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ORIGINAL ARTICLE

Natural epitope variants of the hepatitis C virus impair cytotoxic T lymphocyte activity

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Abstract

AIM: To understand how interactions between hepatitis C virus (HCV) and the host's immune system might lead to viral persistence or effective elimination of HCV.

METHODS: Nucleotides 3519-3935 of the non-structural 3 (NS3) region were amplified by using reverse transcription polymerase chain reaction (PCR). PCR products of the HCV NS3 regions were integrated into a PCR® T7TOPO® TA vector and then sequenced in both directions using an automated DNA sequencer. Relative major histocompatibility complex binding levels of wild-type and variant peptides were performed by fluorescence polarization-based peptide competition assays. Peptides with wild type and variant sequences of NS3 were synthesized locally using F-moc chemistry and purified by high-performance liquid chromatography. Specific cytotoxic T lymphocytes (CTLs) clones toward HCV NS3 wild-type peptides were generated through limiting dilution cloning. The CTL clones specifically recognizing HCV NS3 wild-type peptides were tested by tetramer staining and flow cytometry. Cytolytic activity of CTL clones was measured using target cells labeled with the fluorescence enhancing ligand, DELFIA EuTDA.

RESULTS: The pattern of natural variants within three human leukocyte antigen (HLA)-A2-restricted NS3 epitopes has been examined in one patient with chronic HCV infection at 12, 28 and 63 mo post-infection. Results obtained may provide convincing evidence of immune selection pressure for all epitopes investigated. Statistical analysis of the extensive sequence variation found within these NS3 epitopes favors a Darwinian selection model of variant viruses. Mutations within the epitopes coincided with the decline of CTL responses, and peptide-binding studies suggested a significant impact of the mutation on T cell recognition rather than peptide presentation by HLA molecules. While most variants were either not recognized or elicited low responses, such could antagonize CTL responses to target cells pulsed with wild-type peptides.

CONCLUSION: Cross-recognition of CTL epitopes from wild-type and naturally-occurring HCV variants may lead to impaired immune responses and ultimately contribute to viral persistence.

Key words: Epitopes; Human; T Cells; Cytotoxic; Anergy; Viral

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INTRODUCTION

Hepatitis C virus (HCV) is one of the leading causes of chronic liver disease[1] arising from persistent infection that lasts decades despite evidence of humoral and cellular immune responses[2]. Chronic infection occurs in up to 85% of patients[3]. The mechanisms responsible for high rates of HCV persistence are unknown, but are thought to involve a complex interplay between the host immune system and viral diversity[4], which may lead to viral escape through the mutation of epitopes recognized as targets of the immune response[4,5]. The combination of a very high rate of HCV replication, estimated at 10^{12} virions per day[6], and an RNA-dependent RNA polymerase that lacks proofreading ability[7] sets the stage for Darwinian selection of variant or mutant viruses *via* pressure mediated by humoral and cellular immune responses[8].

Selective pressure appears to be applied by all elements of the immune response including antibody-producing B-cells and both $CD4^+$ and $CD8^+$ T cells. Such persistence of HCV infection has been particularly associated with mutations in epitopes encompassed within the hypervariable region 1 of HCV envelope glycoprotein 2, recognized by both antibodies and $CD4^+$ helper T cells[9,10]. Studying a class Ⅱ restricted immunodominant epitope within the non-structural 3 (NS3) protein region of HCV, we have identified a highly significant variation that correlated with escape from $CD4^+$ T cell responses[11-14]. Other sequence variations in epitopes recognized by $CD8^+$ cytotoxic T lymphocytes (CTLs) have been identified in chimpanzees[4,15] and humans[16-22] with chronic HCV infections.

CTLs recognize peptide fragments of cellular or viral proteins in the form of short peptides comprising 8-11 amino acids presented in association with major histocompatibility complex (MHC) class I molecules on the surface of infected cells[23-27]. These peptides are usually derived from intracellular viral protein pools and associated in the lumen of the endoplasmic reticulum with MHC class I molecules, after which the MHC-peptide complex is transported to the cell surface and recognized by a specific T cell receptor (TCR) located on the surface of the $CD8^+$ killer T cell.

Variation within a viral epitope can lead to a total or partial loss of functional recognition by CTL. Substitutions occurring at key anchor residues may alter peptide affinity for MHC class I molecules and thereby interfere with antigen presentation and effector T-cell mediated clearance of infected cells. Other variations, primarily in solvent-accessible residues, may abrogate TCR recognition altogether or alter it in such a way that critical activation signals are not transmitted to the cytotoxic T cells resulting in attenuated responses or even anergy[10,16-18]. Examples of attenuated responses have also been found with HIV and HBV[28-32]. It is thought that by antagonizing T-cell responses to native epitopes, viruses expressing mutant epitopes might aid in the survival of infected cells producing wild-type viruses, which would otherwise be recognized and destroyed by CTL. Although parts of this issue have been examined in chronic HCV infection[15-18,33], unresolved questions remain, including whether naturally occurring variants antagonize CTL responses to wild-type epitopes that are found within the same host. To address this, viral sequences were examined by assessing CTL activity in three epitopes that were previously identified by Koziel *et al*[34]. Comprising amino acids 1073-1081, 1131-1139, and 1169-1177, these 9-mer epitopes are restricted by human leukocyte antigen (HLA)-A*02 and recognized by specific CTL clones. Initially, we examined epitope heterogeneity in the viral sequences from an A*02-positive patient applying polymerase chain reaction (PCR) technology. This was followed by the synthesis of peptides corresponding to observed variations in these epitopes and subsequently used to sensitize A*02-expressing target cells. Results illustrate allele-specific viral evolution and escape from a dominant $CD8^+$ CTL response. While most variants were either not recognized or elicited low responses, such could antagonize CTL responses to target cells pulsed with wild-type peptides. The ability to interfere with CTL function was independent of the ability of variant peptides to bind MHC molecules. Overall, we view this study as a bridge to understanding how interactions between HCV and the host's immune system might lead to viral persistence or effective elimination of HCV.

MATERIALS AND METHODS

Human subjects

Peripheral blood samples were collected from a patient B3019 with chronic HCV at approximately 12 mo (B3019.1), 28 mo (B3019.3), and 63 mo (B3019.5) after infection. This patient never received any therapeutic intervention during the 5 years chronic HCV infection. The presence of HCV-specific antibodies and HCV RNA in the patient's serum was determined as described previously[11]. Blood was collected in acid citrate dextrose anticoagulant, centrifuged at 400 × *g* for 15 min, and divided into plasma and buffy coat fractions. After isolation of peripheral blood mononuclear cells (PBMC) over Lymphocyte Separation Medium (Organon/Teknika), plasma and PBMC were stored at -70℃ or in liquid nitrogen, respectively.

Amplification of the HCV NS3 region using reverse transcription PCR

Total HCV genomic RNA was isolated from 140 μL of B3019.1, 3019.3 and 3019.5 sera using the QIAamp® Viral RNA Mini Kit (QIAGEN, Inc.). The cDNA

was synthesized using reverse transcriptase (RT) from Moloney's murine leukemia virus and random hexadeoxynucleotide primers (Invitrogen). HCV cDNA was then amplified using nested primer sets ("outer" sense prime: 5'-GGCCTCCTAGGGTGTATAATCACC-3'; "outer" antisense primer: 5'-GAGGAGTTGTCCGTGAACAC-3'; "inner" sense primer: 5'-CAGATCGTGTCAACTGCTAC-3'; "inner" antisense primer 5'-CCACAGGGATAAAGTCCACC-3') specific for nucleotides 3519-3935 of the NS3 region. Primers were created based on the previously reported HCV sequence from genotypes 1a, (isolate H77), which generated a final PCR product of 417 bp.

Initial PCR was performed using the outer primer set starting with heat-activation of the ProofStart DNA Polymerase at 95℃ for 5 min, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and extension at 72℃ for 1 min. A final extension step at 72℃ for 10 min was introduced to increase the pool of full-length products. Two microliters of the first amplification product was transferred into the second nested PCR reaction mixture containing the "inner" primer pair. The second round of amplification was performed for 35 cycles under equal conditions. For all PCR amplifications, ProofStart DNA Polymerase (QIAGEN) with proofreading capabilities was used. PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide. As described elsewhere[35], all measures were taken to avoid contamination of PCR samples.

As a control strategy for polymerase errors, two different experimental approaches were applied. In a first experiment, the plasmid pT7 TOPO-TA/NS3 from the Hutchinson strain (1a) of HCV[36] was diluted to 10^{-14} g/mL and amplified using the same PCR procedure described above. In an alternative experiment, reverse transcription PCR (RT-PCR) was used to amplify an RNA template derived from the pT7 TOPO-TA/NS3 plasmid. The RNA template was obtained utilizing a T7 RNA polymerase (USB) according to the supplier's instructions. The resulting transcript was treated with DNase I for 15 min at room temperature and RNA was extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN, Inc.). Reverse transcription and amplification of this control RNA was carried out as described above.

Cloning and sequencing

PCR products of the HCV NS3 regions were integrated into a PCR® T7TOPO® TA vector (Invitrogen, Carlsbad, CA). Ligations and transformations were executed according to the manufacturer's instructions. Recombinant clones were then screened for positive PCR product integration by using a PCR amplification procedure detecting HCV NS3 inserted fragments. Plasmid DNAs with confirmed inserts were purified with QIAprep® spin miniprep kit (QIAGEN, Inc.) according to the standard protocol from Qiagen and further analyzed. Thirty independent clones for each sample of B3019.1, B3019.3 and B3019.5, as well as 23 and 17 independent clones for each control strategy, respectively, were sequenced in both directions using an automated DNA sequencer (373A, Applied Biosystems). The sequencing results were analyzed using GCG SeqWeb package (V2.0.2). The polymerase error rate under applied conditions was calculated as [(No. of sporadic changes)/[(No. of clones) × (sequence length) × (PCR cycles)] as described by Smith *et al*[37].

Peptide synthesis

Peptides with wild type and variant sequences of NS3 1073-1081, NS3 1131-1139 and NS3 1169-1177 were synthesized locally using F-moc chemistry and purified by high-performance liquid chromatography (HPLC). Peptide powder was dissolved in a drop of DMSO and adjusted to approximately 1 mg/mL with RPMI 1640 tissue culture medium before being used to stimulate PBMC in CTL cloning, cytotoxicity and antagonist assays. For the competition assay procedure, the FITC-labeled peptide was commercially synthesized by Synpep (Dublin, CA) using solid-phase strategies and purified with reverse-phase HPLC. For this procedure, NS3 peptides were originally dissolved in 100% DMSO at a concentration of 10 mmol/L. Subsequent dilutions were done in 1 × bovine γ globulin in PBS (BGG/PBS; 0.5 mg/mL; 0.05%; Sigma; St. Louis, MO).

Fluorescence polarization-based peptide competition assay

To determine relative MHC binding levels of wild-type and variant peptides, fluorescence polarization (FP)-based peptide competition assays were performed as described[38,39]. Initially, the four components of the binding reaction (competitor peptide, tracer, sHLA and β2m) were prepared as concentrates. The fluorescent-labeled tracer peptide (pFITC P5), ALMDKVL-K(FITC)-V, and the sHLA-A*0201 component of the reaction were diluted to appropriate 8 × and 2 × solutions, respectively. The β2m component (Fitzgerald Industries International; Concord, MA) was prepared as an 8 × mix and always added in a 2 × molar excess of the used sHLA concentration. Each competitor peptide was prepared at various dilutions and added as 4 × solutions. For all preparations, 1 × BGG/PBS was used as buffer. Next, each individual well of a black 96-well LJL HE PS microplate (Molecular Devices) was loaded with 5 μL of the prepared 8 × β2m, 10 μL of each competitor solution, and 5 μL of 8 × pFITC. To start the peptide exchange procedure, the 2 × sHLA mixes was activated by incubating at 53℃ for 15 min before adding 20 μL to the previously loaded wells reaching a final volume of 40 μL. All reagents were added to the wells of the microtiter plate sequentially using manual pipettors. The plates were then read at room temperature using an Analyst AD (Molecular Devices; Sunnyvale, CA) until no further increase in polarization was observed indicating that equilibrium was reached (24-48 h). Data analysis was performed using the software package Prism (GraphPad), by direct fit to the

appropriate models by computer- aided, nonlinear regression analysis.

CTL cloning

Specific CTL clones toward HCV NS3 wild-type peptides 1073-1081 (wt1073), 1131-1139 (wt1131) and 1169 (wt1169) were generated. Briefly, PBMCs were thawed, diluted rapidly at 4℃, washed twice by centrifugation at 400 × *g* for 10 min, resuspended and plated into wells of 96 well flat bottom plates (200 μL) at a density of 2×10^6 cells/mL. Cells were then stimulated individually with wild-type NS3 peptides 10 nmol/L at 37℃ in a 5% CO_2 incubator. After 7 d of incubation, 20 U/mL rhIL-2 (ENDOGEN) was added to the cultures. On day 14, cells were screened for the ability to lyse target cells pulsed with NS3 wild-type peptide. Functional cells were further subcloned by limiting dilution (at cell densities of 10, 3, 1 and 0.3 cells/well) in 96 well round bottom plates which contained 10^5 irradiated, autologous PBMCs, 20 U/mL rhIL2, and 10 nmol/L HCV NS3 wild-type peptide. Positive clones were further expanded and re-stimulated in 24-well plates with 10^5 irradiated (3000 rad) autologous PBMCs, in the presence of 10 nmol/L wild-type peptide and 20 U/mL rhIL-2 in RPMI 1640 medium supplemented with 25 mmol/L HEPES buffer, 2.0 mmol/L L-glutamine, 50 U/mL penicillin, and 100 mg/mL streptomycin, 5.0 mg/mL gentamicin, 10 U/mL sodium heparin, 1.0 mmol/L sodium-pyruvate, and 10% pooled AB human serum (complete RPMI-10 AB). Finally, cells were tested for cytolytic recognition of B-LCL targets pulsed separately with wild-type peptides. 14 d after the last stimulation, specific CTL clones were maintained in a long term culture in T-25 flasks by re-stimulating 2×10^6 cells every 2 wk with 1×10^6 irradiated (3000 rad) allogeneic PBMC feeders and 50 U/mL rIL-2 in complete RPMI-10 AB media. Not immediately used clones were frozen in liquid nitrogen for later usage.

Tetramer staining and flow cytometry

The CTL clones specifically recognizing HCV NS3 wild-type peptides were washed with 10% FCS in PBS followed by staining with the HCV NS3 1073-1081 peptide MHC class I tetramer complexes as described previously[40]. The following antibodies and tetramer complexes were used: Anti-CD8-FITC, anti-mouse IgG FITC (Pharmingen, San Diego, CA, USA), HLA-A*02-restricted HIV p17 epitope tetramer PE, HLA-A*02- restricted HCV NS3 1073-1081 tetramer PE. Specific CTL clones were incubated with the antibodies and tetramer reagents for 45 min at room temperature in the dark, then washed with 1 × PBS and resuspended in 500 μL of 1 × PBS. Samples were analyzed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA). The data were analyzed by WinMDI 2.8 software, kindly provided by Dr. Joel Trotter. Both the HCV NS3 1073-1081 peptide-MHC class I tetramer complex and the negative control tetramer complex HIV p17 peptide SLYNTVATL were made by the NIAID tetramer Facility, Emory University Vaccine Center (Emory University, Atlanta, GA, USA).

Cytotoxicity assay

Cytolytic activity of CTL clones was measured using target cells labeled with the fluorescence enhancing ligand, DELFIA EuTDA (Perkin-Elmer Life Sciences, Norwalk, CT) according to the manufacturer's instructions. As target cells, the HLA-A*02 positive Epstein-Barr virus (EBV) transformed B-cell line (L.B3019) was labeled with DELFIA BATDA reagents at 37℃ for 20 min, washed three times, and incubated with the indicated concentration of peptide for 1 h. After three additional washes, effector cells were added at various concentrations and incubated for 2 h in 96-well round-bottom plates (5000 target cells per well) at 37℃ in 5% CO_2. After centrifugation at 500 × *g* for 5 min, 20 μL of supernatant was transferred to corresponding wells of a flat bottom plate and 200 μL of europium solution was added. Fluorescence was measured using a Wallace Victor2 Multilabel Counter (Perkin-Elmer Life Sciences). Percent specific release was calculated according to the following formula: Percent specific lysis = 100 × [(experimental release - spontaneous release)/(maximum release - spontaneous release)]. Results were reported as the means of duplicate wells.

Antagonist assay

To measure the ability of each variant peptide to antagonize CTL responsiveness against wild-type peptide, an antagonist assay was performed using the method described by Jameson *et al*[41] under slightly modified conditions. Briefly, target cells were labeled with DELFIA BATDA reagents at 37℃ for 20 min, washed three times with RPMI 1640 medium, and then pulsed for 1 h with 10 nmol/L wild-type peptide. After removal of the wild-type peptide by another wash step (3 ×), the cells were pulsed a second time using varying concentrations of variant or control peptide for another hour. After a final wash step, specific CTL effector cells were added at various concentrations and incubated for 2 h. After incubation, the cytolytic activity of CTL was measured as described above. Percent inhibition of lysis was calculated as {% inhibition = 100 × [(A-B)/A]} where A is the percent specific lysis in the absence and B is the percent lysis in the presence of the variant peptide under investigation. Each point represents the mean of duplicate wells.

RESULTS

Mutations in the HCV NS3 region

The majority of RNA viruses produce RNA polymerases that lack proofreading activity, and thus introduce mutations into the viral genome. In the presence of immune selection pressure exerted by CTLs against wild-type virus, this genomic diversity could facilitate preferential expansion of mutant progeny encoding altered

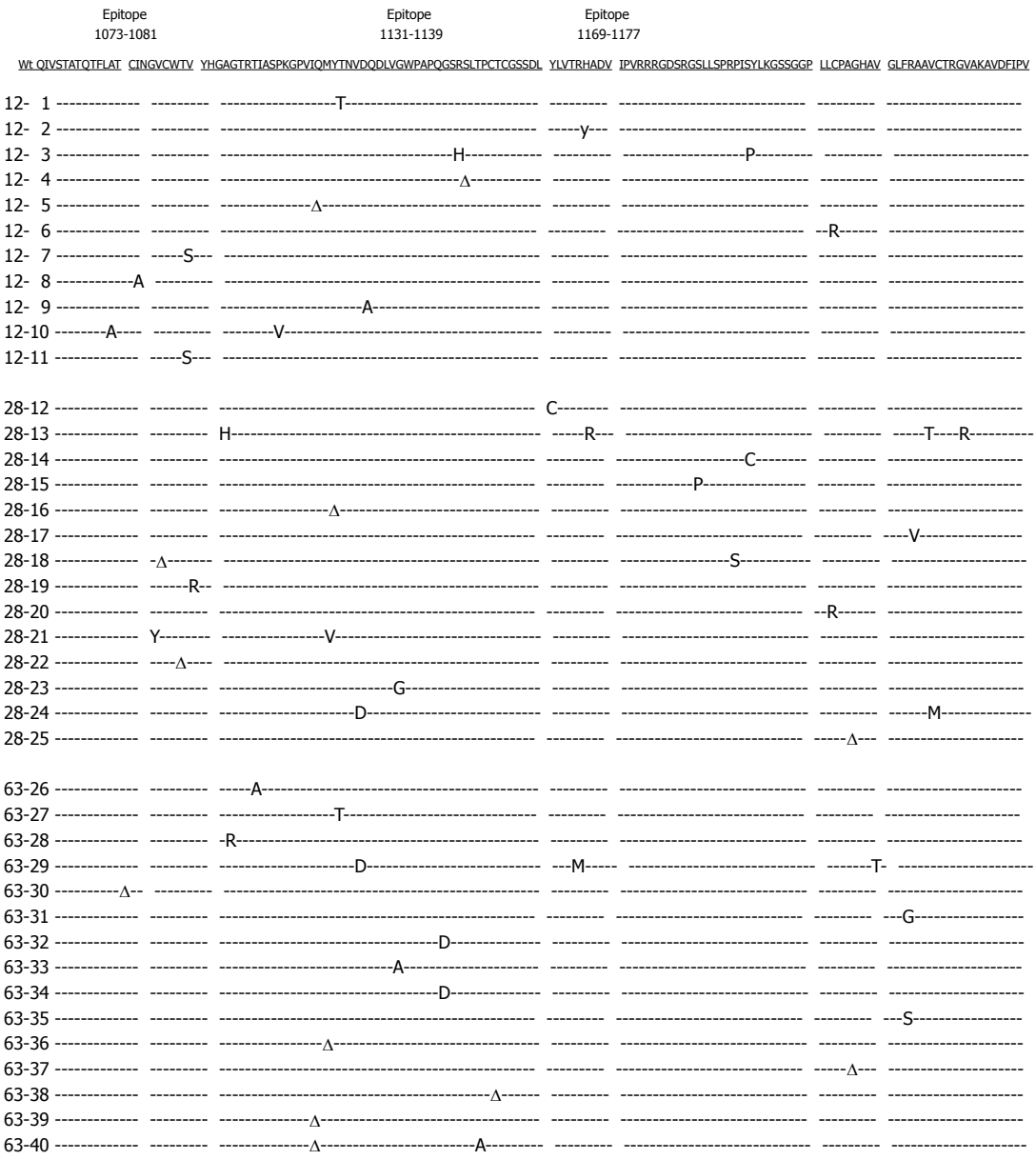

Figure 1 Summary of sequence changes detected over time within the hepatitis C virus (HCV) non-structural 3 (NS3) fragment of a single patient. HCV RNA extracted from patient sera at months 12, 28 and 63 was amplified by reverse transcription polymerase chain reaction (RT-PCR). A panel of molecular clones spanning the NS3 fragment was aligned with the wild-type consensus sequence which is shown on top (underlined). From a total of 51 nucleotide substitutions found, 39 were nonsynonymous, causing amino acid alterations (capital letters). The amplified NS3 fragment encompasses epitopes NS3 1073-1081, 1131-1139 and 1169-1177. Dashed lines indicate identity to the consensus sequences, whereas positions of synonymous mutations not causing amino acid alterations are marked with the greek delta sign (Δ).

epitopes that evade recognition by effector T cells. To monitor HCV genetic diversity within a fragment of the NS3 region, three different sera samples of a single subject with chronic HCV, collected at approximately 12, 28, and 63 mo after infection, were investigated by sequence analysis. In particular, the region 1060-1198 was selected for analysis because of the inclusion of previously identified HLA-A*02 restricted epitopes comprising amino acids 1073-1081, 1131-1139, and 1169-1177. An overall sequence comparison to the wild-type sequence revealed a set of 40 out of 90 individually sequenced clones (44.4%) carrying one or more mutations (Table 1). Within the 40 alternative sequences, a total of 51 nucleotide substitutions were detected of which 12 (23.5%) were synonymous (SYN) silent mutations and 39 (76.5%) were nonsynonymous (NSY) mutations, respectively, leading to specific amino acid alterations within this NS3 fragment (Figure 1). The frequency of sporadic substitutions was calculated as 1.9×10^{-5} according to the formula described by Smith *et al*[37]. Furthermore, the frequency of mutations observed at 3 different time points of disease progression for the same patient was not significantly different.

To determine if observed mutations are consistent with a positive Darwinian selection model as described in other reports[13,14,20], we compared the ratios of NSY/

Table 1 Mutations in the HCV NS3 region

Sample	Month 12	Month 28	Month 63	Total
No. of clones tested	30	30	30	90
Clones with mutations	11	14	15	40
Total mutations	13	20	18	51
SYN mutations	2	4	6	12
NSY mutations	11	16	12	39

SYN: Synonymous; NSY: Nonsynonymous; HCV: Hepatitis C virus; NS3: Non-structural 3.

SYN mutation for the NS3 fragment covering amino acid 1060-1198. As defined earlier for positive Darwinian selection, the rate of NSY substitution usually exceeds the SYN substitution rate and heterogeneity increases more quickly, whereas at sites subject to negative selection, the NSY/SYN ratio is < 1 and the heterogeneity will be much lower[37,42]. Among the total 51 nucleotide substitutions, 18 occurred in the first codon position, 18 at the second and 15 at the third position. According to the methods used by Nei *et al*[43] and Wang *et al*[14], theoretical values for SYN and NSY mutations were calculated as 11.7 and 39.3, respectively, which closely match our observed values of 12 for SYN and 39 for NSY. The total NSY/SYN ratio was 3.3, which is consistent with the positive Darwinian selection theory. Furthermore, we also compared our observed mutation values from different sections of the amplified region within NS3 with theoretical values for NSY mutations (Table 2). As seen in Figure 2, we found a significantly higher frequency of NSY mutations for section 1070-1119, 1130-1139, 1150-1159 and 1170-1189, earlier described as variable regions and also harboring the known HLA-A*02 epitopes. As expected, these findings are in contrast to the frequencies found within the so-called conservative regions covering sections 1060-1069 and 1120-1129 and showing a much lower mutation rate. It is notable that the 10-fold higher mutation rate within the epitope region 1073-1081, 1131-1139 and 1169-1177 compared to the conservative regions, with a NSY/SYN ratio of 2.8, suggests a high level of positive selective pressure on these immunogenic regions. No mutations were found in sections 1140-1149, 1160-1169 and 1190-1198 potentially carrying sequences not under immunological pressure within this patient. Variants do not seem to accumulate within CTL epitopes and most NSY are transient.

To ensure that the observed mutations were not due to nucleotide misincorporations introduced by the RT or polymerase during the amplification reaction, two control experiments were carried out: In the first experiment, 0.01 pg of a known NS3 plasmid template was subjected to PCR amplification in the usual two step protocol and subcloned. Sequence analysis of 23 independent clones showed absolute identity compared to the parental clone. In a second experiment, 0.01 pg of the same NS3 plasmid template was linearized and *in vitro* transcribed with T7 RNA polymerase. Limiting amounts of the RNA transcript was subjected to the

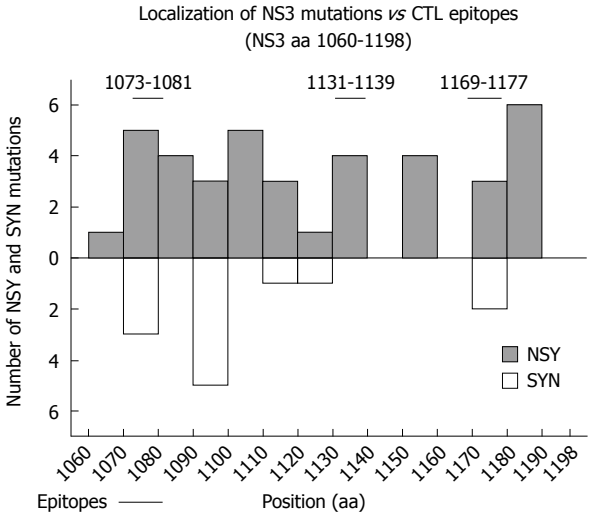

Figure 2 Localization of NS3 mutations in the proximity of three cytotoxic T lymphocyte (CTL) epitopes. Sequence alterations within the NS3 region between amino acid (aa) 1060-1198 are graphically visualized. Nonsynonymous (top) and synonymous (bottom) mutations are shown separately. NS3 CTL epitope regions 1073-1081, 1131-1139 and 1169-1177 are indicated.

complete RT-PCR amplification procedure, and the amplified product was subcloned and sequenced. Out of 17 independent clones sequenced, only one demonstrated a single nucleotide change. An error rate of 2.02×10^{-6} was calculated according to the formula described by Smith *et al*[37] and found to be well in line with the Pfu DNA polymerase error rate reported by others[44,45]. Conclusively, these findings not only highlight the fidelity of the polymerase used for our viral genome amplification procedure, but also confirm the necessity of utilizing proofreading DNA polymerases to prevent false interpretation of mutations in genetic diversity studies of RNA viruses. Taken together, our results suggest that the mutations identified by our approach primarily represent naturally occurring mutations in the HCV RNA genome, rather than artificial PCR errors.

In addition, particular attention was given to the analysis of sequence changes encompassing epitopes recognized by CTL responses[34]. Changes that emerged within stretches of sequence containing CTL epitopes are shown in Figure 1. As seen, sequence data from the epitopes defined within the NS3 region clearly reveal evolution in these target regions. By month 63 after transmission, bulk sequence data from the single targeted regions illustrates the development of mixed viral populations while still maintaining the original dominant sequence. Complete replacement of the initial virus population with viruses bearing nonsynonymous sequence changes within one or more epitope-containing regions could not be observed. For epitope CINGVCWTV (1073-1081), three mutations developed at residues 1, 6 and 7, whereas 4 different mutations were noted within epitope YLVTRHADV (1131-1139) with changes at residues 1, 4, and 6. Only 2 alterations were observed for epitope LLCPAGHAV at residues 3 and 8, which may indicate a less immunologically pressured epitope. These

Table 2 Nonsynonymous and synonymous mutations in different regions of HCV NS3

Region (aa)	Length (bp)	PCR cycles	No. of clones	Mutation		*P*	Mutation	
				Expected sporadic	NSY		SYN	NSY/ SYN
1060-1069	30	70	90	0.25	1	> 0.050	0	1/0
1070-1079	30	70	90	0.25	5	< 0.001	3	5/3
1080-1089	30	70	90	0.25	4	< 0.001	0	4/0
1090-1099	30	70	90	0.25	3	< 0.001	5	3/5
1100-1109	30	70	90	0.25	5	< 0.001	0	5/0
1110-1119	30	70	90	0.25	3	< 0.001	1	3/1
1120-1129	30	70	90	0.25	1	> 0.050	1	1/1
1130-1139	30	70	90	0.25	4	< 0.001	0	4/0
1140-1149	30	70	90	0.25	0	NS	0	0
1150-1159	30	70	90	0.25	4	< 0.001	0	4/0
1160-1169	30	70	90	0.25	0	NS	0	0
1170-1179	30	70	90	0.25	3	< 0.001	2	3/2
1180-1189	30	70	90	0.25	6	< 0.001	0	6/0
1190-1198	27	70	90	0.23	0	NS	0	0
1073-1081[1]	27	70	90	0.23	4	< 0.001	2	4/2
1131-1139[1]	27	70	90	0.23	4	< 0.001	0	4/0
1169-1177[1]	27	70	90	0.23	3	< 0.001	2	3/2

[1]Epitope region. Expected sporadic mutations = ER × L × Nc × N × P/Ns, where ER = error rate of polymerases (2.02×10^{-6}), L: Nucleotide length of compared region; Nc: Number of PCR cycles; N: Number of clones sequenced; P: Proportion of sporadic mutations expected to produce amino acid substitutions (2/3), Ns: Number of sample (1) according to Smith *et al*[37] (1997). PCR: Polymerase chain reaction; NS: No significant.

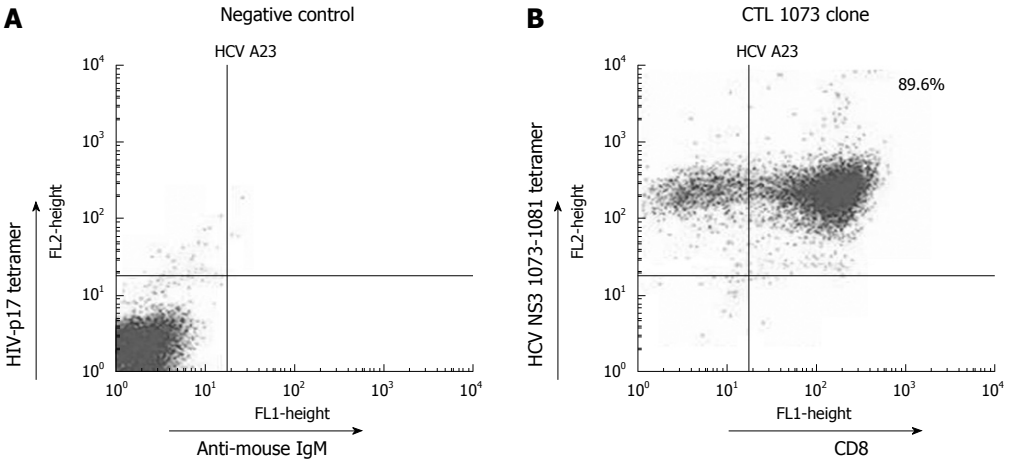

Figure 3 HCV NS3 1073-1081 tetramer staining using a CTL clone specific for wt1073. A: As a negative control, CTL clone 1073 was stained with the HIV-p17 tetramer reagent and anti-mouse IgM FITC. As expected, no cell shift was observed; B: The same CTL clone was stained with the HCV NS3 1073-1081 tetramer and anti-CD8$^+$-FITC. A positive HCV NS3 1073-1081 tetramer staining is shown with tetramer/CD8$^+$ cells located in the upper right quadrant. The origin of cells seen in the upper left quadrant could not be identified and need further investigation. All staining experiments were repeated three times with similar results. Identical results were obtained with HCV NS3 1073-1081 tetramer staining using other CTL clones for wt1073 (data not shown).

numbers are small, but consistent with the possibility of CTL-mediated selection.

Cytotoxicity of wt-specific CTL clones against various natural epitope variants

Next, we examined the phenotypic effects of these changes upon CTL recognition. For this reason, we initially produced CTL clones specifically recognizing target cells pulsed with wild-type peptides. CTL clone 1073 was generated recognizing MHC complexes loaded with wt1073, whereas CTL 1131 and CTL1169 were made to bind complexes harboring wt1131 and wt1169, respectively. Their specificity was confirmed in a separate set of experiments, where CTL clones were stained with MHC class I tetramers loaded with the corresponding HCV NS3 wild-type peptide. Figure 3 shows the result for the CTL clone 1073.3 containing tetramer positive CD8$^+$ cells in the upper-right quadrant. Additional staining experiments were performed using clones 1073.2 and 1073.4 with similar outcome (data not shown).

Subsequently, the cytolytic activity of these CTL clones was determined by incubating them with target cells pulsed with either variant or wild type peptides as reference. Results obtained for CTL 1073 (Figure 4A) showed a more than 80% cytolytic activity, confirming the capability of this CTL clone to recognize wild-type peptide wt1073 presented by the HLA-A*02 positive EBV transformed target B-cell line (L.B3019). In all instances,

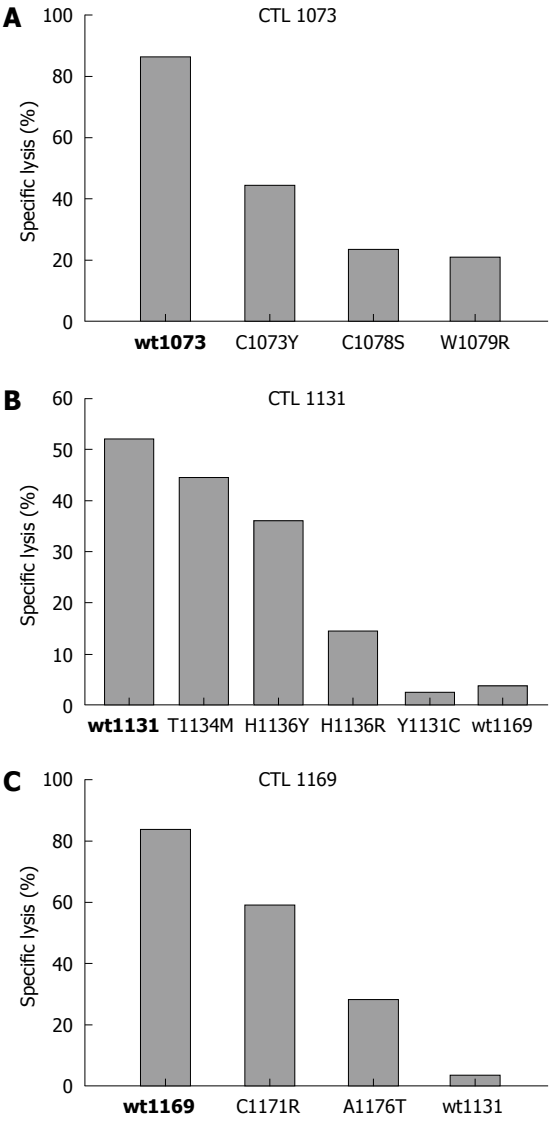

Figure 4 Effect of amino acid changes on epitope-specific CD8$^+$ CTL responses. The relative ability of synthetic peptides corresponding to the indicated wild type (bold) and variant epitope sequences to sensitize autologous target cell lines for lysis by CTL clone 1073 specific for wt1073 (A), clone 1131 specific for wt1131 (B) and clone 1169 specific for wt1169 peptide (C) was determined using *in vitro* cytotoxicity assays. The results shown are the specific lysis (%) of target cells incubated with each wild type (bold) and variant peptide at a concentration of 10 nmol/L. As expected, the highest cytotoxicity was found against wild-type peptides followed by various degrees of lysis for epitope variants. Target cells used in these assays were prepared from the B cell line (L.B3019) with an effector to target ratio of 20:1.

the amino acid changes found within this epitope region (1073-1081) showed reduced ability of the variant peptides to sensitize target cells for CTL lysis. Changes from cysteine (C) to serine (S) at position 6 and tryptophan (W) to arginine (R) at position 7 were found to be more effective than changes from a cysteine (C) to tyrosine (Y) at position 1 thus showing only a 50% reduction of specific lysis. Similar results were found for the epitope region 1169-1177 using CTL1169 (Figure 4C). The cytolytic activity for the parental peptide wt1169 again was over 80%. Variant C1171R, containing an amino acid change from cysteine (C) to an arginine (R) at position 3 could still be recognized with a 60% lytic activity, whereas only a minor activity level was noted for A1176T, showing a change from alanine (A) to threonine (T) at position 8. The control experiment using unrelated wild-type peptide wt1131 did not produce any cytolytic response confirming the specificity of CTL recognition. Finally, CTL clone 1131 (covering epitope region 1131-1139) (Figure 4B) was also, as expected, able to sensitize target cells for CTL lysis when using the wt peptide (wt1131). Target cells loaded with variant peptide T1134M and H1136Y with mutations at position 4 [threonine (T) to methionine (M)] and position 6 [histidine (H) to tyrosine (Y)], respectively, seemed nearly unaffected, showing only minor loss of cytolytic activity compared to the wild-type response, suggesting that the mutations seen in these particular cases did not abolish CTL recognition. In contrast, CTL 1131 completely failed to respond to variant peptide Y1131C and showed only minor lytic activity using target cells loaded with peptide H1136R harboring an amino acid substitution at position 6 [histidine (H) to arginine (R)]. This result suggests that the mutations within variant peptide Y1131C from a tyrosine (Y) to a cysteine (C) at position 1 was highly efficient to abrogate CTL recognition in contrast to the observation made for CTL 1073, where the opposite mutation at the same position from a cysteine (C) to tyrosine (Y) had much less impact on the lytic capability of the CTL clone. Overall, a decrease in specific lysis could be demonstrated for the majority of variant peptides compared to wild-type responses indicating that most of our identified HCV NS3 epitope variants were able to escape or lower specific CTL recognition to various degrees.

Impact of variant peptides on MHC class I binding

To investigate directly whether these amino acid changes within the variant peptides were due to impairment of peptide binding to MHC and/or to alteration of recognition of the peptide-MHC complex by T cells, we performed a series of peptide binding assays to determine if our identified viral peptide epitope variants lost their capacity to form stable class I major histocompatibility complexes compared to their wild-type counterpart. After peptides representing putative escape variants were synthesized, binding affinities of both wild-type and variant peptide epitopes were assessed using serial dilutions of the peptides in FP-based peptide competition assays utilizing soluble HLA (sHLA)-A*0201 molecules as the binding entity. Each peptide tested generated its own binding isotherm from which IC_{50} values were extracted. Figure 5A-C present multiple reaction curves obtained from the competition experiments, whereas Table 3 summarizes assessed IC_{50} values for the peptides along with their exact amino acid sequences. Using previously obtained results[39], we used other assay systems as guidelines to define an FP-based classification system, where peptides with an FP-based IC_{50} value of 5000 nmol/L and lower were considered high affinity binding, 5000-50 000 nmol/L IC_{50} values were considered medium-affinity binding, 50 000-1 000 000 nmol/L

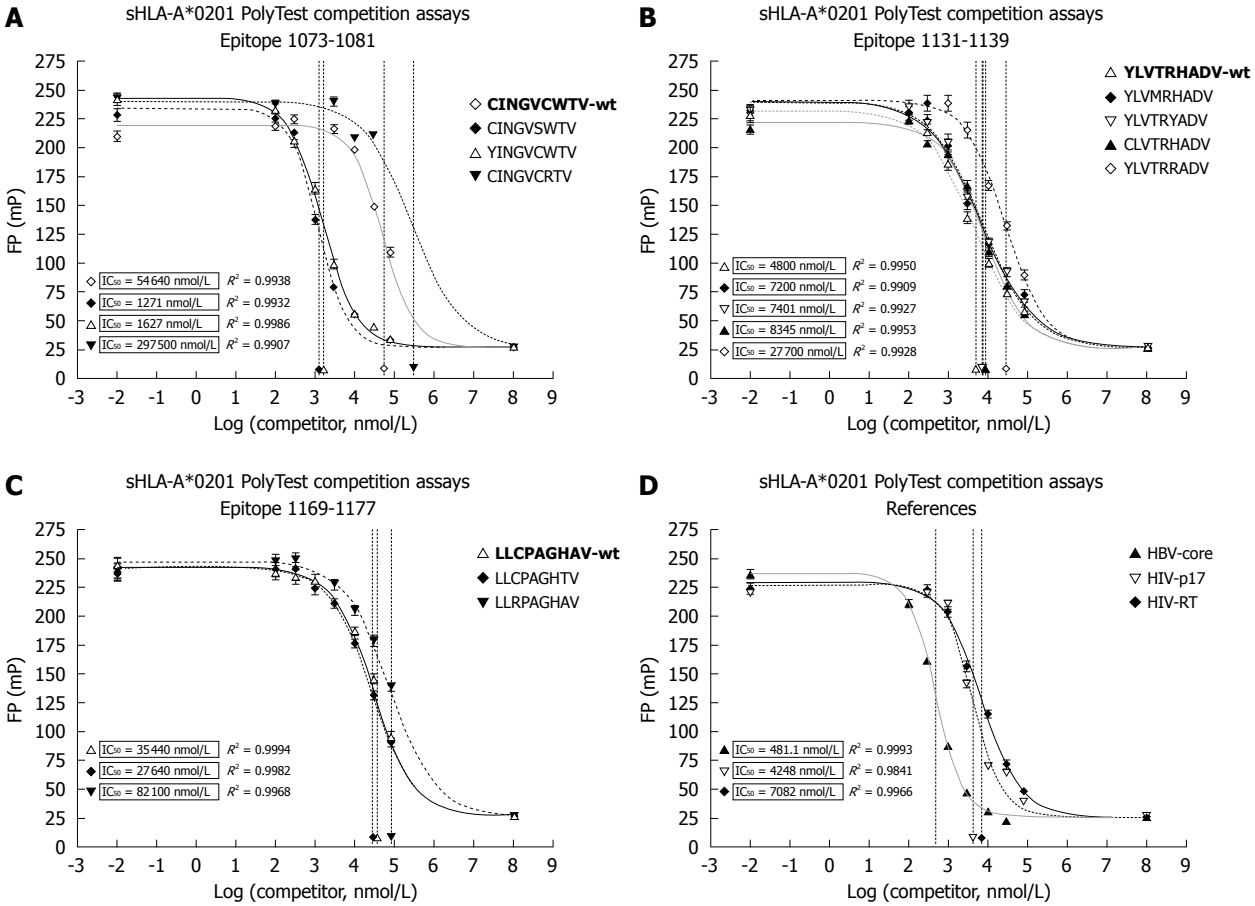

Figure 5 Soluble human leukocyte antigen (HLA)-A*0201 competition assays determining the binding capacity (IC_{50}) of natural HCV epitope variants. The affinity of three wild type HCV peptide epitopes (bold) wt1073 (A), wt1131 (B), wt1169 (C) derived from three locations within the NS3 region and their variants C1073Y, C1078S and W1079R (A), Y1131C, T1134M, H 1136Y and H1136R (B), C1171R and A1176T (C) was determined. Control peptides HBV-Core, HIV-p17 and HIV-reverse transcriptase (RT) were included to provide reference values to other viral systems. Their affinities are presented in graph (D). For each experiment, 8 serial dilutions (100 nmol/L-80 μmol/L) of the unlabeled viral peptide were used to compete against a FITC-labeled tracer peptide. After reaching equilibrium, IC_{50} values for all peptides were determined by fitting the data to a dose-response model using the software Prism. R^2 values indicate the goodness of fit.

Table 3 Binding capacity, cytotoxicity and antagonicity of HCV NS3 peptides

Sequence	Name	Peptide origin	Position	Peptide length (aa)	IC_{50} (nmol/L)	R^2	Cytotoxicity 10 nmol/L (%)[1]	Inhibition 1:1 (%)
CINGVCWTV	Wt1073	HCV NS3	1073-1081	9	54640	0.994	86.74	-
CINGV<u>S</u>WTV	C1078S	HCV NS3	wt1073 (C6S)	9	1271	0.993	24.57	59.30
<u>Y</u>INGVCWTV	C1073Y	HCV NS3	wt1073 (C1Y)	9	1627	0.999	45.21	44.70
CINGVC<u>R</u>TV	W1079R	HCV NS3	wt1073 (W7R)	9	297500	0.991	21.02	54.50
YLVTRHADV	Wt1131	HCV NS3	1131-1139	9	4800	0.995	52.03	-
YLV<u>M</u>RHADV	T1134M	HCV NS3	wt1131 (T4M)	9	7200	0.991	44.53	36.75
YLVTR<u>Y</u>ADV	H1136Y	HCV NS3	wt1131 (H6Y)	9	7401	0.993	35.56	92.54
CLVTRHADV	Y1131C	HCV NS3	wt1131 (Y1C)	9	8345	0.995	2.01	67.89
YLVTR<u>R</u>ADV	H1136R	HCV NS3	wt1131 (H6R)	9	27700	0.993	14.53	47.46
LLCPAGHAV	Wt1169	HCV NS3	1169-1177	9	35440	0.999	84.56	-
LLCPAGH<u>T</u>V	A1176T	HCV NS3	wt1169 (A8T)	9	27640	0.998	27.75	61.42
LL<u>R</u>PAGHAV	C1171R	HCV NS3	wt1169 (C3R)	9	82100	0.997	59.98	44.39
FLPSDFFPSV	HBV-Core	HBV Core	(18-27)[99]	10	481.1	0.999	NS	NS
SLYNTVATL	HIV-p17	HIV-1 p17	(77-85)[100]	9	4248	0.984	NS	NS
ILKEPVHGV	HIV-RT	HIV-1 Pol	(476-484)[47]	9	7082	0.997	NS	NS

[1]Means of two experiments. Variant substitutions are expressed in underlined letters.

IC_{50} values were judged low-affinity binding, and IC_{50} values above 1 mmol/L were regarded as no binders. Additionally, low affinity binders were further subdivided into a low (50000-350000 nmol/L) and very low affinity category (350000-1000000 nmol/L). To provide better correlation between peptide binding affinity and

immunogenicity, binding results from peptides known for their ability to induce potent and specific CTL responses were presented (Figure 5A). Among our viral controls, the HBV-derived epitope FLPSDFFPSV was found to display very high affinity values. As one of the more referenced peptides found in literature, FLPSDFFPSV is known for high affinity binding to A*0201[46-48] as well as for inducing potent CTL responses[49]. Additional controls were the peptides SLYNTVATL and ILKEPVHGV, two other well-studied HIV-derived CTL epitopes[50-52].

According to our FP-based classification system, wild-type peptides wt1131 and wt1169 were found to be of high and medium affinity, respectively, matching with the high cytolytic activity level of these CTL clones (Figure 4B and C). Somewhat unexpected was the low affinity binding observed for wild-type peptide wt1073, showing a very high cytolytic response when used in combination with CTL clone 1073 (Figure 4A). However, it has to be noted that this wild-type peptide has two cysteine residues within its sequence, allowing for disulfide bond formation. Potentially, this characteristic could lead to a reduced availability of intact peptides during the assay procedure directly affecting IC_{50} determination, ultimately causing an underestimation of its binding capacity. As the two variant peptides within this group, C1078S and C1073Y, have only a single cysteine in their sequence and demonstrate very high affinity thus strongly supporting our hypothesis of a cys-cys interference.

In reviewing the binding results found for each variant peptide, none of the amino acid changes identified within each epitope region abolished peptide binding. Motif analysis of these variants showed that none of the anchor positions (defined at position 2 and 9 for nonameric peptides and critical for high affinity A*0201-related binding) contained any amino acid alterations, consistent with an escape mechanism affecting mainly peptide regions responsible for TCR recognition rather than MHC binding. More specifically, variants T1134M, H1136Y and Y1131C covering epitope 1131-1139 remained in close affinity range of the wild-type peptide indicating that mutations at positions 1, 4, and 6 had practically no effect on their binding capacity. An exception within this epitope region is variant peptide H1136R, whose binding capacity dropped 5.8 fold compared to the wild-type peptide probably caused by the introduction of a positive charge derived from the arginine (R) residue replacing the original histidine (H) residue at position 6. Similar observations were made in variant peptides W1079R and C1171R, in which the introduction of an arginine (R) at position 7 and 3 resulted in a more dramatic decrease in affinity. W1079R shifted to a 5.4 and C1171R to a 2.3 fold lower binding capacity. It is noteworthy that variant W1079R, like parental peptide Wt1073, possesses a disulfide bond potentially causing interference in binding thus also causing an underestimation of its binding capacity. Nevertheless, these arginine substituted variant peptides were still able to bind A*0201. Interestingly, in some cases such as C1078S, C1073Y and A1176T, the binding capacity actually increased compared to the wild-type peptide.

CTL antagonism

Because peptide variants of class I -restricted epitopes potentially could antagonize naturally occurring epitopes[31], we explored this possibility by using different ratios of variant to wild-type peptide concentrations (V:W). In these antagonist assays, target cells were pre-pulsed with wt1073 (Figure 6A), wt1131 (Figure 6B) or wt1169 (Figure 6C) peptides for 1 h and pulsed a second time after excessive washing with variant or control peptide for another hour. Notably, results showed that all of the specific clones were antagonized by their corresponding variant peptides generally providing inhibition values above 35% for the V:W ratio 1:1 (Figure 6). This observation is in good agreement with the obtained IC_{50} data suggesting that all variant peptides are capable of binding HLA-A*0201. In more detail, NS3 variant peptides C1073Y, C1078S and W1079R behaved as strong antagonists for CTL 1073 and inhibited lysis of target cells at all three ratios tested (Figure 6A). An exception is variant peptide W1079R, which could not significantly inhibit the lysis of target cells at the low V:W ratio 0.1:1. This is most likely due to its much lower affinity compared to the other variant peptides within this epitope region. However, the fact that W1079R performed well as antagonist above the 1:1 ratio supports our earlier concern of reduced activity within the peptide binding assay which seems not to influence this cell-based antagonist assay. Equal results were obtained for variant peptides Y1131C, T1134M, H1136R and H1136Y testing antagonistic effects using CTL 1131 (Figure 6B). However, because of an insufficient amount of cell material obtained for CTL clone 1131, experiments were only conducted at a single V:W ratio of 1:1. Nevertheless, all of the variant peptides acted as antagonists for this CTL with highest inhibition of target lysis seen for H1136Y (92%), followed by Y1131C (68%), H1136R (47%), and T1134M (37%). The low inhibition results obtained for T1134M was somewhat unexpected considering its much higher binding capacity and cytotoxicity compared to the other variants within this group. This observation seems to indicate the presence of other factors involved to successfully antagonize CTL responses within this experimental setup such as peptide stability towards degradation and also uptake and transport mechanisms of the target cell potentially influencing peptide availability within the cell. Furthermore, both variant peptides C1171R and A1176T also demonstrated strong capabilities to antagonize CTL 1169 responses as shown in Figure 6C. Moreover, it was found that control peptide wt1131 (specific to CTL clone 1131), with high HLA-A*0201 binding affinity, was unable to inhibit CTL recognition of the wild-type peptides for CTL 1073 and 1169, respectively (Figure 6A and C). A similar result was obtained for wt1169 together with CTL 1131, suggesting that those natural variant peptides do not

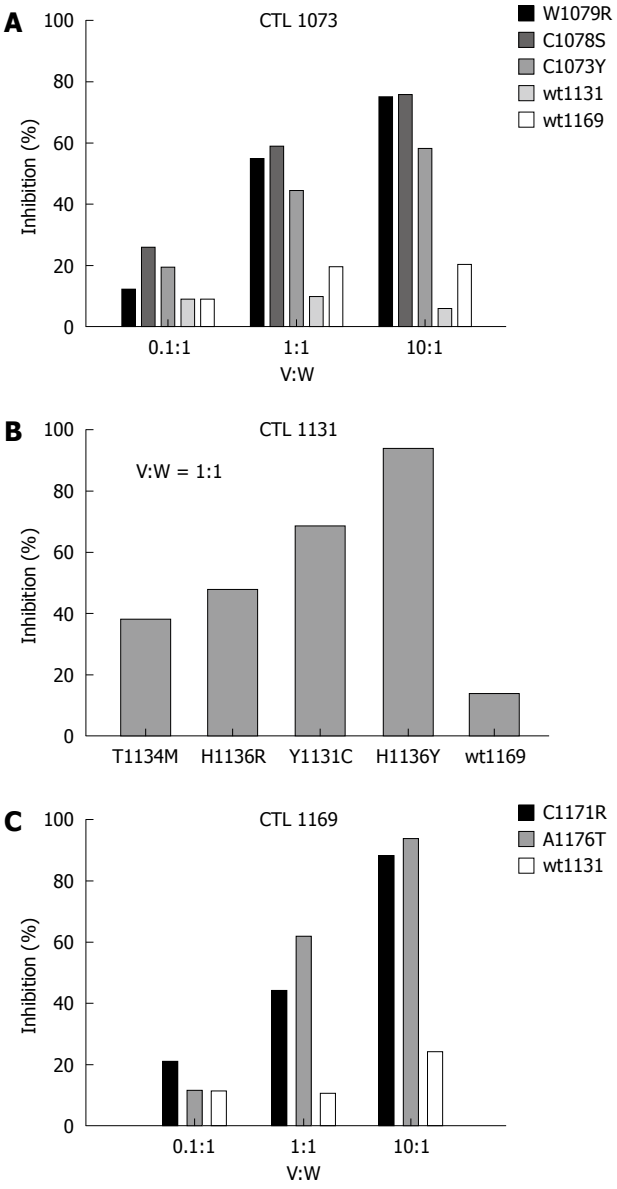

Figure 6 Antagonistic effect of NS3 peptide variants inhibiting specific cytolytic activity of specific CTL clones. A detailed inhibition profile is shown for CTL clones 1073 (A) and 1169 (C) in which target cells were pre-pulsed with 10 nmol/L of wild-type peptide, and then incubated at indicated ratios of variant to wild-type peptide concentrations (V:W). Wild-type peptides wt1131 and wt1169 were used as negative controls for experiments using CTL clone 1073, where only a single control (wt1131) was used for tests involving CTL clone 1169. Both data sets were determined at effector to target ratios of 20:1. Because of lack of material, a more simplified profile is shown for CTL clone 1131 (B) testing only a V:W ratio of 1:1 with wt1169 as negative control. All experiments were performed in duplicates.

simply exert their inhibitory activity by competing with wild-type peptides for HLA binding as recently described for hepatitis B epitopes by Bertoletti *et al*[28].

DISCUSSION

In recent years, there has been an increasing interest in HCV vaccine approaches that elicit CTL, which recognize and eliminate cells infected with HCV. Unlike antibodies, effective CTL responses can be directed against epitopes derived from any viral protein, raising the possibility that CTLs can be targeted to regions that are more conserved than the viral envelope. Current vaccine modalities can elicit potent CTL responses against multiple viral epitopes. Indeed, many lines of evidence indicate that cell-mediated immunity plays a major role in restraining HCV infections. Several studies have suggested an association between certain MHC class I and class II alleles in the control of viral replication. Strong HCV-specific $CD4^+$ and $CD8^+$ T cell responses against multiple viral epitopes have been associated with clearance of HCV during acute infection[1,15,53-60], and thought to be important contributors to protective immunity[58,61,62]. A typical example of replication control by CTL was recently presented for HIV, showing antibody-mediated depletion of $CD8^+$ cells in infected macaques, which resulted in dramatically increased virus loads in both acute and chronic infections[63-65]. However, despite the presence of specific $CD8^+$ CTL responses, more than 80% of individuals develop a persistent HCV infection. Although there is increasing evidence for the importance of HCV-specific T cell responses in the resolution of HCV infection, reasons for the failure of the immune system to eradicate the virus are less clear[66]. Functional impairment of the antigen-specific CTL responses has been observed by several investigators and is thought to be possible reasons for viral persistence despite measurable T cell responses in HCV[66-69]. Under normal circumstances $CD8^+$ T-cells contribute to the control of viral infections by recognizing peptides of viral proteins presented by MHC class I molecules on infected cells. If HCV peptides are presented to the immune system, why does the virus persist? One potential explanation for this phenomenon is that HCV seems to accumulate mutations in both its structural and NS proteins[36,70], and escape mutants may emerge under the presence of immune selection pressure exerted by CTLs against wild-type virus. Despite the importance of the CTL epitope viral mutation for immune evasion, in HCV infection many highly targeted epitopes have a low mutation frequency. Epitopes such as HLA-A2 restricted NS3 1073-1081 are consistently targeted by $CD8^+$ T cells, but amino acid mutations facilitating immune evasion are rarely observed[16,71]. Since the NS3 protein shares both protease and NTPase-dependent helicase functions, it has been proposed that mutations in these epitopes may be lethal to the virus[72]. Additionally, another study indicates that CTL escape mutations emerging early in infection are not necessarily stable, but are eventually replaced with variants that achieve a balance between immune evasion and fitness for replication[73].

CTLs contribute to the control of viral infections by recognizing peptides of viral proteins presented by MHC class I molecules on infected cells. Some viruses have developed strategies to evade recognition by CTL and one of these strategies involves antigenic variation in CTL epitopes. The emergence of $CD8^+$ escape variants has been demonstrated in numerous other viral infections chronically infecting their host like HBV, HIV, or SIV[74-78]. In HCV infection, a strong association between viral persistence and the development of escape mutations has

been demonstrated in the chimpanzee model[4,15,62]. In this study, animals with persistent infections developed mutations in multiple regions of the viral genome encoding known epitopes and were largely confined to the MHC class I restriction element expressed by these animals. Further, such mutations correlated with abrogated CTL function. In addition to studies in acute hepatitis, other sequence variations in epitopes recognized by $CD8^+$ CTLs have been identified in humans with chronic HCV infections[16-20,22]. By focusing on single MHC class I alleles, Tsai *et al*[18] observed variant epitope sequences with CTL antagonist activity within an A*02-restricted HCV E1 epitope in two patients who developed chronic infections, whereas Timm *et al*[20] described the development of CTL responses against a single HLA-B*08-restricted epitope within the NS3. Furthermore, Ray *et al*[22] used the unique approach of comparing the sequences of viruses from 22 humans with chronic hepatitis C with the sequence of the single common virus. The expression of HLA-B*07, HLA-B*35, or HLA-B*37 alleles were found to be linked to the presence of mutations in epitopes presented by these alleles, indicating a likely role for CTL-mediated pressure in driving viral evolution. All these manuscripts constitute a critical mass of evidence for CTL mutations in MHC class I -restricted epitopes of HCV, which may play an important role in evasion of the antiviral CTL response. CTL activities *in vivo* may be impacted by cross-recognition with HCV-related or unrelated epitope sequences found in humans. For example, there exists partial sequence homology between the NS3 1131-1139 and NS4 1585-1593 epitopes and between NS 1073-1081 epitope and influenza-A neuraminidase, a common human pathogen.

Study of viral evolution throughout the course of HCV infection has hence proved extremely difficult in the past. Much effort has therefore recently been directed to the monitoring of HCV evolution. Such analyses allow definitive assessment of changes within the viral genome, which are critical in determining the role of immune selection pressure in viral evolution. Since the high rate of chronicity after acute HCV infection is difficult to explain in the presence of a multi-specific CTL response[18], we sought to identify mechanisms favoring viral persistence. As noted earlier, we have previously described an immunodominant T-cell epitope restricted by HLA-DR15 in HCV NS3, NS3 358-375, for which epitope variants evolve through immune selection and stimulate not only attenuated levels of proliferation and IL-2 production, but also higher levels of type 2 cytokines[13,79]. We reasoned that if $CD8^+$ CTL exerts selection pressure on the virus, then the frequency of amino acid replacement should be higher in class I MHC restricted epitopes as well, potentially altering the outcome of infection by preventing or delaying clearance of infected hepatocytes by T lymphocytes and thus contribute to persistent HCV infection.

In order to identify escape mutations in a single patient with chronic infection, we monitored the genetic diversity in a region of the HCV NS3 protein that contains HLA-A*02-restricted CTL epitopes, NS3 1073-1081[34,54,80], NS3 1131-1139[81,82], and NS3 1169-1177[60,83]. Sequence analysis presented extensive variations in this region along with significant substitutions in segments encoding the class I restricted epitopes. Furthermore, results showed that the ratio of nonsynonymous base substitution (which changed the amino acid encoded) to synonymous base substitution (which left encoded amino acids unchanged) in these NS3 epitopes was 10-fold higher than in flanking sequences. This is comparable with our previous observations and consistent with the model for a positive Darwinian selection pressure expected for immune-mediated selection of escape variants at the epitope level[13,14,21]. Genetic variation is inherent to all RNA viruses but has been best characterized for HIV-1, which is the result of a high number of errors made by the RT enzyme[84], the absence of an RT proofreading mechanism during replication[85,86], the fast turnover of virions in HIV infected individuals[87-89] and selective immunological pressure from the host. Since HCV replication is directed by an error-prone RNA-dependent RNA polymerase encoded by the viral *NS5b* gene, which due to its propensity to introduce mutations into the viral genome, seems to provide the same selective advantage enjoyed by HIV, facilitating preferential expansion of the mutant progeny that potentially evade immune recognition. As a result, the virus population in an infected patient does not consist of a single uniform sequence but rather a distribution of different variants or quasispecies. The generation of new antigenic variants that escape the current immunological attack may lead to a persistent infection that culminates in the development of chronic infections. However, the massive heterogeneity observed in the worldwide epidemic of HIV-1 originated from a rapid viral turnover in HIV infected individuals, and seems to be much less extensive in chronic HCV infected individuals where the initial highly homogeneous virus population changes with much slower kinetics towards a mixed viral population.

Indeed, this process of immune evasion through mutation that characterizes infection with HCV viruses is a substantial barrier to the development of successful vaccines and therapeutic interventions based on manipulation of the T cell response. From this perspective, it is essential to gain a more integrated picture of the controlling influences that underlie the complex relationship between HCV and $CD8^+$ T cell immunity. Due to the observed viral evolution in the NS3 region, we hypothesized that the newly discovered variant sequences may resemble mutations capable of escaping from the original CTL response against the wt epitope. For this reason, we examined the relationship between cloned CTL responses and variant viral peptide sequences derived from the three NS3 epitopes. In total, 5 out of the 9 CTL responses studied here were not recognized by specific CTL and another four variant peptides dramatically reduced the cytolytic activity of CTL. Nonetheless, comparison of features of CTL suggested that both

quantitative and qualitative factors may play a role in determining the pressure exerted by individual epitope-specific CTL responses on *in vivo* viral replication. Considering that HLA-A*02 represents the most frequent allele in the Northern American population with a gene frequency of 27.2% in Caucasians, 23.0% in Hispanics, 22% in Natives and 12.3% in African Americans[90], we assume that, in the absence of reversion, certain variants can be expected to be present at least in some HLA-A*02-negative subjects with chronic infection. Another factor that likely has an important impact on the extent and kinetics of viral escape from epitope-specific CTL responses is the cost of escape to intrinsic viral fitness. Several papers[91,92] provide examples of high costs to intrinsic viral fitness preventing a lasting impact on overall viral evolution of particular epitopes. If CTL escape constitutes a common and significant means of immune evasion in HCV infection, vaccination strategies should be designed to elicit a response that will have the minimal chance of being escaped after infection. One way in which escape can be reduced is by induction of a T cell response that exerts balanced pressure against multiple viral epitopes (e.g. composed of multiple epitope-specific responses of similar magnitude and efficacy). A vaccine-generated host immune response that attacks the primary viral strain and subsequent mutants that arise during replication would possibly circumvent persistence through elimination or drive viral evolution towards defective mutants with high fitness cost and which lack the ability to infect new host cells or replicate in infected cells.

With respect to cellular immune responses, mutations can have effects other than loss of binding to MHC or TCR molecules. Studies in HLA class I restricted systems have demonstrated that altered peptide ligands (APLs) may antagonize the immune response or lead to antigen-specific anergy not only in CTL responses to HCV[10,16-18] but also to the inhibition of CTL responses to native antigens in other viral systems[28,31,32,93,94]. Based on our previous work with the class II restricted epitope NS3 358-375[13,14], we were interested to know whether class I restricted variant epitopes for NS3 could also act as APLs and thus antagonize CTL function. Indeed, most variant peptides for each of the three CTL epitopes were capable of acting as antagonists and suppressed CTL recognition of wild-type peptide epitopes. For some of the variant peptides, inhibition was detectable at antagonist concentrations as low as 1 nmol/L, which is similar to physiological levels of natural peptides within infected cells according to Christinck *et al*[95]. Thus, it is interesting to speculate that escape and antagonism may together serve to blunt the CTL response to multiple HCV epitopes. Ultimately, antagonism may play an important role in the persistence of HCV and other viral infections, where mutant viruses harboring antagonist APL epitopes may aid in the survival of wild-type viruses which otherwise would be recognized and destroyed by CTL. Furthermore, these observations are likely due to TCR antagonism as opposed to MHC blockade, which is supported by the fact that experiments using wild-type control peptides with high HLA-A*02 binding affinity failed to show inhibition of CTL recognition of targets pulsed with a CTL corresponding wild-type peptide. This represents a different mechanism than that reported earlier by Bertoletti *et al*[28]. In terms of the practical implications of this phenomenon, reported antagonism of T lymphocyte activity in a vaccine study is of particular concern because the antagonism was identified in a patient that became infected following vaccination[96]; antagonism may therefore represent a potential mechanism for vaccine failure and requires further careful consideration.

Overall, our data are correlative and it is important to emphasize that the coexistence of virus encoding wild type and variant epitopes does not prove that such are selected for by *in vivo* CTL responses. In the absence of a convenient animal model for HCV infection, the causal relationship between blunted CTL responses and a variant viral peptide sequence in HCV infection cannot be tested directly[97,98]. Nonetheless, the presence of variant peptides that are not recognized by or are able to antagonize specific CTL in the same patient is consistent with the notion that CTL pressure on a mutable virus such as HCV can result in the selection of escape or APL variants, similar to proposals by others with respect to HCV[16,18] and other viral infections[31,74]. The fact that all variant peptides in the NS3 1073-1081 epitope were able to antagonize two CTL clones specific for the wild-type sequence suggests that immune selection for variants, if it exists, may be very strong. In line with this is the fact that similar results were obtained with variant peptides located in epitopes NS3 1131-1139 and NS3 1169-1177 although clones were differentially susceptible to the inhibitory activity of certain variant peptides. Alternatively, if such variants arise from quasispecies variations present in the initial inoculums, they would need to be maintained without sacrificing viral fitness in a significant way. This raises a question, currently unanswered, as to whether mutation of the HCV genome recapitulates quasispecies diversity within a single patient. To our knowledge this has not been investigated, but the answer would have important ramifications for understanding the immuno-pathogenesis of an HCV infection.

In summary, the findings presented here illuminate the potential mechanisms that underlie observed patterns of mutational immune escape. Analyses of MHC binding data suggest that amino acid substitutions in the bound peptide preferably impact TCR recognition, rather than MHC binding, as a consequence of continuous shifts in antigen topography that exemplify adaptive viral evolution to the individual host environment. In addition, the ability of naturally occurring variant forms to antagonize CTL clones, as suggested within this study, is increasingly recognized in chronic infections of other viruses. In this light, persistence of HCV seems to be facilitated by viral evolution not only enabling the escape from prominent CTL responses but also through antagonistic effects triggered by

viral variants. It can be imagined that various variant peptides, which were found to have similar binding affinities to the MHC, compete against the wild type and therefore help to maintain wild-type virus by lowering the number of wild-type peptides to be recognized by patient CTLs. In any event, simultaneous analysis of the viral nucleotide sequence and the CTL response to multiple CTL epitopes in the same individual is needed to determine the potential contribution of CTL escape variants to HCV persistence. Information on heterogeneity in a single carrier seems very important in understanding immunopathogenic processes that may be influenced by viral genomic changes; such goes beyond a simple paradigm of viral escape from strong and multi-specific CTL responses against various immunodominant epitopes and should be considered as a potential determinant of HCV persistence. However, variants do not seem to accumulate within CTL epitopes but occur in early infection. Other minor species may be present but not detected and/or arise at time points other than those examined. We suggested that HCV escape mutants occurring are transient, but are eventually replaced with variants/or wild type that may seek a balance between avoiding recognition by host immune cells and reducing fitness for replication. Ultimately, viral mutants that escape immune recognition are a formidable challenge to the design of an effective HCV vaccine.

COMMENTS

Background

The mechanisms responsible for high rates of hepatitis C virus (HCV) persistence are unknown, but may involve viral escape through the mutation of epitopes recognized as targets of the immune response. It is thought that by antagonizing T-cell responses to native epitopes, viruses expressing mutant epitopes might aid in the survival of infected cells producing wild-type viruses, which would otherwise be recognized and destroyed by cytotoxic T lymphocytes (CTLs). Although parts of this issue have been examined in chronic HCV infection unresolved questions remain, including whether naturally occurring variants antagonize CTL responses to wild-type epitopes that are found within the same host.

Research frontiers

Variation within a viral epitope can lead to a total or partial loss of functional recognition by CTL. Substitutions occurring at key anchor residues may alter peptide affinity for major histocompatibility complex (MHC) class I molecules and thereby interfere with antigen presentation and effector T-cell mediated clearance of infected cells. Overall, the authors' study showed the way to understanding how interactions between HCV and the host's immune system might lead to viral persistence or effective elimination of HCV.

Innovations and breakthroughs

Mutations in MHC class I -restricted HCV epitopes that affect T-cell receptor recognition by $CD8^+$ CTLs have also been correlated with the severity and persistence of HCV infection. The authors examined viral evolution in three immunodominant HLA-A*02-restricted NS3 epitopes in a patient chronically infected with HCV. Results obtained provide convincing evidence of immune selection pressure for all epitopes investigated. Mutations within the epitopes coincided with the decline of CTL responses, and peptide-binding studies suggested a significant impact of the mutation on T cell recognition. Results illustrate allele-specific viral evolution and escape from a dominant $CD8^+$ CTL response.

Peer review

The authors have examined the pattern of natural variants within three HLA-A2-restricted NS3 epitopes in one patient with chronic HCV infection at 12, 28 and 63 mo post-infection.

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