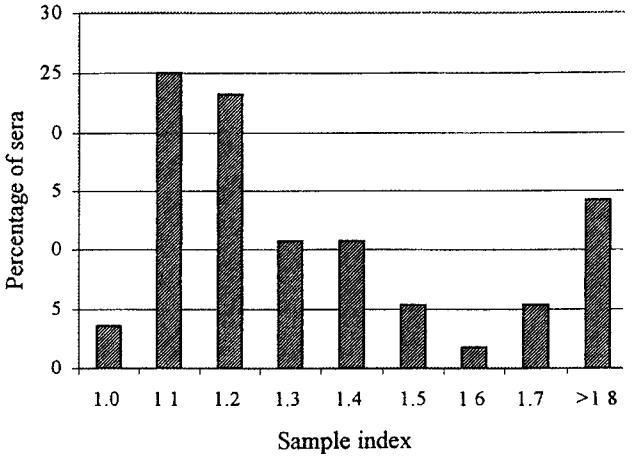

Fig. 2
Distribution of sample index values of a representative set of sera positive in PBEIA with the E2/NS1 peptide

Table 4. Number of HCV antibody-positive sera assayed by PBEIA with the E2/NS1 peptide alone and in combination with other peptides

E2/NS1 alone	Combination		
	E2/NS1+core peptide	E2/NS1+NS2 peptide	E2/NS1+NS3 peptide
50	32	31	27

Total number of sera was 50. All the sera tested were positive for HCV RNA and HCV antibodies assayed by commercial EIA and by PBEIA with the E2/NS1 peptide alone.

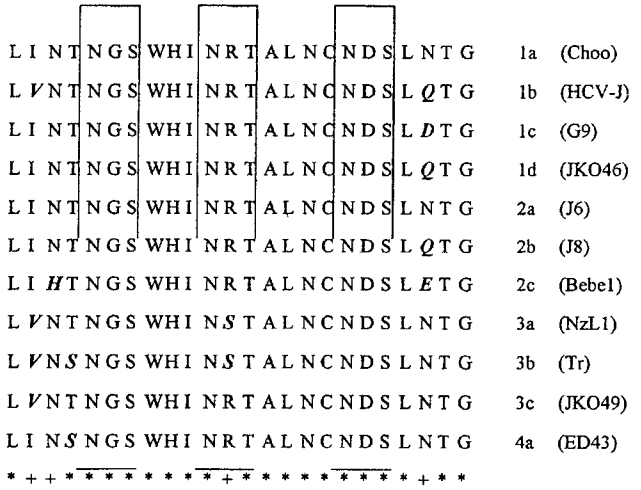

Fig. 3
Comparison of amino acid sequences of the E2/NS1 peptide (aa 413–436) in various HCV genotypes and isolates
Changes concerning the in-house peptide sequence are indicated in bold italic. Boxes indicate potential glycosylation sites. (*) = well conserved, (+) = relatively conserved. Genotypes are given from 1a to 4a Isolates are indicated in parentheses.

Table 5. Prevalence of antibodies to major proteins of HCV

Disease status	Envelope proteins (group I)	Core protein (group II)	Non-structural proteins (group III)
AH (n=28)	23 (82.14%)	8 (28.57%)	14 (50%)
CH (n=21)	16 (76.2%)	14 (66.67%)	9 (42.85%)
CIR (n=58)	49 (84.5%)	45 (77 58%)	45 (77.58%)
HCC (n=6)	6 (100%)	6 (100%)	6 (100%)
Total (n=113)	94 (83.18%)	73 (64 60%)	74 (65 48%)

Data for this table were generated by grouping individual data from Table 2. Differences in the percentage of prevalence were caused by the fact that the sera missed out by one peptide were detected by other peptides in that group. All the sera were positive for HCV RNA.

data that the number of positives decreased drastically when the peptides were used in combination as compared to the number of positives detected with individual peptides. A possible explanation for this could be peptide-peptide interactions, owing to differences in their isoelectric points (Table 1), resulting in masking linear epitopes. Conformational studies on these peptides will be helpful for a better understanding of these interactions.

Predominance analysis

As the peptides used in the present study represent all the predicted or identified proteins of HCV, grouping the individual peptide data (Table 2) would give a better insight into the antibody response to each category of proteins, namely envelope, core/capsid and non-structural proteins. Consequently, the individual peptide data have been grouped as the envelope protein group (group I) comprising peptides from envelope proteins, the core/capsid protein group (group II) consisting of the peptide from the core/capsid proteins, and the non-structural protein group consisting of the peptides from the non-structural proteins (group III). A patient was defined as having antibodies to the envelope proteins if he scored positive for at least two peptides from the envelope proteins. Similarly, a patient was defined as having antibodies to the non-structural proteins if he scored positive for at least three peptides from the non-structural proteins. These data were used to generate Table 5. It is evident from the data that antibodies to envelope proteins occur in a higher percentage of the infected population than the antibodies to other proteins, and the persistence of HCV infection appears to evoke antibody response in equal amounts to almost all the viral proteins. These observations confirm our hypothesis that in an HCV infection antibody responses might be directed mostly against the outermost structural proteins of the virus, namely the envelope proteins, and only at a later stage to the other proteins.

Discussion

In this study, we showed that the antibodies to the envelope proteins, in general, and to the E2/NS1, in particular, predominate over the antibodies to other proteins in various disease statuses of HCV infection. We also demonstrated that a single peptide alone is sufficient for effective and reliable diagnosis of HCV infection, not only in infected symptomatic individuals but also in apparently healthy individuals.

Earlier studies of other workers have shown that antibody response to HVR1 is more rapid in patients with acute self-limiting hepatitis or resolving infection than in patients with chronic hepatitis (Van Doorn *et al.*, 1995; Allander *et al.*,

1997; Zibert *et al.*, 1997). More recently it has been demonstrated that antibodies in this category of patients are directed against HCV envelope proteins outside of HVR1 (Lechnar *et al.*, 1998). The peptides used by these authors correspond to the peptides Pep3 and Pep4 used by us in the present study.

The sequence of the E2/NS1 peptide used by us corresponds, in part, to the 43 amino acids long peptide used in a study carried out by Allander *et al.* (1997), who have shown that this peptide can recognize HCV infection regardless of genotype. The region of concern corresponds to the C-terminus of HVR1; it consists of three potential glycosylation sites and is probably heavily glycosylated *in vivo*. Inference gathered from other similar studies (Van Doorn *et al.*, 1995; Allander *et al.*, 1997; Lechnar *et al.*, 1998) and from our present study indicates that the viral receptor for entry might be present in this region. This may explain conservation of this amino acid stretch in various HCV isolates.

Our results also show that there is poor antibody response to the peptide from the HCV core region in acute hepatitis and that the prevalence of antibodies to this peptide increases in persistent infection. This observation is consistent with that of Zhang *et al.* (1995). Two earlier studies (Van der Poel *et al.*, 1991; Katayama *et al.*, 1992) have suggested that the serum titers of antibodies to HCV core proteins in patients with chronic hepatitis and HCC are higher than those to any other HCV gene products. However, these studies were carried out using a four-antigen RIBA (recombinant immunoblot assay) that did not include HCV envelope protein antigens. Our study demonstrates that the prevalence of antibodies to the envelope proteins occurs in higher percentage than that to other proteins in various disease statuses, particularly in acute hepatitis. In addition, Prince *et al.* (1996), have demonstrated that predicted epitopes on E1 and E2 proteins are expressed intact on the surface of the HCV virion.

HCV envelope proteins expressed in recombinant baculovirus and vaccinia virus have been successfully used for the detection of HCV envelope-specific antibodies in 90% of patients with various disease statuses of HCV infection (Inoue *et al.*, 1992; Chien *et al.*, 1993; Lee *et al.*, 1997). In the present study, we report a similar finding using a single peptide (81%), as well as using a mixture of peptides from E1 and E2/NS1 proteins (83.42%). This concurrence of data clearly demonstrates that a single peptide is sufficient for a reliable diagnosis of HCV infection and addition of peptides from other regions causes only a marginal change in the sensitivity of the assay used, a conclusion already made in our earlier publication (Khanna *et al.*, 1998). These findings indicate necessity to employ antigens from HCV envelope proteins in such studies as well as in diagnostics. It has been documented that the E2/NS1 protein sequence is highly variable (Higashi *et al.*, 1993; Zonaro *et al.*, 1994), restricting its usage in diagnostic tests. Despite this, sequencing of various isolates revealed that some regions on the E2/NS1 protein are relatively conserved (data not shown) and can be successfully exploited for diagnostic purpose. One such region is the E2/NS1 peptide used in the present study (Fig. 3).

In the present study, sera from 64 patients were positive with the E2/NS1 peptide but were negative for HCV RNA. A similar situation has been reported recently by Haydon *et al.* (1998), the virus sequestered in the liver was suggestive of latent infection. Nevertheless, the possibility of post infection cases with resolved disease and false positivity, in the absence of reliable confirmatory assays, cannot be excluded.

The 6 HCC cases tested for HCV antibodies in the present study were positive with all the 8 peptides used, confirming that HCC in these patients was caused by HCV. This also suggests that antibodies to all the proteins of HCV in HCC patients can be readily and effectively detected. In addition, as the chronicity of the infection increases, antibodies to all the viral proteins appear in increasing amounts.

The poor performance of the commercial kit used in this study might be due to genotypic variation of the virus in different regions. Genotype 3b seems to be predominant in the geographic regions involved in the present report (data not shown), while genotype 1 is predominant in the USA, country of origin of the commercial kit used in the present study (Bukh *et al.*, 1995; Simmonds, 1995). It should be mentioned at this point that one of the major recommendations of the Pacific Rim Conference held at Bali, Indonesia, in 1998 (M.R. Das, personal communication) was the necessity to develop region-specific diagnostic kits, therapeutic agents and vaccines taking into account genotypic variation of HCV. The commercial kits currently available, as stated earlier, include regions from the HCV core, NS3 and NS4 proteins. The core region and NS4 protein have been and are still used for HCV genotyping in serum (Simmonds *et al.*, 1993; Bhattacherjee *et al.*, 1995), restricting their use in diagnostic immunoassay of HCV. However, as stated earlier, the E2/NS1 peptide used in the present study is from a relatively well conserved region and is capable of detecting various HCV genotypes. Accordingly, the PBEIA based on the E2/NS1 peptide should improve detection of HCV infection in this region.

In conclusion, we demonstrated that antibodies to HCV envelope protein antigens predominate in HCV infection and that these antigens are important for accurate diagnosis of HCV. There is an obvious requirement for use of representative sequences from HCV envelope proteins in tests for a reliable diagnostics of HCV.

Acknowledgements. We thank Drs. M. Renil and T.J. Rasool for their critical comments during preparation of the manuscript. This study was supported by grants from DBT.

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