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Full Length Research Paper

Antigen specific cellular response in patients with hepatitis C virus infection and its association with HLA alleles

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Host genetic diversity is believed to contribute to the spectrum of clinical outcomes in hepatitis C virus (HCV) infection. The present study aimed at finding out the frequencies of HLA class I alleles of HCV infected individuals from Western India (Maharashtra State). Forty-three clinically characterized anti-HCV positive patients from Maharashtra were studied for HLA A, B and C alleles by PCR- sequence specific primer (SSP) typing method and compared with 67 and 113 ethnically matched anti-HCV negative healthy controls from Western India (Maharashtra State). The study's analysis reveals an association of HLA alleles A*03 (OR = 16.69, EF, 0.44, P = 7.9E-12), A*32 (OR = 1474, EF 0.21, P = 1.8E-9), HLA B*15 (OR = 14.11, EF 0.39, P = 2.18E-10), B*55 (OR = 12.09, EF 0.07, P = 0.005), Cw*16 (OR = 7.45, EF 0.12, P = 0.001) and Cw*18 (OR = 402, EF 0.05, P = 0.003), with HCV infection chronicity, while its results suggest that the establishment of viral persistence in patients is due to a failure of the immune response and is associated with HLA class 1 allele (mainly, A*03, A*32, B*15, B*55, Cw*16 and Cw*18 restricted individuals), as indicated by the absence of a significant T-cell response. Thus, this proves that associated haplotype influence HCV infection as a host genetic factor.

Key words: Hepatitis C virus (HCV), human leukocyte antigen - HLA A*03, A*32, B*15, B*55, Cw*18, Western India.

INTRODUCTION

Hepatitis C virus (HCV) is an important blood borne pathogen that is eliminated from the host in approximately 15% of acutely infected individuals, but persists in the remaining 85% (Anuradha et al., 2009). HCV is responsible for a wide spectrum of chronic liver lesions ranging from minimal to cirrhosis or hepatocellular carcinoma (HCC) and fatal outcome. Both virus-related factors such as viral heterogeneity and replicative activity (Silini et al., 1995) and the host determinants such as lack of efficient immune responses (Spengler et al., 1996) are involved in the pathogenesis of chronic hepatitis (Nathalie and Patrick, 2000). Further, the liver damage in HCV infected patients is probably associated with direct cytopathic effects and immune mediated mechanisms (Abdul, 2008). Though the exact basis for the differential clinical presentation of HCV infection is not fully understood, the viral load and genotype have been reported to influence the prognosis. The observation of different clinical presentations despite the same source of infection led to the recognition of the importance of host genetic factors in disease manifestations (Yee, 2004). In an Irish cohort, of the 704 women infected with HCV from contaminated anti-D immune globulin, 390 (55%) became persistently infected (Yee, 2004). MHC class I and class II antigens are central to the host immune response and thus are ideal candidate genes to investigate the associations with HCV. Classes I and II HLA are encoded by the most polymorphic genes that present antigens to CD8+ cytotoxic T cells and D4+ helper T cells, respectively. Polymorphisms in binding regions of these

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molecules determine antigenic specificities and the strength of the immune response to a given pathogen (Wang, 2003). Moreover, during cellular immune response, HLA class I molecules may present HCV epitopes to cytotoxic T cells, resulting in a protective immune response. The human leukocyte antigen is a crucial genetic factor that initiates and regulates immune responses by presenting foreign or self-antigens to T lymphocytes. Certain HLA alleles have been shown to influence the outcome of chronic HCV infections (Just, 1995; Thio et al., 1999)), while various HLA alleles have been linked with either persistence or clearance of the virus. Several studies have aimed to identify the involvement of HLA with different outcomes of HCV infection, but the results have not been consistent. Moreover, the literature review revealed that the prevalence of HCV infection was significantly low in the Indian population (Jain et al., 2003; Chowdhury et al., 2003; Irshad et al., 1995). So far, no data on HLA association with HCV infection have been reported from Western India. Hence, this study was undertaken, to assess specific cellular response against different HCV antigens in the peripheral blood of HCV infected patients to assess whether or not there is any genetic basis for the involvement of the HLA class I alleles with the disease course and outcomes in HCV infection

MATERIALS AND METHODS

Study population

The study was carried out at NIV, Pune. A total of 43 anti-HCV positive individuals selected from different parts of Maharashtra, over a period of three years (2009 and 2010), were included in this study. All these individuals were referred to NIV, Pune for diagnosis of hepatitis C infection by molecular and serological testing. All patients were tested for HLA A, B and C alleles. Of these patients, 33 were HCV RNA positive. Among these positives, 10 were on maintenance haemodialysis, 8 were voluntary blood donors and the remaining 15 were suffering from chronic liver diseases. Furthermore, patients suffering from chronic liver diseases had elevated levels of serum alanine aminotransferase (ALT), leaving aside 8 of 15 patients suffering from chronic liver disease, while none of the anti-HCV individuals were hospitalized. All of them were tested negative for both hepatitis B surface antigen (HBsAg) and anti-HIV antibodies. The control group consisted of 67 healthy unrelated normal Maharashtrian subjects for HLA A, B and C. Controls were negative for anti-HCV antibodies, HBsAg and anti-HIV antibodies.

Serological testing

All the samples were screened for the presence of anti-HCV antibodies using enzyme linked immunosorbent assay (ELISA, Ortho Clinical diagnostics, Inc. IIIrd generation, New Jersey, USA), while anti-HIV antibodies and HBsAg were screened by ELISA (Lab systems HIV EIA and Surase B-96, General Biological Corporation, Taiwan). Serum ALT was measured by a commercial kit (Span Diagnostics Ltd, India) and all tests were done according to the manufacturer's protocol. HCV RNA was detected by reverse transcription nested polymerase chain reaction (RT-PCR) using primers located in the highly conserved 5' NCR region (Bukh et al., 1992).

HLA typing

Genomic DNA was extracted from frozen peripheral blood mononuclear cells by Qiagen Blood mini kit (Germany). Molecular typing was carried out by polymerase chain reaction-sequence specific primer (PCR- SSP) method (Olerup SSP AB, GenoVision, Inc, Sweden) utilizing allele specific primers along with the control primers to identify the respective alleles. The allele specific primers were provided in the kit (Olerup SSP HLA-A-B-C SSP Combi Tray). The primer set contained 5' and 3' primers for grouping the HLA-A *0101 to *8001 alleles, 5' and 3' primers for grouping the B*0702 to *8302 alleles and 5' and 3' primers for grouping the Cw*0102 to Cw*1802 alleles. The amplified products were visualized under UV following agarose gel electrophoresis, and the interpretations of the alleles were based on Helmberg SCORE programme (update V3.118 KIT software GenoVision, Inc, Sweden).

PBMC isolation

Blood samples were drawn in K3 EDTA tubes. Ficoll-hypaque solution was taken as 1:2 proportions in 15 ml tube. The blood sample was layered from vaccutainer on ficoll by adding it to the tube from the wall of the tube. The tube was centrifuged at 2000 RPM for 40 min. After centrifugation was over, 4 distinct bottom layer RBCs were observed, then Ficoll, PBMC (white opaque ring or buffy coat) and the uppermost plasma layers (7 to 8 ml RPMI) were taken with a centrifuge tube. The plasma layer was removed by pasture pipette collected into a tube and kept at +4°C, while PBMC was collected by pasture pipette suspended in RPMI for first washing at 800 to 1000 RPM in the centrifuge for 10 min. After centrifugation, PBMC was removed from the centrifuge and the medium was discarded. Fresh RPMI (7 ml) were added into the tube and the PBMC were re-suspended again and centrifuged for a second wash at 800 to 1000 RPM for 10 min. After the second washing had removed the tubes from the centrifuge and the medium had been discarded, and after 5 ml of fresh RPMI had been added and the PBMC had been suspended in it, 20 µl of this suspension is taken in a micro well plate, and 20 µl of Trypan Blue dye mix is added to this mixture. Subsequently, 10 µl is loaded on Heamocytometer and the PBMC is counted in the WBC chamber under microscope. After counting of gels was completed, in the centrifuge tube at 1000 RPM for 5 min, the supernatant was discarded. PBMC was suspended in complete medium (RPMI 1640 + 10% FBS) in such a manner that each ml contained 10^6 PBMC kept in incubator at 37°C.

Nested PCR of 5' non-coding region of hepatitis C virus

QIAamp Viral RNA Mini Kit combines the selective binding properties of a silica-gel based membrane with the speed of micro spin or vacuum technology and is ideally suited for simultaneous processing of multiple samples. The sample was first lysed under highly denaturing conditions to inactivate RNase and to ensure isolation of intact viral RNA. Buffering conditions were then adjusted to provide optimum binding of the RNA to the QIAamp membrane, and the sample was loaded onto the QIAamp mini spin column. The RNA binds to the membrane, and the contaminants are efficiently washed away in two steps using two different wash buffers. High

Table 1. Primers used for HCV diagnosis.

External forward (JENS1)	5' ACT GTC TTC ACG CAG AAA GCG TCT AGC CAT 3' 5'
External reverse (JENS 2)	CGA GAC CTC CCG GGG CAC TCG CAA GCA CCC 3' 5'
Internal forward (JENS 3)	ACG CAG AAA GCG TCT AGC CAT GGC GTT AGT 3' 5'
Internal reverse (JENS 4)	TCC CGG GGC ACT CGC AAG CAC CCT ATC AGG 3'

Table 2. Reverse transcription PCR mix.

Components	∝l
D W	77
TAQ 10X with 25 mM $MgCl_2$	10
Primer 1 (Jens 1) 10 ∝M	5
Primer 2 (Jens 2) 10 ∝M	5
AMV RT 10 u/ ∝l (Promega)	1
Rnasin 40 u./ ∝l (Promega)	0. 5
TAQ polymerase (Perkin Elmer) 5u./ ∝l	0. 5
dNTP's (Promega) 25 mM	1
Total volume	100

Table 3. Reaction conditions for RT-PCR.

Steps	Temperature (°C)	Time (min)	Cycles
1	94	5.00	
2	94	1.00	
3	55	1.00	
4	72	1.00	For 35 cycles
5	72	3.00	
6	4	Store	

quality RNA is eluted in a special RNase free buffer ready for direct use or safe storage. The purified RNA is free of protein, nucleases and other contaminants and inhibitors. This RNA is used for cDNA preparation and subsequent amplification of the cDNA by PCR. This amplified product is then analyzed by gel electrophoresis. The region amplified (for qualitative or diagnostic PCR) is 5'NCR with 255 base pairs (5' NCR is the conserved region throughout the genome of HCV and is also among the major 6 HCV genotypes available) (Tables 1 to 6).

Reverse transcription PCR

100 ∝l of the RT-PCR was added to the dry pellet mixture. It was flicked to mix well and then spin down. Subsequently, the mixture was transfered to the PCR tubes.

The PCR mixture for nested PCR or 2nd round PCR

10 ∝l of the 1st PCR was added and mixed with the 2nd PCR mixture.

Gel electrophoresis

About 2.0% agarose (Sigma) gel was prepared in 1× TAE buffer (pH 8.0) and 8 ∝l of the PCR product was run by adding 2 µl of 6X loading dye to it. The gel was run by using Power pack (Biorad model 3000 xi) at 60 V for 45 min with 100 bp DNA ladder as marker. The gel was observed on transilluminator (Transilluminator UVP) and gel documentation system (Syngene). However, the use of the machine should be noted with details in the Logbook provided. Nested PCR products (PCR amplicons) were analyzed by electrophoresis on 2% TAE ultra pure agarose (invitrogen) gel and was visualized by ethidium bromide staining on a U. V. transilluminator. Subsequently, the image was captured in a gel documentation system. The gel was sliced from the region showing the band of the PCR product and was used for further processing of the cycle sequencing.

Elispot assay

PVDF membranes of plate were wet with 70% ethanol by adding 50 µl/ well. The plate was incubated for 5 min at room temperature in

Table 4. PCR mixture for nested PCR.

Components	∝l
D W	36.25
Taq 10 X Buffer (promega)	5.00
PRIMER 3 (Jens 3) 10∝M	1.50
PRIMER 4 (Jens 4) 10∝M	1.50
Taq polymerase (promega)	0.25
DNTP's (promega)	0. 5
cDNA from 1st round PCR	5.00
Total volume	50.00

Table 5. Thermal conditions for nested PCR of 5' NCR of hepatitis C virus.

Reaction conditions		
Temperature (°C)	**Time (min)**	**Cycles**
42	60	
94	5	**35**
94	1	
55	1	
72	1	

decant ethanol solution. Plates were washed with 1X PBS thrice and 300 µl/ well were added to the coated HA or IP plate (Millipore Multiscreen) with 100 ul/ well antibody captured in PBS (for example, 5 ug/ml Mabtech anti-human IFN-gamma antibody). The solution was incubated overnight at 4°C in decant antibody solution. Plates were washed with 1X PBS thrice and 300 µl/ well were added to the block with 200 ul/ well of heat inactivated FBS (GIBCO) for at least 4 h at 37°C. When preparing the effector cells, the PBMC from blood sample were isolated by the Ficoll Hypaque density gradient method. They were washed in RPMI 1640 medium and counted by hemocytometer. Later, they were resuspended at a final concentration of $1x10^6$ cells/ml in a complete medium containing RPMI + 10% FBS. The plate out assay was done in decant blocking medium and PBMC (recommendation: $1x10^5$ cells/well) was gently plated out in 100 ul RPMI medium/well, where 50 ul HCV core peptide/ well was added to the solution. It was incubated for 24 h (for example, IFN-gamma assay) in the control sample or 48 h (for example, INF- gamma assay) in the HCV infected individual at 37°C and 5% CO2. Later, the cells were discarded and the plates were washed twice with 1X PBS and 4 times with 0.05% Tween 20+1X PBS (PBST).

A total of 100 ul/well biotinylated detection antibodies (for example, 1 ug/ml Mabtech biotinylated anti- human IFN-g antibody) were added to the PBS/0.5% BSA and were incubated for at least 3 h at room temperature. Afterwards, the biotinylated detection antibodies were discarded and the plates were washed twice with 1X PBS and 4 times with 0.05% Tween 20+1X PBS (PBST). DPBS (10 ml) was taken in Petri dish and 1 drop each of reagents A and B of VECTASTAIN and 10 µl of Tween 20 were added to it to prepare avidin. Subsequently, 10 ml of the mixture was filtered by 0.45 µ syringe filter, while avidin-enzyme-complex was discarded. It was washed thrice with PBS/0.05% of Tween 20, and was followed by a 3 times washing with plain 1X PBS. Afterwards, 100 ul AEC substrate per well was added and incubated for 4 min, while the spot development was stopped under tap water. The substrates were removed under drain, while the excess liquid were removed from wells. The wells were dried thoroughly with a paper towel and the plate was allowed to dry overnight in darkness before the membrane was removed with ELI-Puncher Kit (ZellNet Consulting). Spots were counted (recommendation: KS Elispot Automated Reader System from Carl Zeiss) and the enzyme-complex (recommendation: from Vector Laboratories) was left at room temperature for about 30 min prior to use.

Furthermore, 100 ul of avidin- enzyme-complex/well was added to the solution and incubated for 1 h at room temperature. In preparing the substrate, 2.5 ml DMF was taken in a 15 ml tube cover with aluminium foil and 1 tablet of AEC was added to it. 47.5 ml of the D-Water (SIGMA) was taken in 50 ml tube and 280 µl sodium acetate, 180 µl acetic acid and 25 µl hydrogen peroxide were added to the tube. As a result, 2.5 ml DMF was mixed with AEC in this mixture.

Human leukocyte antigen MHC class 1 ABC -PCR ssp

Major histocompatibility complex (MHC) is the most polymorphic gene cluster known in man. Using conventional serology, one can determine HLA-A, HLA-B and HLA-C specificities. Serology has several limitations including cross reactivity, complement problems, nonavailability of good reagents and difficulties in phenotype assignment in cases where the expression of LA molecule is low or absent. Furthermore, the most available HLA anti-sera are developed in Caucasoid, and often, reagents from other ethnic groups cannot be used with the same degree of accuracy. The development of molecular typing procedures has considerably enhanced the study's capabilities of typing HLA alleles at the DNA level; thus, the HLA specific primers coated in plate amplified the DNA. This shift from serology to sequencing has been rapid. These technologies are more sensitive and allow the typing of several subtypes of commonly known alleles in HLA class 1 and class 2 loci. The molecular techniques are based on polymerase chain

Table 6. HLA distribution in anti-HCV positive individuals and controls from Maharashtra, India.

HLA	Anti-HCV positives (n = 43) AF (%)	Controls (n = 67) AF (%)	OR	Ki2	EF	PF	P value
A*03	47.91	5.22	16.69	12.12	0.44		7.9E-12**
A*11	2.08	11.94	0.15			0.10	0.040
A*24	6.25	23.88	0.21			0.18	0.007
A*26	2.08	0.74	2.82		0.01		
A*31	4.16	6.71	0.60				
A*32	22.91	0.00	1474.00		0.21		1.8E-8**
A*33	2.08	8.95	0.21			0.06	
A*66	6.25	1.49	4.40		0.04		
A*68	6.25	4.47	1.42				
B*07	10.41	9.70	1.08				
B*08	6.25	2.23	2.91		0.03		
B*15	43.75	5.22	14.11	10.42	0.39		2.18E-10**
B*27	4.16	2.98	1.41				
B*40	8.33	23.88	0.28				0.020
B*44	8.33	17.16	0.43			0.17	
B*51	8.33	4.47	1.93			0.09	
B*55	8.33	0.74	12.09	1.81	0.07		0.005
B*57	2.08	0.00	134.00		0.01		0.090
Cw*01	6.25	1.49	4.40		0.04		0.080
Cw*02	4.16	3.73	1.12				
Cw*03	4.16	10.44	0.37				
Cw*04	16.66	16.41	1.01				
Cw*06	6.25	19.40	0.27				0.030
Cw*07	12.50	22.38	0.49				
Cw*08	2.08	0.00	134.00		0.01		0.090
Cw*12	8.33	5.22	1.64				
Cw*14	2.08	2.98	0.69				
Cw*15	14.58	12.68	1.17				
Cw*16	14.58	2.23	7.45		0.12		0.001
Cw*18	6.25	0.00	1.64		0.05	0.02	0.003
Cw*19	2.27	12.68	0.49				

*EF, etiological fraction or attributable risk; PF, preventive fraction; Ki2, Chi-square with Yates correction; OR, odds ratio; **Very highly significant P value, obtained by multiplying with the no. of alleles; AF, allele frequency.

reaction (PCR) which is capable of amplifying a defined stretch of DNA (of several hundreds of base pairs) into several fold copies. The most commonly used PCR based HLA DNA typing techniques in tissue typing laboratories are: PCR based sequence specific oligonucleotide probe (PCR-SSOP) hybridization, PCR based sequence specific primer (PCR-SSP) typing and PCR based restriction fragment length polymorphism (PCR-RFLP).

The sequence based typing (SBT) method and the ARMS-PCR technique, were used for HLA-class 1 typing. Out of these, the PCR based sequence specific primer (PCR-SSP) typing was used.

In order to set up HLA-PCR, DNA was extracted from the blood sample. As a result, the entire blood cell or PBMCs were suspended in 200 ∝l phosphate buffer saline. Then, 200 ∝l of the lysis buffer was added after 40 ∝l of Quiagen protease had already been added. After that, the solution was mixed by vortexing for 15 s and then spinning was done. This was followed by incubation for 10 min at 60°C. Subsequently, 200 ∝l of absolute ethanol was added to it and centrifugation was done briefly to remove drops from inside the lid. Then, the mixture was added to the QIAamp spin column. The cap was closed and centrifugation was done for 1 min at 8000 rpm RT. Consequently, the QIAamp spin column was placed in a 2 ml collection tube containing filtrate. Then, QIAamp spin column was carefully opened and 500 ∝l of AW1 buffer was added to it. The cap was closed and centrifuged for 1 min at 8000 rpm RT. Afterwards, the collection tube containing filtrate was discarded. Further, 500 ∝l of AW2 buffer was added without wetting the rim; however, the cap was closed and centrifugation was done for 3 min at 14000 rpm RT. Again, the collection tube containing filtrate was discarded, and the column was placed in a clean 1.5 ml micro centrifuge tube. The column was opened and 100 ∝l of elution buffer AE was added to it. Incubation was done for 5 min at RT and then centrifugation was done for 1 min at 8000 rpm RT. The eluted solution was collected in an eppendrof and this was followed by agarose gel electrophoresis in order to check the quality of DNA

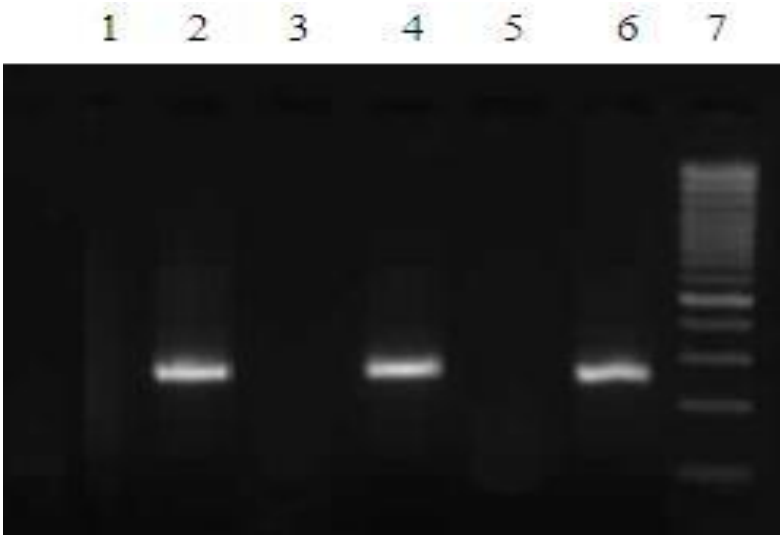

Figure 1. HCV RNA results of hepatitis C patients along with the positive control (Well 7 containing 255 bp band). Lane 1: Negative control 1; Lane 2: Specimen 1; Lane 3: Negative control 2; Lane 4: Specimen 2; Lane 5: Negative control 3; Lane 6: Positive control; Lane 7: 1 kb ladder (Fermentas).

that was thus obtained. For the quantitative analysis, the absorbance of the eluted sample was noted at 260 and 280 nm, respectively. Therefore, the obtained DNA was stored at 4°C and the standard ratio of OD at 260 nm / OD at 280 nm was 1.8. If the ratio is more than 1.8, it can be interpreted that there is a contamination of RNA in DNA, but if it is less than 1.8, it is said that there is protein contamination. However, A260 of 1 denotes 50 ∝g DNA / ml of water. Thus, in order to measure the concentration of the eluted DNA sample at 260 nm, the following formula was used:

Concentration = 50 ∝g/ml * A260 * dilution factor

From this, the total amount of DNA in the study's sample was gotten by multiplying the concentration obtained with the total volume of eluted sample. Then the eluted sample was taken in setting up HLA-PCR. For a case where the PCR reaction mix is required, this contains nucleotides, buffer, glycerol and cresol red.

This PCR mix (Olerup SSP^{TM}) has a total amount of 312 ∝l without Taq. It was transferred to an ependroff and then 8.3 ∝l of the Taq was added to it. A-B-C-SSP Combi tray (coated with primers specific for MHC1 alleles) was taken, and well numbers 24, 72 and 96 were marked as a negative control which contained 7 ∝l of distilled water + 3∝l of PCR mix. From the 320.8 ∝l PCR mix, 311.3 ∝l was taken and added, with 607 ∝l distilled water + amount of DNA, to 7.3 ∝g/100 ∝l of the sample, while 10 ∝l from this mix was added to the remaining wells. This tray was then transferred to thermal cycler, following the adjustment of cycle parameters:

1. Initial denaturation at 95°C for 3 min.
2. First 10 cycles:

(i) Denaturation at 95°C for 30 s.
(ii) Annealing and extension at 65°C for 50 s.

3. Last 25 cycles.
(i) Denaturation at 95°C for 30 s.
(ii) Annealing at 62°C for 50 s.
(iii) Extension at 72°C for 30 s.
Keep the PCR product at 4°C.

To run the PCR product, a 2% agarose gel is prepared and a 96 well gel is cast. After that, a 2 ul of loading dye is added to each well of the combi tray and loaded into the sample. Subsequently, the gel is run at 120 v for 20 min; thereafter, the gel image is saved in the gel doc system.

Statistical analysis

The phenotype frequencies, odds ratios (OR), probability value, Chi-square with Yates correction, aetiological and preventive fraction were estimated using the available data-base and computer programme (Shankarkumar et al., 2002). Since each individual was tested for several HLA alleles and the same data were used for comparing frequency, it was possible that one of the alleles would by chance deviate significantly. To overcome this error, the *P* value was corrected by the use of Bonferroni inequality method (Dunn, 1961), that is, by multiplying with the number of alleles compared. The alleles were determined with the Helmberg SCORE V3.118T software supplied along with the kit.

RESULTS

It was observed that among the HLA A locus, the frequencies of HLA A*03, A*26, A*32 and A*66 alleles increased, although the increase of the first two alleles were highly significant (Figures 1 - 10). Similarly, A*11, A*24 and A*33 alleles decreased among patients when compared to controls. Among the HLA B locus, the frequencies of alleles HLA B*08, B*15, B*55 and B*57 increased, though increase in the allele frequency of B*15 was highly significant. In the same way, allele B*40 decreased. Among the HLA C locus, the frequencies of HLA Cw*01, Cw*08, CW*16 and Cw*18 alleles increased, while Cw*06 decreased in HCV infected individuals when compared to controls. Hence, irrespective of the HCV

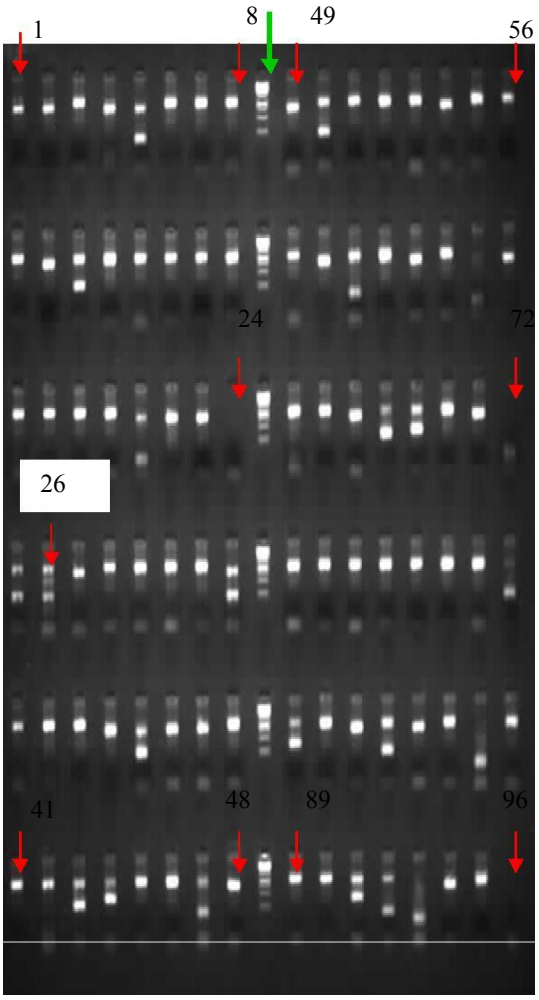

Figure 2. An agarose gel electrophorogram of a control individual. Some well numbers have been mentioned on the picture (numbers in yellow). The wells marked with red arrows are those corresponding to negative controls and have hence not lighted up. The column of wells marked by the green arrow has the 1 Kb DNA ladder. The lower bands in the ladder correspond to the 250 bp mark and the penultimate band corresponds to the 500 bp mark. Bands from 1 to 23 represent HLA alleles; bands from 25 to 71 represent HLA B alleles and bands from 73 to 95 represent HLA C alleles.

RNA status (Figure 1), the entire patients' blood samples were immediately processed for IFN-gamma specific ELISPOT for which viable PBMCs were required. However, HLA Class I allele typing was carried out only for these HCV positive patients.

DISCUSSION

One of the striking features of HCV infection is the very high rate of development of chronicity (Hans and Michael, 1996). Approximately, 15% of infected patients successfully eliminate the virus, while others develop chronic infection with a wide spectrum of disease. Some will remain asymptomatic whereas others may have a more severe course leading to cirrhosis or hepatocellular carcinoma. There are evidences that immune mechanisms contribute to control the HCV infection. In the host immune reaction against viral infections, HLA alleles play a vital role in modulating the immune responses (Chaoyang et al., 2008). Hence, this study was designed to examine the frequencies of HLA

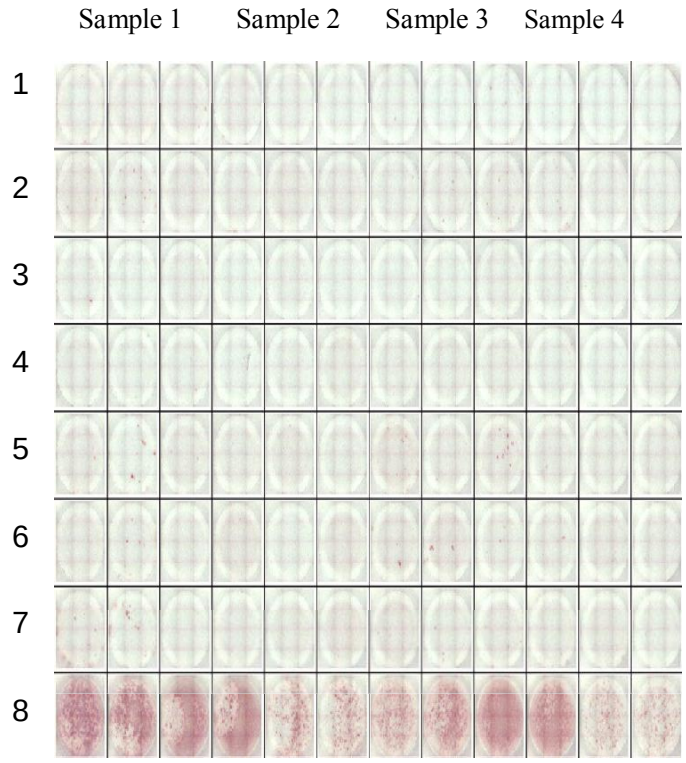

Figure 3. T cell response to various HCV antigen/peptides in healthy individuals as assessed by IFN-γ ELISPOT assay. (1) Negative control; (2) Medium control; (3) NS3 antigen; (4) NS5 antigen; (5) Core antigen ; (6) Pool 7 peptides ; (7) Pool 4 peptides and (8) Positive control.

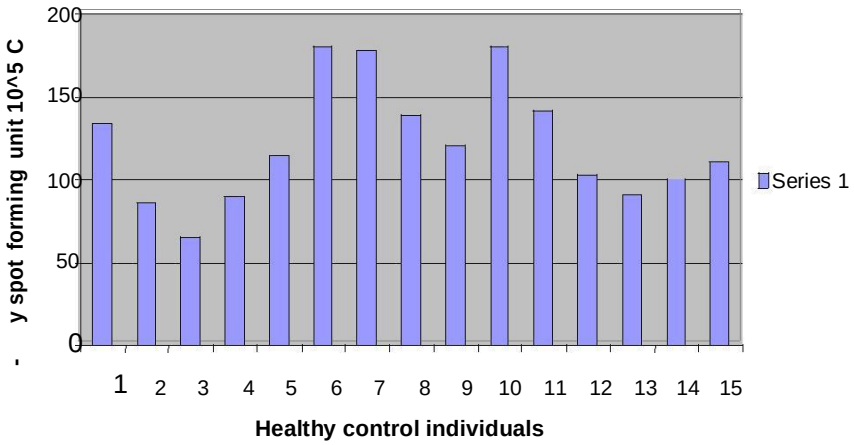

Figure 4. Average spot forming cells in positive control wells of healthy controls.

class I. The Major findings of the present study were a significant increase among the allele frequencies of HLA A*03, A*32, HLA B*15, B*55, Cw*16 and Cw*18. HLA-A3, HLA-B35 and HLA-B46 significantly increased in chronic HCV carriers compared with the controls in the Korean population (Yoon et al., 2005). In an Egyptian population Zekri et al. (2005) observed the HLA class I alleles of A28, A29 and B14 to be significantly encountered in HCV positive cases than negative cases.

An association of the HLA-B 27 with spontaneous HCV clearance has also been reported (Silini et al., 1995). Thio et al. (1999) have reported an association of

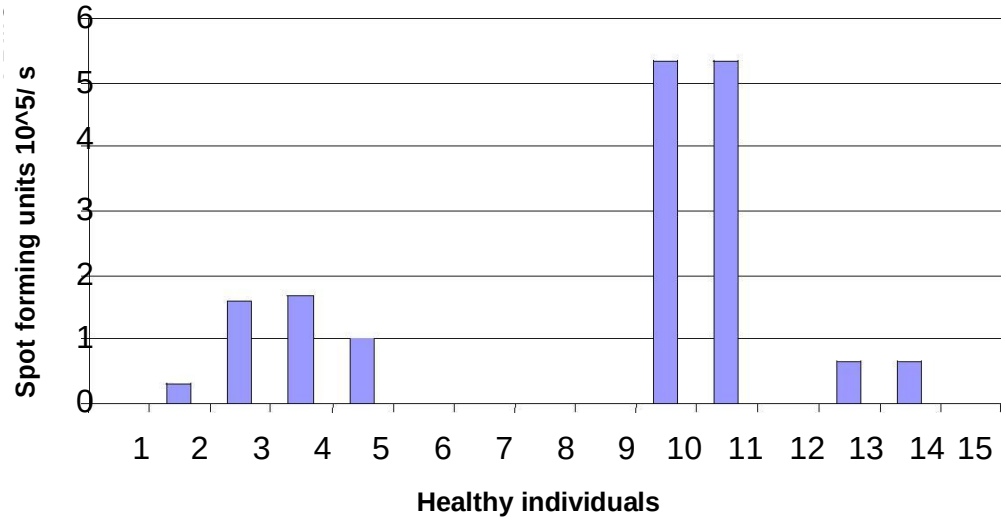

Figure 5. Average spot forming cells in cell control wells of healthy controls.

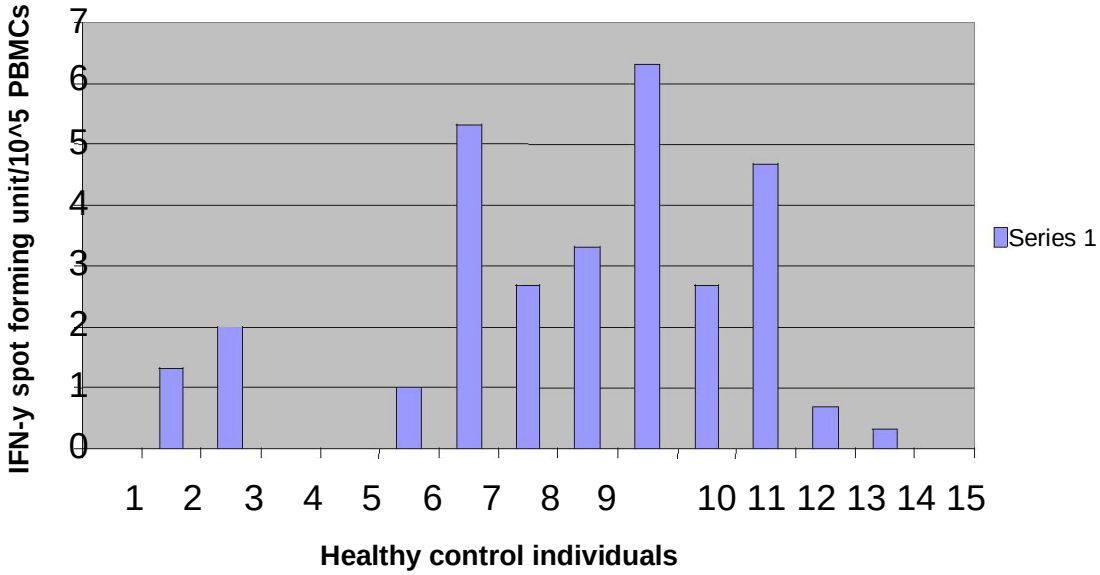

Figure 6. Average SFCs in NS3 antigen added to wells of healthy controls.

Cw*0102 with HCV clearance in Caucasians and of A*2301 and Cw*04 with HCV persistence in both African-Americans and Caucasians. However, allele A*32 was observed among HCV antibody positive individuals from western India. An increased frequency of haplotype HLA A*11 and Cw *04 in viraemic HCV patients was reported in a white population in Ireland (Andrew et al., 2000). Moreover, an association of B*15 allele with HCV infection was observed in this study. In a European population, Romero-Gomez et al. (2003) have reported the association of HLA - B*44 and have sustained HCV response to ribavirin/interferon combination therapy. Among Irish population, McKierman et al. (2004) have reported an increased frequency of B*08 and B*54 in those with chronic HCV infection when compared to those without the infection. These results suggest that HLA association with HCV infection involves both class I antigens. When taken together, the study's results suggest that HLA associations with hepatitis C infection vary in relation to the ethnicity of the population studied. Nevertheless, differences in antigen frequency of selected HLA class I and II alleles between normal subjects and in hepatitis C infected individuals from Maharashtra suggest that a susceptibility factor may contribute towards acquiring hepatitis C virus infection. Most of the healthy individuals had HLA A *03 allele, but

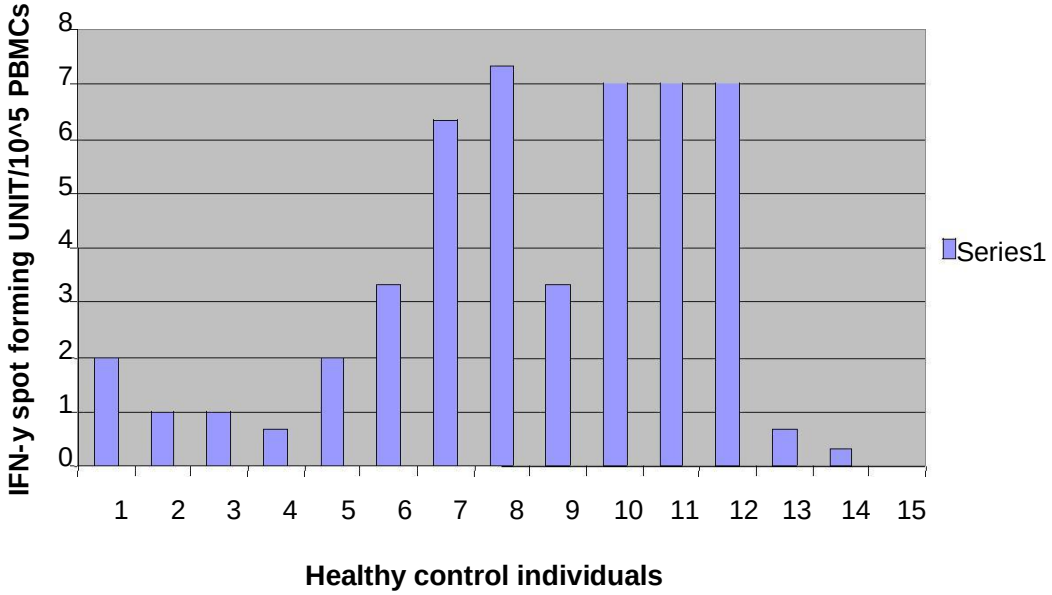

Figure 7. Average spot forming cells in NS5 antigen added to wells of healthy controls.

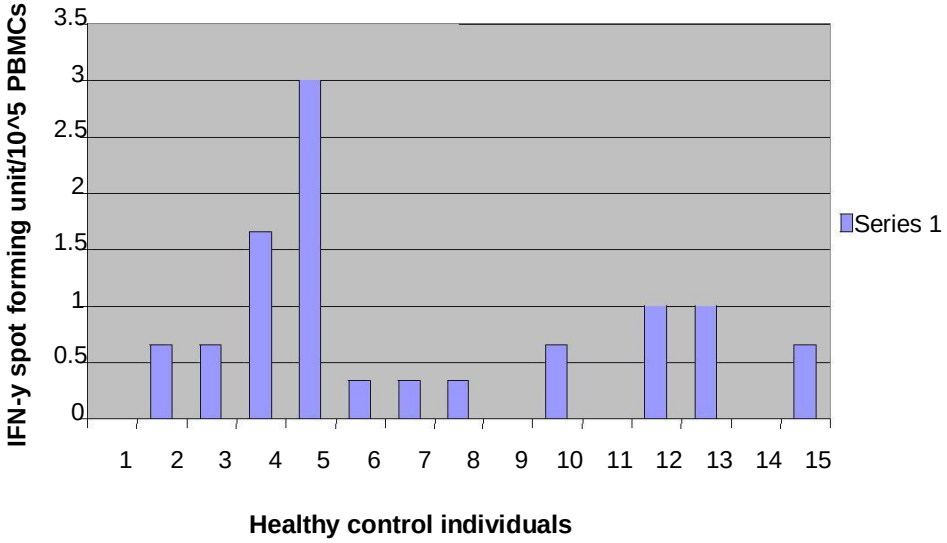

Figure 8. Average spot forming cells in pool 7 peptide added to wells of healthy controls.

none of the healthy individuals had any T cell response against any of the HCV core peptides. Out of the three HCV infected patients, two showed CTL response against the core antigen.

None of the hepatitis C patients responded to any of the core peptides. As such, it is interesting to note here that one of the HCV infected individuals had HLA A*03 allele, thus specifying the hypothesis that people with restricted HLA A*03 show substantial viral clearance and low level of pathogenesis (Silini et al., 1995).

Out of these 4 patients, 3 were positive for HCV RNA and two had a previous history of viral hepatitis. The negative sample could not be screened for anti HCV antibody by Elisa, because of the non-availability of the kit during the study period. One HCV infected patient could have been missed by this way, since there are reports that some hepatitis C cases are only antibody positive.

One of the three confirmed patients was a new case in the dialysis unit who had only recently started undergoing dialysis and who had shown CTL response against core antigen, thus showing that he might be chronically infected. This needs further verification with liver histology and other clinical parameters. The rest two

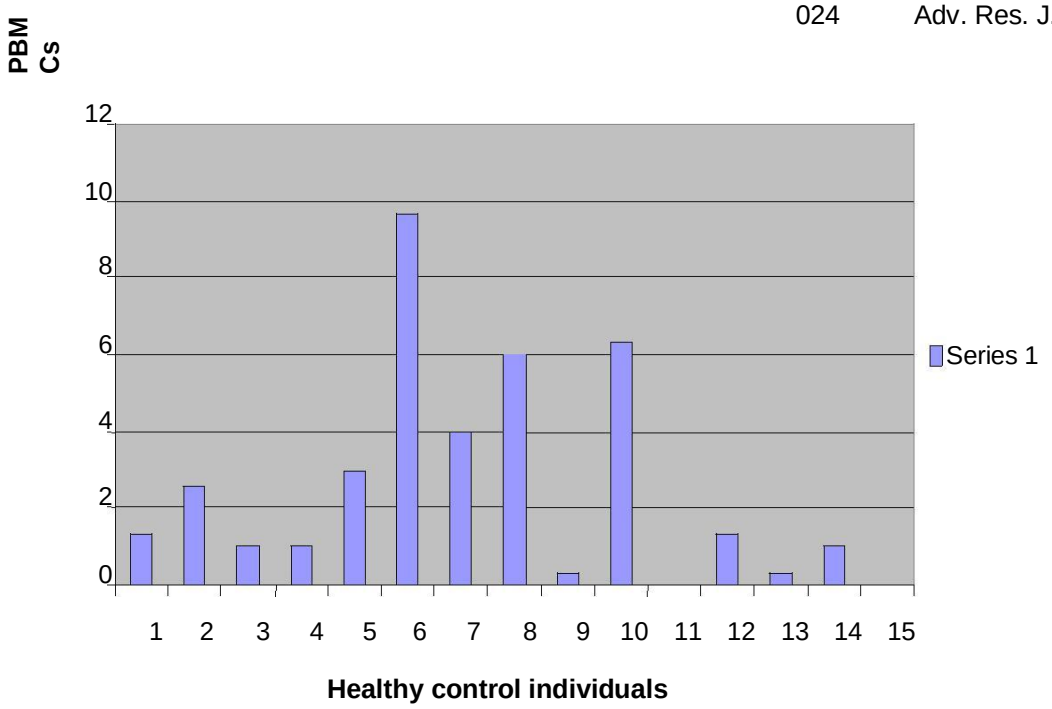

Figure 9. Average spot forming cells in core antigen added to wells of healthy controls.

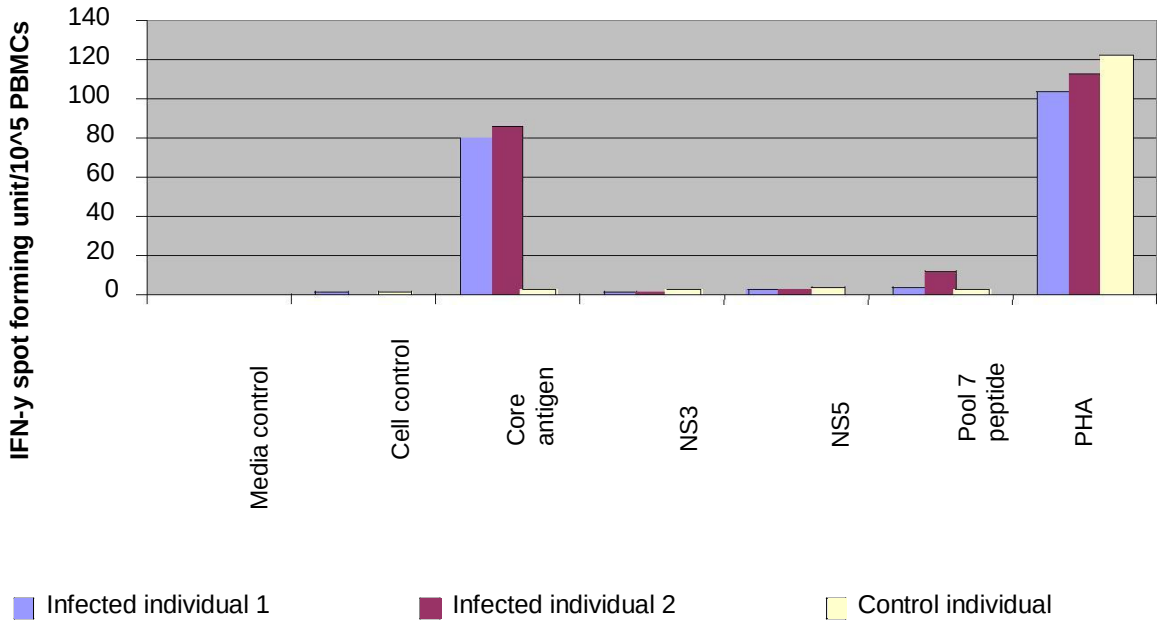

Figure 10. HCV core peptides, NS3, NS5 and core antigen not showing T cell response in healthy controls, but showing reactivity in the HCV infected individual.

patients who were chronically infected did not have CTL response against HCV core peptide, and this might be due to the high viral load that leads to defective T cell response.

There are reports that peripheral blood lymphocytes (PBLs) from HCV-infected patients without cirrhosis respond to NS3 and core proteins, producing predominantly IFN-γ. The study's data partially support the aforesaid report where none of the study's samples had cirrhosis. In contrast, PBLs from HCV-infected patients did not respond to NS3, but to the core protein, suggesting a selectively altered immune state during cirrhosis (Anthony et al., 2001). Also, the study's data provide support for the notion that HCV-specific IFN-γ-producing immunity is important in the pathogenesis of progressing HCV-related disease. Thus, an essential

process for resolution of viral infections is the efficient recognition and elimination of intracellular virus. Recognition of viral antigens in the form of short peptides associated with HLA class I molecule is a major task of $CD8^+$ cytotoxic T lymphocytes (Andrew et al., 2000). In this study, the frequency of the HLA class I alleles have been evaluated in patients with hepatitis C, although HLA-A3, A24, B51, -B52, -B55, - B56, -B61, B70, -Cw1, -Cw3 and -Cw4 are less frequent in patients with chronic hepatitis C(GracËa et al., 1998) The adaptive immune response is crucial for spontaneous resolution of acute hepatitis C virus (HCV) infection, in that it also constitutes the driving force for viral escape (Weseslindtner et al., 2009). For acutely HCV-infected dialysis patients, little is known about the host response and its impact on prognosis. A recent study in the year 2009, with four acutely infected dialysis patients have reported a robust CD4+ and CD8+ T-cell response and its association with transient control of infection, while in the other patients, weak responses correlated with persistently high viremia (Nasser and Paul Klenerman, 2007).

Despite the presence of CD8+ T-cell effectors, the establishment of viral persistence in the special patient group may be due to a failure of the adaptive immune system (Pawlotsky, 2003). This study supports this report as shown by the absence of any T-cell response by two chronic HCV infected individuals. In one of the authors study, it was not appropriate at all to propose/ predict any hypothesis with only three hepatitis C patients. The hepatitis C patient who responded to the core antigen needs to be followed up. The fact that the hepatitis C patient who was a CTL responder did not have HLA Class I allele suggests that this peptide might be an immunodominant CTL epitope that is recognized universally, irrespective of HLA restriction.

Conclusion

There are reports that peripheral blood lymphocytes (PBLs) from HCV-infected patients without cirrhosis respond to NS3 and core proteins, producing predominantly IFN-γ. These data provide support for the notion that HCV-specific IFN-γ-producing immunity is important in the pathogenesis of progressing HCV-related disease and partially supports the aforesaid report where none of the study's samples had cirrhosis. In contrast, peripheral blood lymphocytes from HCV-infected patients did not respond to NS3, but to the core protein, suggesting a selectively altered immune state during cirrhosis. The study's results suggest that the establishment of viral persistence in patients is due to a failure of the immune response and is associated with HLA class 1 allele that are mainly A*03 restricted individuals. As indicated by the absence of a significant T-cell response, the HCV core peptide, HCV core antigen, HCV NS3 antigen and the HCV NS5 antigen could be used for defining the prognosis of an HCV infected individual.

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