ANALYSIS OF HUMAN IMMUNE RESPONSE TO POTENTIAL HEPATITIS C VIRAL EPITOPES

A. KHANNA[1], C.D. PODURI[1], P. MURUGAN[1], S. KUMAR[1], V.S. SUGUNAN[1], K.T. SHENOY[2], M.R. DAS[1]*

[1]Rajiv Gandhi Centre for Biotechnology, Jagathy, Trivandrum 695 014, Kerala; [2]Department of Gasteroenterology, Medical College Hospital, Trivandrum 695 011, Kerala, India

Received March 17, 1998; accepted May 19, 1998

Summary. – A peptide-based enzyme immunoassay (PBEIA) has been developed using synthetic peptides whose sequences were selected from the core, envelope and non-structural regions of the prototype hepatitis C virus (HCV) genome. Results of PBEIA of sera obtained from several patients with various liver disorders were compared to those of commercial enzyme-linked immunosorbent assays (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). A large number of samples, which were repeatedly negative in commercial ELISA, were positive in PBEIA. There was a good correlation between the results of PBEIA and RT-PCR. The developed PBEIA proved to be a sensitive assay that had high specificity and was capable of detecting antibodies in various HCV-related liver disorders including the acute phase of the infection.

Key words: hepatitis C virus; epitopes; antibodies; peptide-based immunoassay; synthetic peptides; ELISA; RT-PCR

Introduction

Synthetic peptides have become an important tool for investigation and detection of viral immunity. Synthetic peptides, selected from immunodominant regions of viral pathogens, have been used successfully for detecting specific retroviral infections (Wang *et al.*, 1988a,b, 1989a,b, 1990). Synthetic peptides can successfully substitute recombinant proteins or viral lysates as solid-phase antigens and have several inherent advantages such as the presence of essential sequences only and a high degree of purity. They permit the formulation of immunosorbents with high signal/cut off ratio for maximum sensitivity and specificity (Wang, 1988b).

HCV, whose genomic structure is similar to that of pestiviruses and flaviruses, is now known to be the etiologic agent of various human liver disorders with a propensity to progress to cirrhosis of liver and hepatocellular carcinoma (HCC) (Choo *et al.*, 1989; Saito *et al.*, 1993; Bianchi *et al.*, 1996). HCV contains a single-stranded positive-sense RNA of approximately 9.4 kb. The viral nucleic acid codes for a 3,000 amino acid long polyprotein which is subsequently proteolytically cleaved to yield smaller functional units, a core (C), two envelope (E1 and E2/NS1) and at least six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Grakoui *et al.*, 1993). In view of the mechanism involved in the generation of these functional proteins, it is expected that all the proteins are made nearly in equimolar amounts.

Despite this, it is possible that the individually processed functional proteins may have different intrinsic decay rates and therefore may be present for varying time periods within the cell or in circulation, and may even elicit immune response with different efficiency.

*Corresponding author.

Abbreviations: HCC = hepatocellular carcinoma; HCV = hepatitis C virus; PBEIA = peptide-based enzyme immunoassay; PBS = phosphate-buffered saline; RIBA = recombinant immunoblott assay; RT-PCR = reverse transcription-polymerase chain reaction; 5'-UTR = 5'-untranslated region

Table 1. Amino acid sequences of HCV peptides

Peptide	Region	No. of amino acids	Sequence
PEP3	E1	27	HTPGCVPCVREGNSSRCWVALTPTLVA
PEP4	E2/NS1	24	LFYTHKFNSSGCPERLASCRSLDP
NS1*	E2/NS1	24	LINTNGSWHINRTALNCNDSLNTG
NS2	NS2	23	HVWIPPLNARGGRDAIILLMCAV
NS3	NS3	22	RHLIFCHSKKKCDELAAKLSAL
NS4	NS4	22	REVLYQEFDEMEECSQHLPYIE
NS5	NS5	23	DEFDPLVAEEDEREVSVPAEILR
HCV-C	CORE	23	PKPQRKTKRNTNRRPQDVKFPGG

Sequence of the peptides are based on the HCV-1 genotype (Choo *et al.*,1989).
*Das MR (personal communication).

The recombinant immunoblot assay (RIBA) (van der Poel *et al.*, 1991) and RT-PCR (Weiner *et al.*, 1990) are currently used to detect HCV infection, but are not practical substitutes for ELISA. As routine screening of blood for HCV infection has now become mandatory in India, our aim was to develop a sensitive and specific enzyme immunoassay for HCV antibodies at an early phase of the infection. To achieve this objective, 8 putative epitopes from core, envelope and non-structural proteins were selected on the basis of a computer algorithm, and synthetic peptides (22 – 27-mers) corresponding to these regions were generated. Reactivities of various patient sera with different case histories to these putative epitopes were assayed by the developed PBEIA. As an independent check, the same sera were subjected to a commercial ELISA and a RT-PCR assay for HCV RNA. The correlation between the RT-PCR positivity and presence of antibodies to any of the putative viral antigens in the PBEIA was very high.

Materials and Methods

Sera negative for hepatitis B surface antigen as well as an anti-hepatitis A IgM marker from patients with various liver disorders were obtained from Army Headquarter Hospital, Bangalore; PVS Hospital, Ernakulam; Medical College Hospital, Trivandrum, and Gastroenterology and Endoscopy Centre, Coimbatore, India. Sera were aliquoted and stored at -20°C until used. HCV antibodies in the sera were detected by commercial ELISA

Peptide synthesis. Potential antigenic sites on core, envelope and non-structural proteins of the HCV genome were identified on the basis of an algorithm. Peptides encompassing these potentially immunogenic regions were synthesised on the solid phase using the Fmoc chemistry. After synthesis, the resin-bound peptides were cleaved off using trifluoroacetic acid. The peptides were purified by reverse phase high performance liquid chromatography (Pharmacia Biotech Akta Purifier) using a Sephasil peptide C-18 column. The purity of each peptide was verified by amino acid analysis. The sequences of amino acids in these peptides are shown in Table 1.

PBEIA. One mg of each purified peptide dissolved in phosphate-buffered saline (PBS) was used to coat multiwell Nunc Maxisorp 96F plates at room temperature for 3 hrs. The wells were blocked with BSA/PBS-T (3% bovine serum albumin and 0.1% Tween in PBS) at 37°C for 1 hr. Plates were subsequently washed 6 times with 0.25% Tween in PBS and incubated with anti-human IgG peroxidase conjugate (Sigma) at a dilution of 1:20,000 at 37°C for 1 hr. After six washes, 50 ml of tetramethyl benzidine solution (Bangalore Genei) was added. After 20 mins, the reaction was stopped with an equal volume of 2N H_2SO_4 and A_{450} was read. Optimal amounts of the antigens and dilutions of the sera were found by titration. The cut-off value represented the mean of a minimum of 10 negative controls (included in every assay) plus 3SD. Any reading above the cut-off value was considered positive.

RT-PCR. HCV RNA was extracted from 200 µl of serum, copied into cDNA by reverse transcription, and amplified by PCR as described elsewhere (Das *et al.*, 1993). The primers used were derived from the 5'-untranslated region (5'-UTR) of HCV RNA (Table 2) (Das *et al.*, 1993) which is highly conserved.

The first round of PCR was carried out with the outer primer pair, while the second round with the internal primer pair. The

Table 2. Primers used for the amplification of 5'-UTR of HCV genome by PCR

Name	Sequence (5'-3')	nt position
DIS	ACT GTC TTC ACG CAG AAA GCG TCT AGC CAT	40–69[a]
D2A	CGA GAC CTC CCG GGG CAC TCG CAA GCA CCC	282–311[a]
D3S	ACG CAG AAA GCG TCT AGC CAT GGC GTT AGT	49–78[b]
D4A	TCC CGG GGC ACT CGC AAG CAC CCT ATC AGG	275–304[b]

[a]External primer pair. [b]Internal primer pair

amplification was carried out in 35 cycles with denaturation at 95°C for 1 min, primer annealing at 50°C for 1.5 min, and extension at 72°C for 3 mins. To preclude false positive results, appropriate positive and negative controls along with one sample of distilled water were included in each assay.

ELISA. HCV antibodies in the patients' sera were estimated with a commercial ELISA Kit SP Nanbase C-96 (GB Biologicals, Taiwan).

Results

A total of 102 sera from patients with various liver disorders have been included in the present study. The distribution of patients with HCV antibodies among different types of hepatic patients is shown in Table 3.

Table 3 Detection of HCV antibodies in HCV patient sera by PBEIA and commercial ELISA

Assay	Liver disorder					
	AH	CH	CIR	HCC	Others	Total of positive
	No. of positives					
PBEIA/PEP 3	6	13	37	6	2	64
/PEP 4	12	12	32	7	4	67
/NS1	10	12	34	7	4	67
/NS2	7	11	33	6	3	60
/NS3	9	9	28	6	2	54
/NS4	7	8	37	7	5	64
/NS5	7	10	37	6	4	64
/HCV-C	8	12	37	7	6	70
ELISA6	9	26	3	5	49	
Total of sera assayed	17	19	48	9	9	102

AH = acute hepatitis; CH = chronic hepatitis; CIR = cirrhosis, HCC = hepatocellular carcinoma. Others: include patients on dialysis, post operative renal transplant and high risk groups like health care workers, etc. For the legend see also Table 1.

The reactivities of the 8 putative epitopes from core (HCV-C), envelope (PEP3, PEP4, and NS1) and non-structural proteins (NS2, NS3, NS4 and NS5) to the sera are also demonstrated. A large majority of them were positive in these assays to one or more of these peptides (Table 4).

As an independent check, the same sera were subjected to RT-PCR to detect the presence of HCV RNA. The correlation between the PCR positivity and antibody response to any of these putative epitopes was very high (Table 5).

Table 5. PBEIA positivity and RT-PCR positivity of the sera

Assay	No. of positives in PBEIA	No. of positives in RT-PCR
PBEIA/PEP 3	64	63
/PEP 4	67	63
/NS1	67	66
/NS2	60	58
/NS3	54	52
/NS4	64	57
/NS5	64	57
/HCV-C	70	63
ELISA	49	48

The PBEIA data were analysed to find if any of the chosen antigens showed selectivity or preference in a disease-dependent manner. The analysis showed that, except for peptides from structural regions (PEP4 and NS1) which displayed marginal preference to acute hepatitis, there was neither significant selectivity nor preference (Table 3).

In the present study, there were 12 sera which scored negative for RT-PCR (Table 6). Eight of these 12 sera were found to be positive in PBEIA. Since 6 of these 8 sera had antibodies to multiple (2 or more) epitopes, it is possible that these patients were exposed to the virus at some stage of the disease. It is also known that in some patients the viremia disappears during the course of time.

Table 4. Presence of multiple antibody types in the sera as determined by PBEIA

Disease status	No. of epitopes									
	8	7	6	5	4	3	2	1	0	Total
AH	3	0	1	2	2	3	3	3	0	17
CH	3	2	1	3	2	3	3	2	0	19
CIR	20	5	9	3	5	5	0	0	1	48
HCC	6	0	0	0	1	0	0	0	2	9
Others	1	0	0	2	0	1	2	2	1	9
Total	33	7	11	10	10	12	8	7	4	102
%	32.35	6.86	10 78	9.80	9.80	11.76	7.80	6.86	3.92	–

To the legend see Table 2.

Table 6. Details of RT-PCR-negative sera

Serum No.	RT-PCR	No. of positives in PBEIA	Disease status
134	–	4	AH
135	–	1	AH
153	–	1	CH
154	–	7	CIR
198	–	3	CIR
199	–	–	CIR
200	–	2	CIR
201	–	2	CIR
209	–	–	HCC
210	–	–	HCC
211	–	4	HCC
202	–	–	other

For the legend see Table 3.

Discussion

We have measured and compared the immunoreactivity of 8 putative epitopes representing the structural (C, E1, & E2/NS1) and non-structural proteins (NS2, NS3, NS4, & NS5). As already stated, these peptides were selected on the basis of a computer algorithm which predicted putative B cell epitopes. Sera from patients with various liver disorders served as a source of antibodies to these epitopes. PBEIA-positives (HCV antibodies) correlated well with ELISA-positives (HCV antibodies) and RT-PCR-positives (HCV RNA).

It should be emphasized that there was a very strong correlation between the PBEIA with peptides from envelope region and the RT-PCR. This is an indication that the antibodies to this region were non-neutralising. It has been reported earlier that a majority (95 – 100%) of the infected individuals had antibodies to the HCV envelope proteins which appeared to be non-neutralising and that the anti-HCV envelope antibodies were readily detected by peptide-based assays (Zhu-Xu Zhang *et al.*, 1995; M.R. Das, personal communication). The first contact between a virus and its host cell occurs via binding of the virus envelope to cell surface receptors. Neutralisation of this interaction is expected to be one of the major infection neutralisation modes. The E2-binding neutralisation has been demonstrated *in vitro* (Rosa *et al.*, 1996). But once the infection is established, the virus is mostly intracellular, and the cell-mediated immunity is supposed to play a major role in protection from the disease. This may be the reason why a majority (95 – 100%) of the patients invariably harbour antibodies to this region. Kotwal *et al.* (1992) reported that the appearance of antibodies to structural proteins coincided with the first elevation of alanine transaminase levels, making them a marker for detecting HCV infection and that they remained at a relatively high titre for several months.

Even though autoantibodies may be present in patients with HCV infection, the Brighton recommendation of 1992 (Johnson *et al.*, 1993) states that the presence of autoantibodies does not preclude the diagnosis of HCV. Furthermore, it has been authenticated that HCV infection can lead to the induction of autoimmune complexes (Magrin *et al.*, 1991). However, in this study, we found an active HCV infection by RT-PCR.

Quite a significant number of sera scored positive for the peptide from NS2 protein. Not much work on NS2 protein as a tool for the detection of HCV antibodies has been done. Antibodies to the non-structural proteins in general and NS2 in particular may be elicited when the cells get lysed and these proteins become exposed. In the present study, we could not find any significant relationship between the NS2 and other non-structural peptides.

It has been reported that antibodies to core and NS3 proteins was found in the majority of the patients irrespective of the genotype, suggesting a high antigenicity of the core and NS3 regions of the prototype HCV (HCV1), and that the proteins from these regions were relatively well conserved among different genotypes (Yuki *et al.*, 1995).

However, in the present study, the antibody response to NS3 peptide was rather low as compared to the peptides from other regions. This contradicts observations reported earlier (Yuki *et al.*, 1992, 1993, 1995; McHutchinson *et al.*, 1992). The peptide sequences used in the present study were also chosen from the prototype HCV. A possible explanation of this poor response may be the fact that the sequence of the NS3 peptide chosen for the present study may not be immunodominant.

Antibodies to the NS4 and NS5 regions have been reported to be genotype-dependent. Epitopes from these regions were used earlier to differentiate serologically various genotypes (Simmonds *et al.*, 1993a,b). The NS4 peptide used in the present study corresponds in sequence to 370-B and 373-C peptides used by Simmonds *et al.* (1993a). This indicates that this genotype might be prevalent in India. Further work is underway to sequence the HCV strains in circulation.

In the present study, we could not find any significant selectivity or preference to any of the 8 epitopes tested in a disease-dependent manner. On the other hand, antibodies were found against almost all the 8 antigens, and there was marginal apparent selectivity to some of the epitopes. Since the number of sera studied here was rather small, further work will be neccessary to delineate clearly whether such marginal selectivity is significant or not.

A significant fraction (30%) of the sera tested contained antibodies to multiple antigens. In case of cirrhosis and HCC, there was significantly higher number of samples scoring positive for all the 8 antigens simultaneously compared to other disease groups. This indicates that the antibody response may be elicited against all the viral proteins in long term infection and the reactivity of a serum depends also

on the stage of the disease in which it was collected. On the other hand, in acute cases where the disease course is short, the antibody response appears to be directed against envelope proteins, particularly E2/NS1 proteins.

In the sera analysed, there were 12 samples in which it was not possible to detect the presence of HCV RNA by RT-PCR assay. However, the PBEIA analysis showed that only three of these samples did not contain HCV antibodies to any of the 8 epitopes tested. Of the remaining 9 samples, six had antibodies to multiple epitopes. Therefore, these samples cannot be taken for negative with respect to HCV exposure. Their RT-PCR negativity could be due to low virus content. These results may therefore suggest a better utility of the PBEIA than RT-PCR when multiple antigens are tested simultaneously.

In conclusion, a single peptide derived from the structural region of viral genome may be sufficient for reliable, early detection of HCV infection by PBEIA. Addition of peptides from core region may broaden the spectrum of PBEIA positives to postinfection cases. Peptides from the non-structural region do not improve the sensitivity of PBEIA, but cause a significant increase of its specificity.

Acknowledgements. We thank Drs. L.C. Padhy, T.J. Rasool, K. Sreekumar and S. Mundayoor for helpful discussions and constructive criticism during the preparation of the manuscript. The help rendered by Dr. M. Renil during the course of this study is gratefully acknowledged.

References

Bianchi E, Steinkuhler C, Taliani M, Urbani A, De Francesco R, Pessi A (1996): Synthetic Depsipeptide substrates for the assay of human HCV protease. *Anal. Biochem.* **237**, 239–244.

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359.

Das MR, Naushad Ali, Aruna B, Rama Durga, Nambiar A, Rehana Z, Shekhar MS, Siddiqui A, Habibullah CM (1993): Indian strains of hepatitis-C-virus: Prevalence and detection. *Curr. Sci.* **65**(6), 477–483.

Grakoui A, McCourt D, Wychowski C, Feinstone S, Rice CM (1993): A second hepatitis C virus-encoded proteinase. *Proc. Natl. Acad. Sci. USA* **90**, 10583–10587.

Johnson PJ, McFarlane IG, Alvarez F *et al.* (1993): Meeting report. International auto immune hepatitis group. *Hepatology* **18**, 998.

Kotwal GJ, Baroudy BM, Kuramoto IK *et al.* (1992): Detection of acute hepatitis C virus infection by ELISA using a synthetic peptide comprising a structural epitope. *Proc. Natl. Acad. Sci. USA* **89**, 4486–4489.

McHutchinson JG, Person JL, Govindarajan S *et al.* (1992): Improved detection of hepatitis C virus antibodies in high – risk populations. *Hepatology* **15**,19–25.

Magrin S, Craxi A, Fiorentino G *et al* (1991): Is auto immune chronic active hepatitis a HCV related disease? *J. Hepatol.* **13**, 56.

Rosa D, Campagnoli S, Moretto C *et al.* (1996): A quantitative test to estimate neutralising antibodies to the hepatitis C virus: Cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. *Proc Natl. Acad. Sci. USA* **93**, 1759–1763.

Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanbe Y, Koi S, Onji M, Ohta Y, Choo QL, Houghton M, Kuo G (1990): Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* **87**, 6547–6549.

Simmonds P, Rose KA, Graham S *et al.* (1993a): Mapping of serotype specific, immunodominant epitopes in the NS4 region of hepatitis C virus: Use of type – specific peptides to serologically differentiate infections with HCV types 1, 2, and 3. *J. Clin. Microbiol.* **31**, 1493–1503.

Simmonds P, Holmes EC, Cha T-A *et al.* (1993b): Classification of HCV into six major genotypes and a series of subtypes by phylogenetic analysis of the NS5 region. *J. Gen. Virol.* **74**, 2391–2399.

Sugitano M, Inchauspe G, Shindo M, Prince AM (1992): Sensitivity of serological assays to identify blood donors with hepatitis C viraemia. *Lancet* **339**, 1018–1019.

van der Poel CL, Cuypers HT, Reesinck HW *et al.* (1991): Confirmation hepatitis C virus infection by new four antigen recombinant immunoblot assay. *Lancet* **337**, 317.

Wang CY (1988a): In Mizrahi A (Ed.): *Advances in Biotechnological Processes*. Liss, New York, **10**, 131–148.

Wang CY, Wang JG (1988b): US patent 4,735, 896.

Wang CY, Wang JG (1989a): US patent 4, 879, 212.

Wang CY, Wang JG, Walters DW (1989b): US patent 4, 833, 071.

Weiner AJ, Kuo G, Bradley DW (1990): Detection of hepatitis C viral sequences in non-A, non-B hepatitis. *Lancet* **335**, 1.

Yuki N, Hayashi N, Hagiwara H *et al.* (1992): HCV RNA and antibody to HCV core protein in Japanese patients with chronic liver disease. *Dig. Dis. Sci.* **37**, 1483–1488.

Yuki N, Hayashi N, Hagiwara H *et al.* (1993): Serodiagnosis of chronic hepatitis C in Japan by second generation recombinant immunoblot assay. *J. Hepatol.* **17**,170–174.

Yuki N, Hayashi N, Mita E *et al.* (1995): Clinical characteristics and antibody profiles of chronic hepatitis C patients: relation to hepatitis C virus genotypes. *J. Med. Virol.* **45**, 162–167.

Zhu-Xu Zhang, Margaret Chen, Anders Sonnerborg, Ola Weiland, Matti Sallberg (1995): Distinguishing acute from symptomatic chronic HCV infection by site-directed serology of the HCV structural proteins. *J. Infect. Dis.* **171**, 1356–59.