

CD8 Epitope Escape and Reversion in Acute HCV Infection

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Abstract

In the setting of acute hepatitis C virus (HCV) infection, robust HCV-specific CD8⁺ cytotoxic T lymphocyte (CTL) responses are associated with initial control of viremia. Despite these responses, 70–80% of individuals develop persistent infection. Although viral escape from CD8 responses has been illustrated in the chimpanzee model of HCV infection, the effect of CD8 selection pressure on viral evolution and containment in acute HCV infection in humans remains unclear. Here, we examined viral evolution in an immunodominant human histocompatibility leukocyte antigen (HLA)-B8–restricted NS3 epitope in subjects with acute HCV infection. Development of mutations within the epitope coincided with loss of strong ex vivo tetramer and interferon γ enzyme-linked immunospot responses, and endogenous expression of variant NS3 sequences suggested that the selected mutations altered processing and presentation of the variant epitope. Analysis of NS3 sequences from 30 additional chronic HCV-infected subjects revealed a strong association between sequence variation within this region and expression of HLA-B8, supporting reproducible allele-specific selection pressures at the population level. Interestingly, transmission of an HLA-B8–associated escape mutation to an HLA-B8 negative subject resulted in rapid reversion of the mutation. Together, these data indicate that viral escape from CD8⁺ T cell responses occurs during human HCV infection and that acute immune selection pressure is of sufficient magnitude to influence HCV evolution.

Key words: HLA footprint • processing mutation • viral evolution • acute hepatitis C • CD8 escape

Introduction

Hepatitis C is a common cause of liver disease (1) with the majority of infected individuals developing persistent high-level viremia, some of whom go on to develop progressive hepatic fibrosis. Clearance of the virus during acute infection has been shown to be associated with a strong and broadly directed cellular immune response mediated by both CD4⁺ and CD8⁺ T cells (2–6) and the importance of

these cell subtypes in the control of viral infection has been demonstrated in the chimpanzee model through T cell depletion studies (7, 8). However, although many individuals mount detectable cellular immune responses in the acute phase of infection (4, 6), the majority of these subjects still progress to chronic disease. Complicating our ability to determine the role of the immune response in control of hepatitis C virus (HCV) is that once viral persistence is established, CD4⁺ and CD8⁺ T cell responses become difficult to detect (9–11). When present in blood, they are typically very weak, but can often be detected after peptide-specific re-

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Abbreviations used in this paper: HCV, hepatitis C virus; ICS, intracellular cytokine staining; ML, maximum likelihood.

stimulation *in vitro* or by using ultra-sensitive tetramer staining techniques (12). The frequency of these cells may be enriched in the liver (13, 14), although even here many patients appear to lack responses (15).

The mechanisms of viral persistence in the face of an activated host immune response in addition to the differences between cellular immune responses in individuals who successfully control the virus versus those who fail to control are poorly understood. Several mechanisms suspected to contribute to failure to contain HCV have been suggested (16), including impairment of cellular effector functions (proliferation, cytokine secretion, and cytolytic activity; references 6, 17, 18), dendritic cell dysfunction (19), or T cell exhaustion (20). In addition, the liver as the main site of HCV infection, and therefore antigen presentation, has the potential to delete antigen-specific T cell responses (21).

Persistence of HCV may also be facilitated by viral evolution over the course of infection, enabling escape from prominent CD8⁺ CTL responses. The emergence of CD8 escape variants has been demonstrated in numerous viral infections (HIV, SIV, LCMV, influenza), although the impact of immune escape on disease progression remains less well understood (22–26). In HCV infection, a strong association between viral persistence and the development of escape mutations has been demonstrated in the chimpanzee model (27). However, in human HCV infection, the findings are less clear. Thus far, most CD8 escape studies have been limited in their analysis by the lack of longitudinal samples from patients with acute infection (28). A correlation between the outcome of acute hepatitis C and the evolution of quasispecies has been described and the selection of site-specific mutations in the envelope gene E2 observed (29, 30), but this has not been linked to specific CD4 or CD8 T cell responses. In longitudinal studies in which selected CD8 responses restricted by common HLA class I molecules were studied, no evolution was found within most targeted epitopes (31), and in some of these cases, exhaustion as a mechanism for T cell failure has been favored (20).

To more precisely address the role of viral escape on CD8 T cell recognition, we examined the relationship between a targeted immunodominant HLA-B8–restricted epitope and *in vivo* viral evolution in two HLA-B8 positive subjects with acute infection. Viral sequences in this

epitope were also examined in a larger cohort of chronic HCV-infected subjects to assess whether HLA-associated mutations could also be detected at the population level. These data illustrate consistent allele-specific viral evolution and escape from a dominant CD8 response in human HCV infection. Moreover, transmission of a CD8 escape mutation in the absence of ongoing selecting immune pressure enabled reversion of the mutation to the original sequence. Together, these data indicate that cellular immune pressures can be exerted during acute HCV infection and can reproducibly influence HCV evolution.

Materials and Methods

Subjects. Subject 99B developed acute hepatitis C after a needle stick injury (Table I and see Fig. 2 A). 5 wk after the injection, the subject continued to have HCV RNA levels $>2.0 \times 10^6$ IU/ml (HCV Roche Amplicor assay, detection limit <600 IU/ml), and therapy was initiated with interferon α -2b. During the ensuing 12 mo of treatment, HCV RNA levels were at or below the level of detection in quantitative assays. After cessation of therapy, viral loads relapsed to a level of 0.5×10^6 IU/ml.

Subject 02J (donor) was identified after donating blood to a recipient (subject 02K, see next paragraph) who developed acute HCV infection (Table I and see Fig. 1). 02J was seronegative at the time of the blood donation but subsequently seroconverted between 7 and 9 wk later (EIA 3.0; Abbott Diagnostics), consistent with infection shortly before the transfusion event. Before treatment, 02J exhibited 0.3 – 0.5×10^6 U/ml viral loads (see Fig. 2 A). Treatment with peginterferon α -2b and ribavirin was started 15 wk after the transfusion, and the HCV RNA level declined to below the level of detection (<600 IU/ml). However, 27 wk after initiating therapy, the HCV RNA became consistently detectable at pretreatment levels, and therapy was stopped.

Subject 02K received a transfusion from 02J (Table I and see Fig. 1) and, 35 d later, HCV plasma RNA was 0.8 – 1.0×10^6 IU/ml (see Fig. 2 A). At the time of presentation, subject 02K was asymptomatic. Treatment with peginterferon α -2b and ribavirin was started 13 wk after transfusion and a virological response with undetectable plasma HCV RNA was achieved. However, the subject had virologic relapse when medication was stopped after 24 wk of treatment. Thereafter, HCV RNA transiently declined between weeks 52 and 64 before again rebounding to pretreatment levels. The immune responses and viral loads over the course of infection of these three subjects have been studied in the context of a larger cohort of acute HCV infection (unpublished data), and are repeated here for clarity.

Table I. Study Subjects and Targeted CD8 Epitopes

Subjects	Route of infection	GT	HLA	Epitopes/HLA restriction	Protein	H77 reference sequence
99B	needle stick	1b	A3, 11 B7, 8 Cw7	1395-1403/B8 1636-1644	NS3 NS4	HSKKKCDEL EVTLTHPITKYIMTCMSA
02J	unknown (donor for 02K)	1a	A1, B8, 44 Cw5, 7	1395-1403/B8	NS3	HSKKKCDEL
02K	transfusion (recipient of 02J)	1a	A31, 68 B40, 44 Cw3, 7	–	–	NA

NA, not applicable.

In addition, 30 treatment-naïve subjects with chronic HCV infection were recruited from the Hepatology Outpatient Clinic of the Massachusetts General Hospital in Boston. Subjects were included if they presented with positive HCV RNA in the serum (range: $0.05\text{--}15 \times 10^6$ U/ml) and no anamnestic evidence of acute hepatitis within the previous 6 mo. Fibrosis was present in 20/23 subjects in liver biopsy. The range of ALT was 22–214 U/ml. All subjects were infected with genotype 1a or 1b (see Table III). The study was approved by the local Institutional Review Board and all subjects gave written informed consent.

IFN- γ ELISPOT Assay. HCV-specific CD8⁺ T cell responses were quantified by ELISPOT assay as described previously (10) using 301 overlapping peptides (20-mer peptides overlapping by 10 amino acids) spanning the entire expressed HCV-H77 genome (genotype 1a), as well as 83 peptides corresponding to optimal described CTL epitopes (32) and autologous virus sequences as indicated. Comparison of autologous and variant epitopes was performed using log₁₀ dilutions of peptides as described previously (10). For quantitation of ex vivo responses, the assay was performed at least in duplicate and background was not >15 spot-forming cells/10⁶ PBMCs. Responses were considered positive if the number of spots per well minus the background was at least 25 SFC/10⁶ PBMCs (33). PHA served as a positive control for T cell stimulation.

⁵¹Cr Release Assay. Cytotoxicity assays were performed as described previously (10). Autologous EBV-transformed B cells were incubated in Na₂[⁵¹Cr]O₄ (New England Nuclear) for 1 h at 37°C in 5% CO₂. The cells were washed three times with cold R10 media (RPMI 1640, 10% FCS, and 10 mM Hepes buffer [all obtained from Sigma-Aldrich] with 2 mM glutamine and antibiotics [50 U/ml penicillin-streptomycin]) and incubated with log₁₀ dilutions of peptides and effector cells at 37°C for 4 h at an E:T ratio of 30:1. Cellular release of ⁵¹Cr into the supernatant was measured using a Top Count Microplate scintillation counter (Packard Instrument Co.), and the percent specific cytotoxicity was calculated by the formula % lysis = [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100. Results are reported as the mean of triplicate values, with a standard deviation of <5%.

Intracellular Cytokine Staining (ICS). ICS for IFN- γ was performed as described previously (10). In brief, 10⁶ PBMCs were incubated with 4 μ g/ml peptide at 37°C and 5% CO₂ for 1 h before the addition of Brefeldin A (1 μ l/ml; Sigma-Aldrich). The cells were incubated for an additional 5 h at 37°C and 5% CO₂. PBMCs were washed and stained with surface antibodies, allophycocyanin-conjugated anti-CD8, and PE-conjugated anti-CD3 (Becton Dickinson) at 4°C for 20 min. After the washing, the PBMCs were fixed and permeabilized (Caltag), and the FITC-conjugated anti-IFN- γ mAb (Becton Dickinson) was added. Cells were washed and analyzed on a FACS-Calibur flow cytometer using CELLQuest software (Becton Dickinson). Data analysis was performed with the FlowJo software package (TreeStar).

HLA Class I-Peptide Tetramer Staining. An HLA class I-peptide tetramer specific for the targeted B8-1395 epitope (HS-KKKCDEL) was synthesized as described previously (34). Tetramer staining was performed for 20 min at 37°C using $0.5\text{--}10^6$ PBMCs as described previously (4). After washing for 5 min with PBS containing 1% FCS at room temperature (RT), cells were pelleted and directly stained with CD8-PerCP (from Becton Dickinson). All staining was performed in PBS in the presence of 10% goat immunoglobulin. Background staining was 0.05% of the CD8⁺ T cell population in an HLA-unmatched control. Staining was considered positive in 99B if tetramer positive cells

formed a cluster distinct from the tetramer negative CD8⁺ T cell population and the frequency of tetramer positive cells was >0.1%. Background was higher for 02J; therefore only stainings >0.4% of the CD8⁺ T cell population were considered positive in this subject.

Bulk Stimulation of PBMCs. To establish CD8⁺ T cell lines, cryopreserved or fresh PBMCs ($4\text{--}10 \times 10^6$) were stimulated with 1 μ g/ml of synthetic HCV peptide and 0.5 μ g/ml of the costimulatory antibodies anti-CD28 and anti-CD49d (Becton Dickinson) in R10 media. Recombinant IL-2 (25 IU/ml) was added on day 2 and twice a week thereafter. Cells were restimulated with 25×10^6 irradiated PBMCs after 2 wk.

Sequencing of Autologous Virus. Viral RNA was extracted from plasma samples using the vRNA extraction kit (QIAGEN). Where necessary, samples were pretreated with 1 U/ μ l heparinase I (Sigma-Aldrich) before RNA isolation. Specific primers were designed for genotypes 1a and 1b based on alignments of all available sequences from the public HCV Database (<http://hcvpub.ibcp.fr>). In a combined reverse transcription and first round PCR step, a 1,288-bp fragment for genotype 1a (primers 4a-F, 5'-AGTGC-CCCAGAGCTTCCAGG-3' and 4d-R, 5'-ACCCAGGTGC-TCGTGACG-3') and a 1,361-bp fragment for genotype 1b (primers 4a-F, 5'-ATGGAACACTACYATGCGG-3' and 4d-R, 5'-CCAGGTGCTVGTGACGACC) were amplified. Nested primers amplifying overlapping internal fragments were used in a second round PCR as follows: for genotype 1a, 4b-F, 5'-GTAA-GAGCACCAAGGTCCC-3'; 4b-R, 5'-GCAGTCTATCAC-CGAGTCG-3'; 4c-F 5'-CGAGGAGGTTGCTCTGTCC-3'; and 4c-R 5'-AGCACAGCCYCGTCATAGC-3'; for genotype 1b, 4b-F, 5'-AAGGACCATCACCACGGG-3'; 4b-R 5'-CATTAGACGCTCTGTTGC-3'; 4c-F, 5'-CTATGGCAAA-GCCATCCC-3'; and 4c-R, 5'-GGTGTATTAGGTAAAGC-CCG-3'. Using the QIAGEN One-Step RT-PCR kit, RT-PCR cycling conditions were as follows: 50°C for 60 min and 95°C for 15 min, followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, 1.5 min at 72°C, and a final extension of 68°C for 20 min. Nested PCR conditions were 35 cycles of 30 s at 94°C, 30 s at 62°C, 1 min at 72°C, and a final extension of 68°C for 20 min using a high fidelity Taq DNA polymerase (titanium Taq DNA polymerase; CLONTECH Laboratories, Inc.). PCR fragments were gel or PCR purified (QIAGEN kit) and the population was sequenced bidirectionally on an ABI 3100 PRISM automated sequencer. When necessary, PCR products were also cloned (TOPO TA; Invitrogen) and sequenced. To avoid quasispecies selection due to primer specificities, clonal data from two different primer pairs were generated resulting in similar clonal frequencies. Sequencher (Gene Codes Corp.) and MacVector 4.1 (Oxford Molecular) software programs were used to edit and align sequences. These sequence data are available from GenBank under accession nos. AY707283-312 and AY712793-937.

mRNA Transfection of B Cells. An 837-bp fragment containing the epitope region was amplified from subject 02J in weeks 7, 15, and 57 (using primers 4b-F and 4c-R) and cloned. These clones served as template for a second round PCR using a forward primer containing a T7 promoter sequence (5'-TAATACGACTCACTATAGGGAGAGCCACCATGGATG-AGTGCCACTCCACG-3') and a reverse primer containing an additional sequence coding for the described HLA-B8-restricted HIV epitope FL8 (FLKEKGG) followed by a stop codon (5'-TCACAGTCCCCCCTTTTCTTTTAAAAAATTGAGTGCG-GCAGACAGC-3'). The constructs (479 bp) were confirmed to yield the correct sequence and served as a template to generate mRNA (mMessage T7 machine; Ambion) with a poly (A)

tail added (poly (A) tailing kit; Ambion). 10^6 HLA-B8–positive, EBV-transformed, immortalized B cells were washed twice with Optimum media and incubated with 20 μ g mRNA or mock for 10 min on ice. Cells were electroporated (300 mV, 0.5 ms; GenePulse; Bio-Rad Laboratories) and directly transferred to a plate containing R10. After 16 h, cells were washed once in R10 and cocultured with effector cells at a 5:1 E:T ratio for 1 h.

HLA Typing. HLA typing was performed by the Tissue Typing Laboratory at the Churchill Hospital in Oxford, and the Massachusetts General Hospital Tissue Typing Laboratory using standard serological and molecular techniques (35).

Phylogenetic and Selection Analysis. Maximum likelihood (ML) phylogenetic trees were estimated by using the PAUP* package (36) as described previously (29). Potential positively selected sites were identified using two phylogenetic methods. The ML approach implemented in the CODEML program (37) allows assessment of the evidence for positive selection and estimation of dN/dS ratios at individual codons, with values >1 suggestive of selection. In a second approach, all amino acid changes were mapped on an ML phylogenetic tree by using the parsimony algorithm implemented in the MacClade program. As described previously (29), sites had to fulfill one out of two criteria to be deemed candidates for positive selection: (a) there were synapomorphic changes, occurring on the internal branches of the tree, which indicates that they have been transmitted through the population, and (b) the site showed mutation of the same amino acid on multiple terminal branches, which is likely to indicate a convergent selective change that has arisen independently in multiple lineages. For sequences derived from subjects with chronic infection, the numbers of sequences with and without a nonsynonymous substitution as aligned to the consensus of all sequences in HLA-B8–positive and HLA-B8–negative subjects was determined and compared using Fisher's exact test.

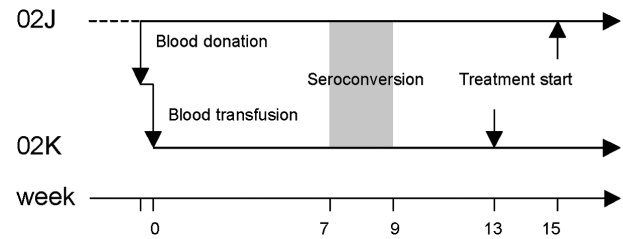


Figure 1. Transmission pair: time course of infection. 02J donated blood that was transfused into 02K 2 d later. Week 0 represents the day of the transfusion event for the transmission pair. Subject 02J had not seroconverted by the time of the blood donation. Both subjects seroconverted subsequently between weeks 7 and 9, suggestive of infection of the donor 02J shortly before the blood donation.

Online Supplemental Material. ML phylogenetic are included in Fig. S1. Using the parsimony algorithm implemented in the MacClade program, tracking mutations that continue to fixation in subject 99B and 02J are tracked. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20041006/DC1>.

Results

Evolution of T Cell Responses to HCV Proteins in Acute Infection. Three HCV positive subjects, 99B (needle stick injury) and a transmission pair 02J (Fig. 1, donor) and 02K (recipient), were screened for cellular responses using an IFN- γ ELISPOT assay (Table I). Screening of fresh and/or

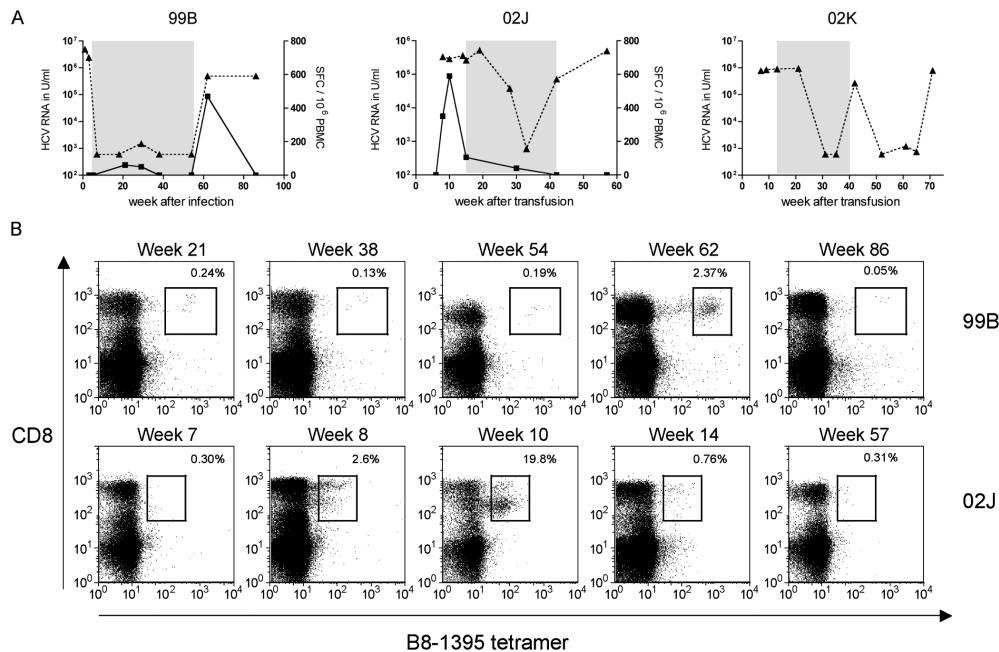


Figure 2. Decline of B8-1395 T cell responses despite persistent viremia. (A) 99B and the transmission pair 02J (donor) and 02K (recipient) presented with acute infection and had persistent high level viremia (HCV-RNA; \blacktriangle). During treatment (shaded area) viral load declined to undetectable limits, but all three subjects either relapsed after cessation of therapy or had a viral breakthrough. Longitudinal results for the epitope B8-1395 (\blacksquare) as determined by ELISPOT are shown for 99B and 02J as spot-forming cells per million (SFC/ 10^6 cells). No CTL response was detected in 02K. (B) A tetramer for the HLA-B8–restricted B8-1395 response was used to follow the response in 02J and 99B longitudinally and is shown here as percentage of all CD8 $^+$ T cells. In 99B, the response expanded after cessation of therapy by week 62 (2.3%) when viral load relapsed, but subsequently declined despite persistent high level viremia. In 02J, the response expanded before treatment by week 10 (19.8%) and declined thereafter despite persistent high level viremia before treatment was started. Staining was considered positive in 99B if tetramer-positive cells formed a cluster distinct from the tetramer-negative CD8 $^+$ T cell population and the frequency of tetramer-positive cells was $>0.1\%$. Background staining was higher in 02J; therefore only stainings $>0.4\%$ of the CD8 $^+$ T cell population were considered positive in this subject.

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Table II. *Viral Evolution within CD8 Epitope B8-1395*

H77	Week	B8-1402 ^a																										
		H	L	I	F	C	H	S	K	K	K	C	D	E	L	A	A	K	L	V	A	L	G	I	N	A	V	A
		<u>B8-1395</u>																										
Bulk seq data^b	99B	5	S	G
		60	R	F	S	G
		191	r	r	f	S	G
		252	F	S	G
	02J	7	t	V	.	.
		15	d	f	V	.	.
		30	d	v	V	.	.
	02K	7	R	t	V	.	.
		14	r	V	.	.
		71	V	.	.
Clonal seq data	99B	5	9/14	G	S	G		
			2/14	S	G	
			1/14	S	G	.	.	V	.	.	.	
			1/14	P	.	.	.	S	G	
		60	1/14	M	.	.	S	G	
			11/12	R	F	.	.	.	S	G	
			1/12	R	F	T	.	.	S	G	
			9/13	F	.	.	.	S	G	
			4/13	R	R	S	G	
		252	16/20	F	.	.	.	S	G	
			1/20	P	F	.	.	.	S	G	
			1/20	F	.	.	.	S	G	F	.	.	T	.	.	
			1/20	F	.	.	.	S	G	.	.	T	.	.	.	
	1/20		F	V	.	.	S	G		
	02J		7	13/26	V	.	.
				7/26	T	V	.	.
		3/26		R	V	.	.	
		1/26		R	T	V	.	.	
		1/26		R	T	V	.	.	
		1/26		
	15	4/8	F	V	.	.		
		4/8	D	V	.	.		
		8/10	V		
		1/10	V	T	.	.		
	02K	7	1/10	R	V	S	.	.		
			06/9	R	V	.	.	
		14	3/9	R	T	V	.	.	
9/19			R	V	.	.		
7/19			V	.	.		
71		3/19	R	T	V	.	.		
		13/14	V	.	.		
		1/14	R	V	.	.		

These sequence data are available from GenBank/EMBL/DDBJ under accession no. AY712793-712937.
^aNote that a second overlapping HLA-B8-restricted CD8 epitope (B8-1402) was not targeted by 99B or 02J.
^bLowercase letters in the bulk data indicate sites of mixed bases coding for different amino acids.

frozen PBMCs at multiple time points between 3 and 80 wk after infection, including the earliest available time point, revealed a dominant T cell response to the identical peptide in both 99B and 02J (Fig. 2 A), as well as one additional T cell response in 99B (Table I). Subjects 99B and 02J both mounted a CD8 T cell response targeting the previously described HLA-B8–restricted epitope B8-1395 (HS-KKKCDEL; reference 38), which was confirmed using HLA-matched B cell lines and ICS assays (not depicted), as well as by tetramer analysis (Fig. 2 B). Ex vivo ELISPOT screening at multiple time points before, during, and after treatment detected no CD8 T cell responses in subject 02K.

MHC class I tetramers were used to follow the B8-1395 (HSKKKCDEL) CD8 T cell responses over the course of infection in subjects 99B and 02J (Fig. 2 B). In subject 99B, the B8-1395 response was not detectable by ELISPOT before treatment (Fig. 2 A), and due to limitations of cell numbers, we were not able to perform a tetramer staining from the first available time point. By week 21, while the subject was undergoing therapy with viral loads at or below detection limit in the quantitative assay, the response was detectable at a low frequency (Fig. 2). During the relapse after cessation of therapy when the viral load rebounded, this response increased to a level of 2.3% of HCV-specific CD8⁺ T cells. However, upon follow-up 6 mo later, the response against B8-1395 became undetectable despite persistently high levels of viremia.

In subject 02J, the HLA-B8-1395–specific response represented the only CD8⁺ T cell response detected. Although this response was not detectable 7 wk after the blood transfusion to 02K, in the following 4 wk during persistently high viremia, this response peaked to levels of >19% CD8⁺ T cells (Fig. 2 B), and then rapidly declined despite high viral loads that eventually prompted initiation of anti-HCV therapy. The HLA-B8-1395–specific response continued to decline during treatment and did not reappear by week 40 or thereafter despite viral breakthrough (Fig. 2 B and not depicted). The same course of the response was observed using longitudinal ELISPOT assays (Fig. 2 A), indicating that specific cells were continuously able to secrete IFN- γ .

Viral Sequencing Reveals Sequence Evolution Coincident with Declining CD8 Responses. Next, we investigated whether viral evolution and immune escape might be responsible for the observed decline in these CD8 T cell responses. Sequence data from the HLA-B8-1395 epitope in subject 99B, aligned to the prototype H77 sequence, revealed evolution in the targeted region (Table II). The sequence before treatment (week 5) largely corresponded to the H77 prototype sequence, with minor variations at the clonal level. Two mutations developed at residues 4 and 9 of the epitope (HSKRKCDEF) by week 60, the first available viremic time point after discontinuation of treatment at week 54. This region continued to evolve over time toward a mixed population by week 191 (HSRRKCDEL and HSKKKCDEF), with the latter variant becoming fixed by week 252. No virus with the original dominant sequence was detectable by clonal analysis at week 60 or any later time point (Table II).

In subject 02J, the dominant infecting sequence within the HLA-B8 epitope was identical to that in subject 99B (Table II). By week 15 after transmission, bulk sequence data from the single targeted region illustrated the development of mixed populations of aspartic acid and phenylalanine mutations in positions 8 and 9 of the epitope (HS-KKKCDf). Clonal data confirmed the presence of two unique populations of virus with individual substitutions at each position (HSKKKCDDL and HSKKKCDEF) occurring with equal frequency and an absence of initial sequences (Table II). Development of these mutations, before treatment initiation, was coincident with the decline of this CD8⁺ T cell response (Fig. 2, A and B), and this region of NS3 continued to evolve toward a valine at position 9 (HSKKKCDEV) ultimately becoming fixed. In the COOH-terminal flanking region of the epitope, an additional change (alanine to threonine) was detectable early in infection as a mixed population that subsequently became undetectable by week 15.

Analysis of the dN/dS ratios yielded values >1 for position 9 of the B8-1395 epitope in subject 99B and positions 4, 6, and 9 of the epitope in subject 02J, suggesting positive selection at these sites (Fig. 3). In a second approach using a parsimony algorithm, the observed mutations were mapped on an ML phylogenetic tree (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20041006/DC1>). This analysis indicated selection within the epitope of the leucine to phenylalanine mutation in position 9 in subject 99B and the leucine to valine mutation in position 9 in subject 02J. Thus, for both of these HLA-B8 positive subjects the dominant CD8 response was directed against the same

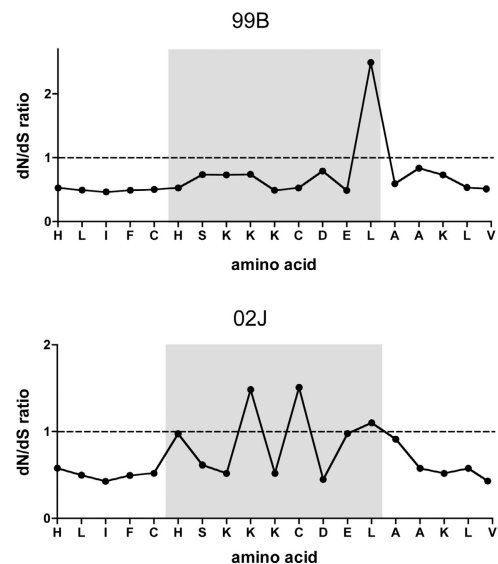


Figure 3. dN/dS values of acute subjects 99B and 02J. Analysis of dN/dS values suggests selective pressure at position 9 in the targeted B8-1395 epitope (shaded area) in subject 99B and positions 4, 6, and 9 in subject 02J (ratio > 1; dotted line). The residues are labeled with amino acids represented in the H77 reference sequence. ML phylogenetic trees were estimated by using the PAUP* package and dN/dS values were calculated using the CODEML program.

Table III. HLA-B8-associated Selective Pressures in B8-1395 at the Population Level

		B8-1402																																						
Genotype		I	K	G	G	R	H	L	I	F	C	H	S	K	K	K	C	D	E	L	A	A	K	L	V	A	L	G	I	N	A	V	A	Y	Y	R	G			
HLA-B8 positive	1a	R	T	
	1a	D	.	.	R	
	1a	R	
	1a	V	
	1a	D	.	S	R	V		
	1a	
	1a	R	
	1a	F	
	1a	R	V	F	.	.	.	
	1a	V
	1a	V
	1a	R
	1a
	1b ^a	R	S	S	.	L	
HLA-B8 negative	1a	V	
	1a	
	1a	V
	1a	V
	1a	V
	1a
	1a	
	1a
	1a
	1a
	1b	S	G	.	L
	1b	S	S	.	L
	1b	S	G	.	L
	1b	S	G	.	L
1b	S	G	.	L	
1b	S	G	.	L	

These sequence data are available from GenBank/EMBL/DDBJ under accession no. AY707283-312.

^aNote that some differences in the COOH-terminal flanking region of the epitope are specific for genotype 1b.

epitope, and in both persons epitopic mutations progressing to fixation were selected.

Evidence for HLA-B8-associated CD8⁺ T Cell-selective Pressures in the B8-1395 Epitope at the Population Level. Due to the observed viral evolution in epitope B8-1395 in each of these two HLA-B8-positive subjects, we hypothesized that escape from this CD8 response in chronic HCV infection may be a common phenomenon. We obtained sequence data from additional randomly selected HLA-B8 positive ($n = 14$) and HLA-B8-negative ($n = 16$) individuals with chronic HCV infection. 8 out of 14 (57%) HLA-B8-positive individuals exhibited sequence variation within the B8-1395 epitope relative to the H77 genotype 1a reference sequence (Table III). In contrast, none of the 16 HLA-B8-

negative subjects (0%) showed any sequence variation, suggestive of HLA-B8-mediated selective pressure against this region of NS3 ($P < 0.001$). The most frequent variant (4/14) was an arginine in position 4 (HSKRKCDEL). In addition, there was a modest increase in polymorphisms in the COOH-terminal region of the dominant HLA-B8 epitope, which is the location of a second partially overlapping HLA-B8-restricted epitope (B8-1402; ELAAKLVAL). Together, these data suggest that sequence polymorphisms within this region of NS3 in persons with chronic HCV infection are associated with HLA-B8-restricted immune pressure.

Impact of Variant Peptides on MHC Class I Binding and T Cell Recognition. The in vivo decline of these B8-1395-specific responses, coincident with sequence evolution, sug-

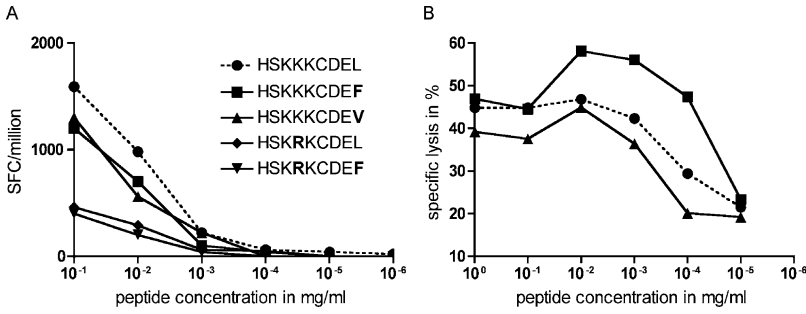


Figure 4. Impact of variant peptides on IFN- γ secretion and cytotoxicity. Variant peptides derived from the sequence data were synthesized and tested in log₁₀ dilutions in an IFN- γ ELISPOT (A) and ⁵¹Cr release cytotoxicity assay (B). Data are shown as spot-forming cells (SFC) per million and specific lysis in percentages. The most frequently detected variant in the chronic subjects (HSKR~~RK~~CDEL; ◆) and the first emerging variant in 99B (HSKR~~RK~~CDEF; ▼) was less efficient in stimulating IFN- γ secretion compared with the wild-type sequence (HSK~~KK~~CDEL; ●). Unexpectedly, fixed variants from later time points of 99B and 02J (HSK~~KK~~CDEF; ■ and HSK~~KK~~CDEV; ▲) did not have any substantial impact on IFN- γ secretion or cytotoxicity.

gested a significant impact of the mutation on T cell recognition. To test this, peptides representing putative escape variants were synthesized and tested in ELISPOT and ⁵¹Cr-release assays using serial dilutions of peptide and B8-1395-specific CD8⁺ T cell lines. The most frequently observed variant in chronically HCV-infected B8-positive subjects (HSKR~~RK~~CDEL) was less efficient than the parental sequence in stimulating IFN- γ secretion and cytotoxicity consistent with a CD8 escape mutation (Fig. 4, A and B). Using the first emerging variant in subject 99B by week 60 (HSKR~~RK~~CDEF), IFN- γ secretion was similarly reduced. However, unexpectedly, peptides representing the fixed variants from subjects 02J and 99B (HSK~~KK~~CDEV and HSK~~KK~~CDEF, respectively) were recognized as well as the initial sequence (Fig. 4, A and B). Binding assays revealed a reduction of the affinity of one variant (HSK~~KK~~CDEV) for the HLA-B8 molecule compared with the prototype sequence (64% reduction of binding), whereas the other observed mutations did not alter MHC binding (unpublished data). These results suggest that the variant peptides could bind sufficiently to HLA-B8 when presented exogenously and that neither MHC binding nor T cell receptor recognition was substantially compromised by the mutations.

Evidence for Impaired Recognition of Endogenously Processed Antigen. To address more physiologically whether the mutations arising in subjects 02J and 99B might be affecting

antigen processing, the wild-type and variant B8-1395 sequences were expressed endogenously to allow for normal processing and presentation of the epitopes within the cytosol and ER of the cell. To accomplish this, mRNA containing the epitope region derived from autologous virus of subject 02J at weeks 7, 15, and 57 was designed. Different clones with the prototype sequence (HSK~~KK~~CDEL) with variant sequences (HSK~~KK~~CDEF and HSK~~KK~~CDEV) and one additional clone harboring an A-T change in the COOH-terminal flanking region (HSK~~KK~~CDEL~~T~~) served as a template. The precise composition of these PCR products was confirmed by sequencing. The template also included on the 3' end a nucleotide sequence coding for the known HLA-B8-restricted HIV nef epitope FL8 (FLKEKGGL) as a positive control. mRNA was transfected into HLA-B8 positive B cells that served as target cells in an ICS assay. B cells transfected with the prototype sequence mRNA (HSK~~KK~~CDEL) were able to stimulate substantially more IFN- γ secretion from the B8-1395-specific T cell line (16.0%) compared with the variant mRNA constructs HSK~~KK~~CDEF and HSK~~KK~~CDEV (1.8 and 1.9%, respectively), suggesting that the variant sequence was interfering with the ability of the B8-1395 epitope to reach the cell surface and be presented to the T cell (Fig. 5). The observed A-T change in the COOH-terminal flanking region in 02J in week 7 (HSK~~KK~~CDEL~~T~~) did not

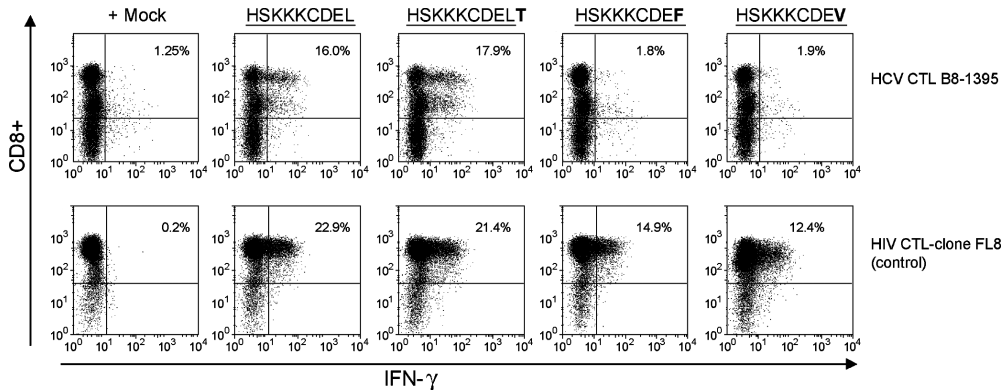


Figure 5. Variant sequence derived from 02J compromises processing and presentation of the B8-1395 epitope. B cells were transfected with mRNA derived from subject 02J representing the acute prototype sequence (HSK~~KK~~CDEL), variant sequences (HSK~~KK~~CDEF and HSK~~KK~~CDEV) and one additional construct with a mutation in the COOH-terminal flanking region (HSK~~KK~~CDEL~~T~~). The transfected constructs included the HIV nef FL8-epitope on the 3' end. In the positive control, the FL8 clone recognized B cells using

all four constructs for transfection. B cells transfected with the prototype sequence (HSK~~KK~~CDEL) stimulated substantially more IFN- γ when cocultured with a B8-1395-specific cell line compared with B cells transfected with the variant sequences (HSK~~KK~~CDEF and HSK~~KK~~CDEV). Numbers in each top right quadrant represent the percentage of IFN- γ -positive CD8 cells.

alter IFN- γ secretion (17.9%). As a control, B cells transfected with all mRNA constructs stimulated IFN- γ secretion when cocultured with the FL8-specific clone, indicating that all constructs were expressed though expression of the two variant constructs was slightly less efficient. After normalizing for transfection efficiency, IFN- γ secretion was reduced between five- and eightfold using the variant sequence in three repeated experiments. These data suggest that the COOH-terminal mutation was altering the normal antigen processing of this epitope by preventing its efficient presentation at the surface of an infected cell.

Reversion of a Transmitted CD8 Escape Mutation. Clinical data indicated that subject 02K was infected through a blood transfusion from subject 02J, which was confirmed by early sequence data from both subjects derived 7 wk after transmission (unpublished data). This provided a unique opportunity to examine whether HCV epitopes differentially evolve in the setting of two different host genetic backgrounds. Surprisingly, comparison of the full-length sequences of these two viruses by bulk sequencing at this first available time point revealed only three nucleotide differences, two of which resulted in altered amino acids located in NS3 at position 1398 and in NS5 at position 2413 (unpublished data). Therefore, one of the nonsynonymous substitutions in 02K resided within the B8-1395 epitope observed previously to escape in subject 02J (Table II) and represented one of the potential sites under selective pressure in the donor 02J (Fig. 3). These data suggested that a viral escape mutant from this B8 epitope in 02J was transmitted to 02K and represented the establishing infecting strain in 02K. In fact, clonal sequence data from donor 02J at the first available time point, 7 wk after the transfusion event, revealed a minor proportion (1/26) of this variant (Table II). Follow-up analysis in 02K revealed that, over time, this transmitted arginine mutation was replaced by the lysine residue that represents the consensus sequence of the HCV 1a strain. These data suggest that the virus infecting 02K harbored the HLA-B8-1395 escape mutation HS-KR_KCDEL, and that continuous replication of this virus in the absence of HLA-B8-restricted CTLs enabled reversion of this mutation back to wild-type sequence.

Discussion

The means by which HCV is able to establish a persistent infection in the face of an activated host immune response are still unclear. It is believed that strong and broadly directed CD4⁺ and CD8⁺ T cell responses play a role in containment of the virus in the acute phase of infection (2–6). Therefore, one possible mechanism of immune evasion is the emergence of escape variants that impair recognition by established CTL responses as described for other viral infections (22–25). Here, we demonstrate escape from a dominantly targeted CTL epitope in two subjects with acute HCV infection, resulting in the rapid *ex vivo* loss of these responses. These data indicate that the cellular immune response is capable of exerting strong selective pressure on the virus during the acute phase of HCV infection.

A strong association between persistence of HCV viremia after acute infection and the emergence of escape variants has been shown in the chimpanzee model (27). However, direct evidence of viral escape and selective pressure of CTL in humans with HCV infection has been more difficult to illustrate. The current paper provides convincing evidence of immune selection pressure in both the acute and chronic phases of infection for an epitope that is a dominant target of the initial immune response. Although the mutations did not appear to impact MHC class I binding or TCR recognition, intracellular expression of the variant epitope was able to illustrate an impact of these mutations upon antigen processing and presentation.

Recent studies in HIV-1 directly illustrate that mutations that alter antigen processing can be selected *in vivo* (39, 40), and one paper demonstrated that these mutations can also reside within the epitope (41), suggesting that the frequency of escape mutations might be underestimated using synthetic peptides. A recent paper also now demonstrates for an HCV epitope altered processing by a mutation in the COOH-terminal flanking region of a frequently targeted HLA-A2-restricted epitope (42). In the present case, presentation of endogenously processed antigen revealed the important impact of an immunologically selected mutation. The power of assays using endogenously synthesized antigen is clear, but previous studies have been limited largely because they relied on the cumbersome generation of recombinant vaccinia virus constructs for infection of B cells. Here, we apply a novel method based on transfection of target cells with synthetic mRNA encoding a portion of HCV NS3 derived from clinical isolates before and after antigenic escape. Our method is based on a technique originally developed for delivery of tumor antigens to dendritic cells (43). With this simple and rapid technique, we were able to demonstrate that the observed sequence variation in the B8-1395 epitope compromised its presentation and recognition.

In addition to the acute immune escape observed within the B8-1395 epitope, chronically infected patients expressing the HLA-B8 allele also demonstrated sequence polymorphisms within B8-1395. This was in stark contrast with all other HLA-B8 negative subjects who expressed the prototype H77 sequence. This analysis demonstrates that selective pressure leading to escape in this region was associated with the HLA-B8 allele. More importantly, these data suggest that mounting of this response in HLA-B8 positive subjects must also be relatively common. In our cohort, no corresponding B8-1395-specific CTL response was detectable in the studied HLA-B8 positive subjects with chronic infection, even after a single round of peptide-specific stimulation (unpublished data). Assuming there was a CTL response that has selected for the escape mutation, the fate of this T cell population is unclear. It has recently been shown in SIV-infected macaques that an escape mutation in a CD8 epitope that rapidly reverts in the absence of the restricting HLA allele is maintained in the presence of a barely detectable CD8 response (44). These data and the fact that the mutations were stable in chronic HCV-infected subjects suggest that selective pressures are still

present in the chronic phase and simply not detectable with our assays in PBMCs.

Recent studies in HIV-1 and SIV suggest that CD8 escape mutations can be unstable and may revert upon transmission to a new host in which the original selecting force is absent (39, 44, 45). The data presented here suggest that a similar phenomenon can occur in HCV infection. In all three subjects with acute infection, the K-R mutation in position 4 of the B8-1395 epitope was unstable and eventually replaced by the consensus residue for this position. However, in both HLA-B8-positive subjects, a second escape mutation became predominant in position 9 (L-V or L-F, respectively) that continued to fixation. These data suggest that the mutation in position 9 that affects presentation of the endogenously processed epitope has a selective advantage over the K-R mutation in position 4. One may speculate that the observed compromised T cell recognition by the K-R mutation in position 4 provides a short-term benefit, but is only transiently selected until the more advantageous processing mutation in position 9 develops. However, as the K-R mutation is the most frequently observed polymorphism in subjects with chronic HCV infection, it therefore seems unlikely that it is generally unstable in all subjects. Transient escape mutations, arising before fixation of another mutation, have also been described in the HCV-infected chimpanzee model as well as for other viral infections, such as HIV (27, 39, 46).

Similarly, the data from subjects with chronic HCV infection illustrate strong purifying selection pressure on this region of NS3. HLA-B8 represents a relatively frequent allele in the Northern American population with a genotype frequency of 10% in Caucasians and 5% in African Americans (47). Thus, in the absence of reversion, variants would be expected to be present at least in some HLA-B8-negative subjects with chronic infection. The complete lack of sequence polymorphisms in all HLA-B8-negative subjects supports reversion of mutations in the B8-1395 epitope in the absence of T cell-mediated selection pressures. The implications of the observed reversion are twofold. First, they suggest that despite viral escape and transmission of these mutations, some mutations may not continue to accumulate on a population level, which might otherwise result in deletion of that epitope over the course of the epidemic. Second, they suggest that some CD8 escape mutations may exact a fitness cost to the virus, much like HIV-1-associated drug resistance mutations (48) that require the virus to revert where possible to the more fit wild-type sequence (49).

It was surprising that a variant clone present only at low frequency in the donor 02J predominated in the recipient 02K early in infection. Although ELISPOT and tetramer analysis of the first time point of subject 02J did not detect CTL responses in PBMCs, selective pressure may have already been underway but below the detection limit in the peripheral blood. The dominance of this low frequency clone from the donor in the early phase after transmission might be caused by an advantage of the variant clone during primary infection as has been described previously during

vertical transmission (50). However, a recent paper investigating quasispecies complexity after transmission through a blood transfusion did not observe this phenomenon (51). A second possible explanation is that the frequency of the transmitted variant was higher 7 wk earlier at the time the transfusion took place. Unfortunately, no sample of the inoculum was available to further address this question.

Although the present data indicate reproducible selective pressure on HCV mediated through the HLA-B8 allele, and clearly show that CD8 selection pressure influences viral evolution, the overall impact of immune escape on failure to contain HCV in humans is unclear. The limited magnitude and breadth of HCV-specific CTL responses as compared with other viruses such as HIV is striking (33); thus, other mechanisms such as failure to prime a response or secondary failure due to exhaustion or impaired effector function may play a role. The effector and maturation phenotype of the B8-1395-specific cells (CD27⁺/CD28⁺; unpublished data) was not substantially different from previously published studies, where chronically infected subjects as well as subjects that continued to resolve infection were analyzed (4, 52). As described for HIV, clearly not all epitopes escape during the course of infection, some are conserved despite consistently detectable CTL responses over time (26). This likely reflects the balance between the strength of the immune response and the ability of the viral protein to accommodate sequence polymorphisms, and each of these forces is undoubtedly shaping the global sequence diversity of HCV.

In conclusion, these data provide support that virus-specific CD8⁺ T cell responses drive viral evolution during the acute phase of HCV infection in humans and illustrate that viral escape in acute HCV infection is one mechanism that may be contributing to viral persistence. Moreover, the results display the power of analyzing the presentation of endogenously synthesized antigen, which revealed a functional impact of the emerging mutants. The reproducible evolution within a targeted CD8 epitope on the population level suggests that more detailed studies are needed to broadly examine viral evolution in hepatitis C and its impact on CTL recognition.

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