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The Outcome of Hepatitis C Virus Infection Is Predicted by Escape Mutations in Epitopes Targeted by Cytotoxic T Lymphocytes

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Summary

$CD8^+$ cytotoxic T lymphocytes (CTL) are thought to control hepatitis C virus (HCV) replication and so we investigated why this response fails in persistently infected individuals. The HCV quasispecies in three persistently infected chimpanzees acquired mutations in multiple epitopes that impaired class I MHC binding and/or CTL recognition. Most escape mutations appeared during acute infection and remained fixed in the quasispecies for years without further diversification. A statistically significant increase in the amino acid replacement rate was observed in epitopes versus adjacent regions of HCV proteins. In contrast, most epitopes were intact when hepatitis C resolved spontaneously. We conclude that CTL exert positive selection pressure against the HCV quasispecies and the outcome of infection is predicted by mutations in class I MHC restricted epitopes.

Introduction

Spontaneous resolution of hepatitis C virus (HCV) infection is kinetically associated with a robust, durable cytotoxic T lymphocyte (CTL) response targeting multiple HCV epitopes (Cooper et al., 1999; Gruner et al., 2000; Lechner et al., 2000b; Takaki et al., 2000). Why this response fails to contain HCV replication in 70%–80% of the individuals who develop persistent viremia is not clear. Chronic infection is characterized by low frequencies of $CD8^+$ T cells in peripheral blood (Battegay et al., 1995; Cerny et al., 1995; He et al., 1999; Hiroishi et al., 1997; Rehermann et al., 1996; Shirai et al., 1994), but they are not necessarily absent from the liver. Indeed, HCV-specific CTL were detected at higher frequencies in liver than blood using soluble, tetrameric class I MHC molecules (Grabowska et al., 2001; He et al., 1999). Generation of HCV-specific $CD8^+$ CTL lines from the liver of some chronically infected humans and chimpanzees is consistent with this observation and suggests that their elimination is not a requirement for virus persistence (Erickson et al., 1993; Kowalski et al., 1996; Koziel et al., 1992; Wong et al., 1998). Survival of HCV in an organ infiltrated with virus-specific $CD8^+$ CTL might be explained by a defect in cytolytic activity or production of cytokines that limit virus replication. Some $CD8^+$ T cell populations in the blood of chronically infected subjects were described as stunned because they didn't produce γ-interferon when stimulated in vitro with cognate HCV epitopes (Gruener et al., 2001; Lechner et al., 2000b). Why some $CD8^+$ T cells develop a nonresponsive phenotype, and whether this mechanism alone can account for their failure to control HCV replication, is unknown.

Persistence might also be facilitated by mutation of key epitopes targeted by $CD8^+$ CTL. The combination of a very high HCV replication rate, estimated at 10^{12} virions per day (Neumann et al., 1998), and an RNA-dependent RNA polymerase that lacks a proofreading mechanism (Bukh et al., 1995) appears to favor Darwinian selection of variant viruses by humoral and cellular immune responses (Manzin et al., 2000). Dominant B cell epitope(s) located in the hypervariable region 1 (HVR-1) of envelope glycoprotein 2 (E2) mutate and this was recently linked to persistence of HCV infection (Farci et al., 2000). Mutation of class I or II MHC restricted epitopes could also alter the outcome of infection by preventing or delaying clearance of infected hepatocytes by T lymphocytes (Eckels et al., 2000). Amino acid substitutions that inhibit $CD8^+$ CTL recognition have been observed in the HCV quasispecies from chimpanzees (Weiner et al., 1995) and humans (Chang et al., 1997; Kaneko et al., 1997; Tsai et al., 1998) with chronic hepatitis C. The role of these mutations in establishing and maintaining the persistent state remains controversial. One argument against this mechanism is that mutation of several epitopes, perhaps simultaneously, would be required for survival of the virus in the face of a multispecific CTL response. Statistical evidence for positive selection pressure against multiple class I MHC restricted epitopes is also lacking in chronic HCV infection.

Animal models are useful for investigating escape mutations because the nucleotide sequence of the challenge virus is known and genetic polymorphism within the MHC complex can be controlled. Recent studies in a family of SIV-infected rhesus macaques provided the first convincing proof that positive selection pressure by $CD8^+$ CTL drives mutation in class I MHC restricted epitopes in lentivirus infections (Evans et al., 1999). We recently reported that spontaneous resolution of acute hepatitis C in chimpanzees was kinetically associated with an intrahepatic $CD8^+$ T cell response targeting multiple HCV epitopes (Cooper et al., 1999). The goal of this study was to compare viruses from animals with resolved and chronic HCV infections for evidence of immune selection pressure.

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A Class I MHC haplotype of study animals

Animal	Status	Patr A		Patr B	
CB0603	chronic	A0101	A0901	B0101	B1602
CB0609	chronic	A0101	A0401	B0101	B1701
CH503	chronic	A1401	A0401	B1601	B1701
CH497	resolved	A0402	A0601	B2001	B1701

B HCV-1 epitopes in resolved hepatitis C.

Restriction	Epitope	Sequence
A0402	$E1_{306}$	306-CSIYPGHITG-315
	$NS2_{1066}$	1066-INGLPVSARRGREIL-1080
A0601	$E2_{651}$	651-RCDLEDRDRSELSPL-665
	$P7_{781}$	781-KWVPGAVYTFYG-792
	$NS5_{2055}$	2031-MWSGTFPINAY-2041
B1701	$NS4_{1629}$	1629-GAVQNEITL-1637
B2001	$E2_{621}$	621-TINYTIFKI-629
C0601	$E1_{366}$	366-GNWAKVLVVL-375

C HCV-1 epitopes in chronic hepatitis C.

Restriction	Epitope	Sequence
A0101	$NS2_{1011}$	1011-LLGPADGMVSK-1021
	$NS3_{1631}$	1631-VQNEITLTH-1639
A0401	$E2_{588}$	588-KHPDATYSR-596
A0901	$NS4_{1963}$	1963-QWISSECTTPC-1972
B0101	$NS3_{1444}$	1444-YTGDFGSVI-1452
B1601	$NS3_{1446}$	1446-GDFDSVIDC-1454
	$NS5_{1989}$	1989-SDFKTWLKA-1997
B1701	$NS3_{1629}$	1939-DAAARVTAIL-1948
	$NS4_{1939}$	1629-GAVQNEITL-1637

Figure 1. Class I MHC Haplotypes and Epitopes Targeted by Chimpanzees with Acute Resolving and Chronic Hepatitis C

Results

Epitope Variation in a Self-Limited HCV Infection

Spontaneous resolution of acute hepatitis C in chimpanzee CH-497 (Todd) was observed approximately 11 weeks after experimental challenge with the HCV-1/910 virus (Cooper et al., 1999). CTL targeting most structural and nonstructural HCV proteins were detected in serial liver samples immediately before termination of viremia and through 96 weeks of follow-up. $CD8^+$ T cell clones derived from liver at weeks 11 and 78 postinfection recognized eight different epitopes presented by Patr-class I A, B, and C molecules (Cooper et al., 1999) (see Figures 1A and 1B). HCV RNA isolated from a plasma sample taken at week 7 immediately before clearance of viremia (Cooper et al., 1999) was examined for mutations in these epitopes. Sequencing of multiple molecular clones revealed that only the Patr-B1701 restricted $NS3_{1629}$ epitope (GAVQNEITL) contained a mutation that was fixed in the quasispecies mastercopy (Figure 2A). The position seven (P7) isoleucine (I1635) to threonine (T) substitution in this epitope prevented target cell lysis by CTL lines generated from the liver of CH-497 at weeks 78 (Figure 2B) and 11 (Figure 2C) postchallenge. These data suggest that escape mutations could arise rapidly in a limited subset of epitopes in individuals with acute resolving hepatitis C. However, at least in this case, resolution was associated with a multispecific response targeting seven other epitopes that did not change.

Evolution of CTL Epitopes in Chronic Infection

We predicted that persistent HCV replication was facilitated by mutation of multiple class I MHC restricted epitopes. Therefore, the evolution of HCV epitopes was studied in three chimpanzees chronically infected with HCV-1/910 for several years. They included CBO603 (Briggsie), CBO609 (Hulk), and CH503 (Ross).

HCV-specific CTL lines derived from the liver or blood

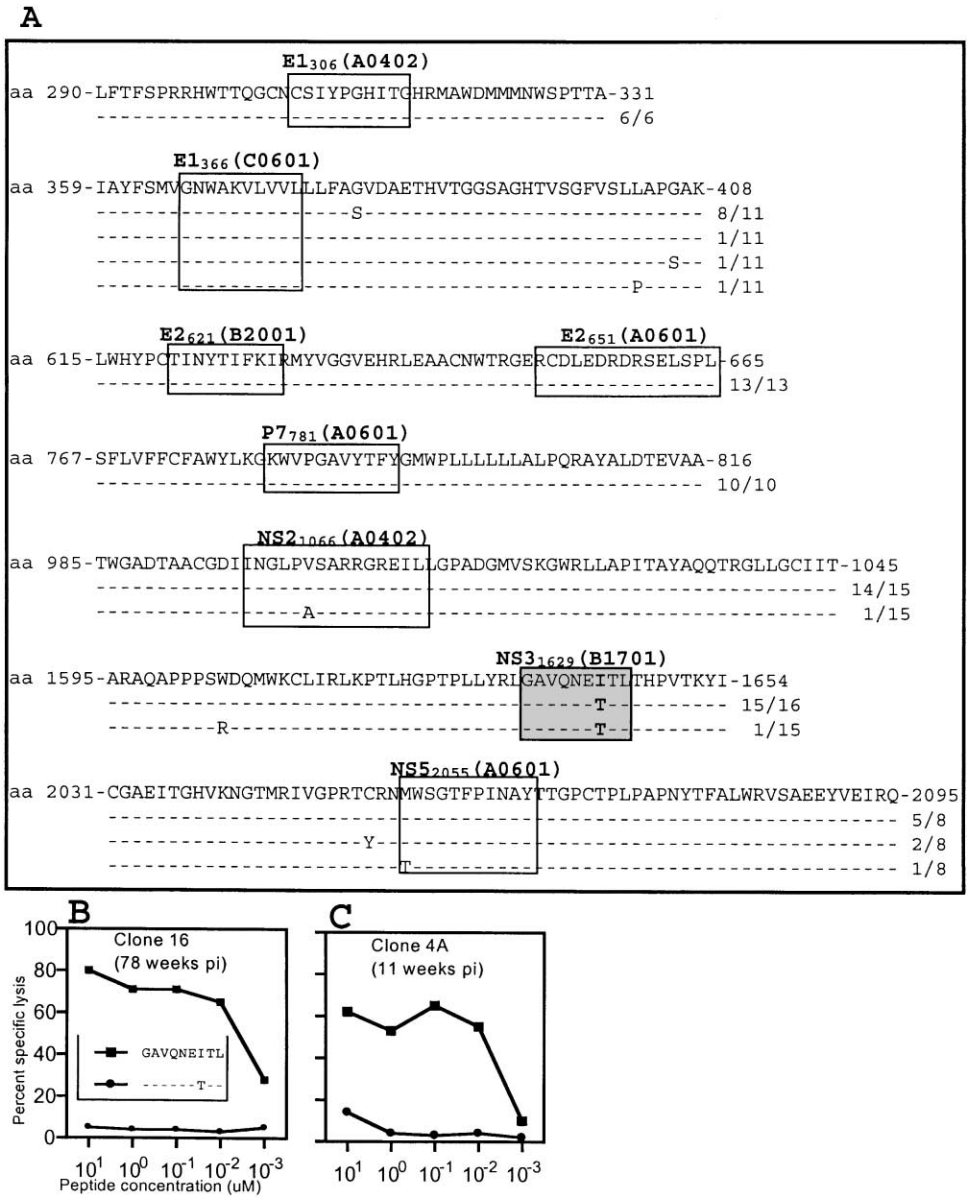

Figure 2. Sequence of Epitopes in Resolved HCV Infection

A plasma sample was collected from CH-497 at week 7 postinfection, the last time point positive for HCV RNA before infection was terminated at week 11 postinfection (Cooper et al., 1999).

(A) Seven regions of the HCV genome encoding eight different CTL epitopes targeted by this animal were amplified by PCR and multiple molecular clones were sequenced. The deduced amino acid sequence of genomes and their frequency are shown. Epitopes are highlighted.

(B) CTL line generated from liver at 78 weeks postinfection was tested for recognition of the $NS3_{1629}$ epitope or the I1635T variant.

(C) CTL line generated from liver at 11 weeks postinfection was tested for recognition of the $NS3_{1629}$ epitope or the I1635T variant.

of chimpanzee CBO609 after 5 years of chronic infection targeted at least five discrete epitopes located in E2 and nonstructural proteins 2, 3, and 4, of the HCV-1/910 virus. The sequence and MHC restriction of these epitopes are shown in Figure 3A. Significantly, all four epitopes examined contained mutations by month 4 (week 16) postinfection that were still fixed in the quasispecies approximately 5 years later (Figure 3A). Mutation of these epitopes was also temporally associated with a narrowly focused intrahepatic CTL response (Cooper et al., 1999). We previously reported that $CD8^+$ T cells expanded from the liver of CBO609 (Hulk) between weeks 14 and 36 postinfection killed target cells expressing NS3 and NS4 but not other structural or nonstructural proteins (Cooper et al., 1999). Interestingly, this CTL activity was predominantly restricted by the Patr-B1701 class I molecule (data not shown) that presented two of the mutated epitopes ($NS3_{1629}$ and $NS4_{1939}$) (Figure 3A). Thus, mutation of NS3 and NS4 epitopes during the first 16 weeks of infection (Figure 3A) coincided with potent but narrowly focused intrahepatic CTL activity.

A CBO609

	A0401	A0101	B0101	B1701	
	$E2_{588}$	$NS2_{1011}$	$NS3_{1444}$	$NS3_{1629}$	$NS4_{1939}$
Month	KHPDATYSR	LLGPADGMVSK	YTGDFDSVI	GAVQNEITL	DAAARVTAIL
4	nt	------R----(12)	--------V(12)	--------T(11)	---G------(11)
9	nt	------R----(12)	--------V(11)	--------T(10)	---G------(13)
60	---S-S-A-(11)	------K----(11)	--------V(10)	--------T(14)	---G------(9)

B CH503

	A0401	B1601		B1701	
	$E2_{588}$	$E1_{233}$	$NS5_{1989}$	$NS3_{1446}$	$NS3_{1629}$	$NS4_{1939}$
Month	KHPDATYSR	GNASRCWVA	SDFKTWLKA	GDFDSVIDC	GAVQNEITL	DAAARVTAIL
3	---------(10)		---------(13)	---E-----(10)	--------P(11)	---V------(13)
10			---R-----(11)		--------S(11) --------P(2) ------T--(1)	-------T--(11)
16	---------(13)	---------(13)	---R-----(14) ---------(4)		--------S(13) ------TA-(1)	--S-------(18)
21	-------T-(9)				--------S(15)	--S-------(8)
42	-------T-(12)	---------(7)	---R-----(8) ---------(1)			
82	-------T-(18)	---------(15)	---R-----(19) ---R---E-(1)	---E-----(20)	--------S(8)	--S-------(19)

C CBO603

	A0901	B0101	B1601/02
	$NS4_{1963}$	$NS3_{1444}$	$NS5_{1989}$
Month	QWISSECTTPC	YTGDFDSVI	SDFKTWLKA
2.5	----------(13)	-----E---(12)	---------(14)
4	----------(11)	-----E---(12)	---------(17)
63	----------(8) ------S----(2) --L-----A--(1) --L--------(13)	-----E---(11)	----V----(23)

Figure 3. Evolution of Class I MHC Restricted Epitopes in Three Chronically Infected Chimpanzees

HCV RNA genomes extracted from serial plasma samples at the indicated time points were amplified by PCR and multiple molecular clones were sequenced and compared with the HCV-1/910 consensus (top row). All epitopes displaying variant sequences are shown. The number of clones of each representative sequence is shown in brackets.

Mutation of epitopes in the other two animals occurred sequentially during the first few months of virus replication. CTL lines generated from the blood and liver of chimpanzee CH-503 (Ross) at month 82 targeted six different epitopes (Figure 3B). We previously reported that the sequence of the Patr-B1601 restricted epitope $NS3_{1446}$ (GDFDSVIDC) was intact after 1 month of virus replication in this animal (Weiner et al., 1995). However, by month 4, a P4 aspartic acid (D1449) to glutamic acid (E) mutation (D1449E) was fixed in the HCV quasispecies mastercopy (Weiner et al., 1995). Analysis of additional plasma samples taken at 3 and 82 months postinfection

confirmed that the same D1449E substitution was fixed in the quasispecies for a very long period of time (Figure 3B). Two additional epitopes of the six examined also mutated within the first 3 months of infection. Evolution of the $NS3_{1629}$ epitope (GAVQNEITL) was complex. The L1637 residue at P9 was replaced with proline (L1637P) by month 3. However, this substitution was remarkably short-lived, since only 15% of the genomes displayed the L1637P change by month 10. The remaining 85% contained the L1637S escape mutation that was completely and permanently fixed in the quasispecies by month 16 (Figure 3B). Similar complexity was observed in the evolution of the second Patr-B1701 restricted epitope, $NS4_{1939}$ (DAAARVTAIL). Substitutions at P4 (A1942V) and P8 (A1946T) were observed at months 3 and 10, respectively, but by month 16 the P3 (A1941S) replacement was fixed in the quasispecies mastercopy (Figure 3B). Changes appeared much later in the $NS5_{1989}$ and $E2_{588}$ epitopes, at 10 and 21 months postinfection respectively (Figure 3B). Only one epitope, $E1_{233}$, did not change even though CTL-specific for this Patr-B1601 restricted peptide were detected in his liver at month 82 postinfection (Figure 3B).

One epitope ($NS3_{1444}$) was mutated at 2.5 months postinfection in chimpanzee CBO603 (Figure 3C). Mutations in two other epitopes ($NS4_{1963}$ and $NS5_{1989}$), detected at month 63 postinfection, must have occurred gradually because they were not present in a plasma sample collected at month 4 (Figure 3C). This might be explained by the weak or undetectable intrahepatic CTL activity in the liver of this animal during the acute phase of infection (Cooper et al., 1999).

These results indicated that in contrast to spontaneous resolution of hepatitis C, persistent replication of the virus was associated with mutation of multiple class I MHC restricted epitopes. Therefore, we analyzed the distribution of mutations in epitopes and flanking regions of the HCV quasispecies from these animals as well as their susceptibility to CTL recognition. All three shared class I MHC haplotypes (Figure 1A), and these have the potential to present several common epitopes (Figure 1C).

Envelope Glycoprotein 2

The Patr-A0401-restricted epitope $E2_{588}$ (KHPDATYSR) located in the E2 glycoprotein (Erickson et al., 1993; Kowalski et al., 1996) was mutated in CH-503 and CBO609, the only two animals that expressed this class I MHC molecule (Figure 4A). Eight independent $E2_{588}$-specific CTL lines established from the liver of CH-503 failed to recognize the S595T substituted epitope (KHPDATYTR, Figure 4A) expressed by viruses circulating in his plasma. Data for one representative CTL line are shown in Figure 4B. The quasispecies mastercopy from chimpanzee CBO609 displayed three amino acid substitutions at positions 4, 6, and 8 of the $E2_{588}$ epitope (KHPSASYAR; Figure 4A). CTL expanded from his peripheral blood with the $E2_{588}$ index peptide did not recognize the triply substituted variant (Figure 4C). CBO603 was the only Patr-A0401 negative animal and the $E2_{588}$ sequence was still intact after five years of HCV-1/910 replication (Figure 4A).

Nonstructural Protein 2

CTL lines targeting $NS2_{1011}$, a Patr-A0101 restricted epitope in nonstructural protein 2 (LLGPADGMVSK), were established from the liver of CBO609. The HCV quasispecies circulating in this animal displayed a glycine (G1017) to lysine (K) substitution (LLGPADKMVSK) (Figure 4D) that prevented CTL recognition of the epitope (Figure 4E). The second Patr-A0101 positive animal (CBO603) did not have detectable $NS2_{1011}$-specific CTL activity in blood or liver (data not shown) and this epitope did not contain any amino acid substitutions that were fixed in the quasispecies mastercopy after 5 years of HCV-1/910 replication (Figure 4D).

Nonstructural Protein 3

The C terminus of the NS3 protein contains a set of overlapping epitopes (Figure 5A). One is a Patr-A0101 restricted epitope, designated $NS3_{1631}$ (VQNEITLTH), that was still intact in CBO603 after 5 years of virus replication even though $NS3_{1631}$-specific CTL were recovered from his liver. The other is a Patr-B1701 restricted $NS3_{1629}$ epitope (GAVQNEITL) targeted by animal CH-497 with acute resolving hepatitis C (Figure 2), and two animals, CH-503 and CB0609, with chronic hepatitis C. The C-terminal leucine (L1637) residue was replaced with a serine (S) and threonine (T) in CH-503 and CBO609, respectively (Figure 5A). All P9 substituted peptides displayed at least a 400-fold reduction in Patr-B1701 binding (Figure 5B), including the variants that dominated in the early (L1637P) and late (L1637S) phases of infection in CH-503 (Figures 3B and 5A). $NS3_{1629}$-specific CTL from the blood of CH-609 did not efficiently recognize target cells pulsed with these P9 substituted peptides (Figure 5C). It is noteworthy that the I1635T escape mutation observed in the HCV quasispecies that was cleared from CH-497 (Figure 2) retained the ability to bind Patr-B1701 (Figure 5B).

NS3 contains a second set of overlapping epitopes designated $NS3_{1444}$ (YTGDFDSVI) and $NS3_{1446}$ (GDFDSVIDC) presented by the Patr-B0101 and Patr-B1601 class I molecules, respectively (Kowalski et al., 1996) (Figure 5D). Both were examined for evidence of CTL selection pressure in the three persistently infected animals.

CH-503

As shown in Figure 3B, the Patr-B1601 restricted epitope $NS3_{1446}$ acquired a P4 D1449E substitution within the first 3 months of infection, and was the only change fixed in this region of the HCV quasispecies almost 7 years later (Figure 5D). Remarkably, a CTL line cloned from the liver of CH-503 at the 7 year point recognized the wild-type $NS3_{1446}$ epitope, but not the D1449E variant (i.e., GDFESVIDC) (Figure 5E). This result is consistent with our previous observation that $NS3_{1446}$-specific CTL lines, established after 4, 9, and 24 months of infection, did not recognize the P4 substituted variant that replicated in his liver (Weiner et al., 1995).

CBO603

Remarkably the same D1449E substitution was also observed in the HCV quasispecies from CBO603 (Figure 5D) even though he did not express the Patr-B1601 class I molecule necessary for presentation of the $NS3_{1446}$ epitope. However, this mutation interfered with presentation of the overlapping $NS3_{1444}$ epitope by Patr-B0101.

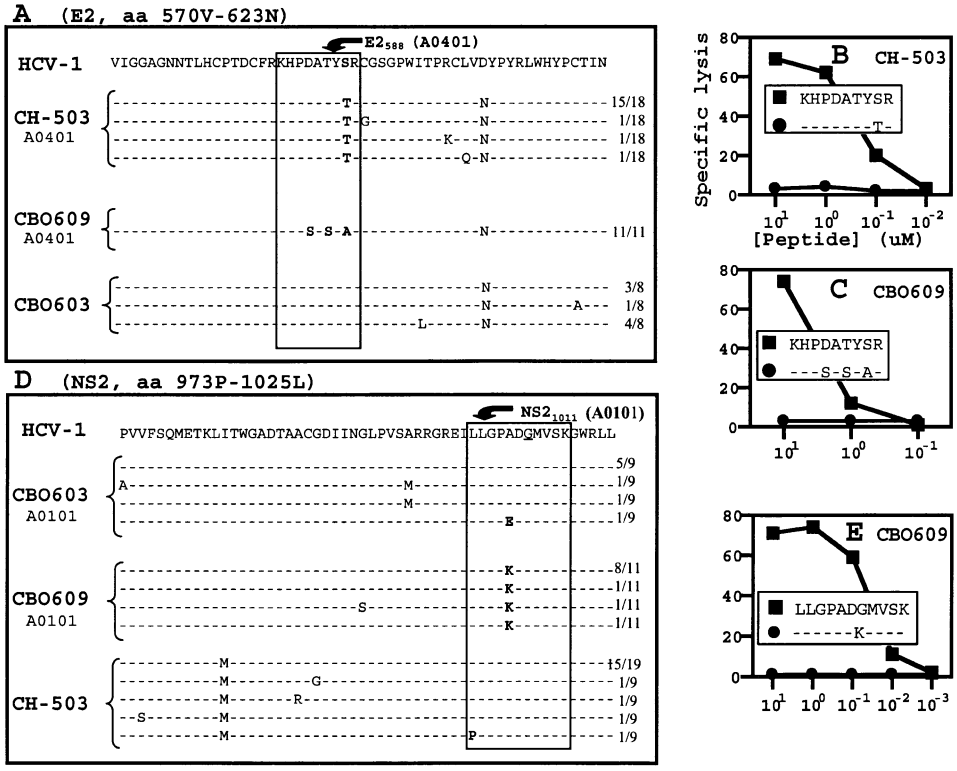

Figure 4. HCV-1 E2 and NS2 Sequences in Chronically Infected Chimpanzees

(A) A region of the HCV-1 E2 gene encoding amino acids 570V–623N was amplified using nested PCR primers from plasma of chimpanzees infected 5 (CBO603 and CBO609) or 7 (CH-503) years earlier with HCV-1/910. Deduced aa sequences and frequencies of individual molecular clones from each animal are shown. Dashes indicate identity with the published consensus sequence of the HCV-1/910 virus used to infect the animals. The Patr-A0401 restricted epitope $E2_{588}$ is outlined and animals that expressed this class I molecule are indicated.

(B) CTL line derived from the liver of CH-503 was tested for lysis of autologous BLCL targets pulsed with varying concentrations of the index HCV-1 $E2_{588}$ epitope or peptides representing the dominant viral sequence from their plasma.

(C) CTL line derived from the blood of CBO609 was tested for recognition of $E2_{588}$ and variant peptides.

(D) A region of the NS2 gene (amino acids 973P–1025L) containing a Patr-A0101 restricted epitope ($NS2_{1011}$) was amplified from the plasma of all three chronically infected chimpanzees.

(E) A CTL line established from the liver of CBO609 after 5 years of chronic infection was tested for lysis of autologous BLCL targets sensitized with peptides representing the $NS2_{1011}$ index epitope or the dominant G1017K substituted variant detected in a contemporaneous plasma sample.

Replacement of D with E at P6 (YTGDFESVI; Figure 5D) impaired recognition by $NS3_{1444}$-specific CTL expanded from peripheral blood (Figure 5F) and caused a 6-fold reduction in Patr-B0101 binding (Figure 5H).

CBO609

All HCV genomes sequenced from this second Patr-B0101 positive animal had an isoleucine to valine replacement at P9 of the $NS3_{1444}$ epitope (YTGDFDSVV) (Figure 5D), which reduced Patr-B0101 binding by approximately 150-fold (Figure 5H). $NS3_{1444}$-specific CTL were present in his peripheral blood but did not efficiently recognize target cells pulsed with the P9 substituted peptide (Figure 5G).

Nonstructural Proteins 4B and 5A

Next, we examined the region flanking the NS4b/NS5a processing site for mutations in three nonoverlapping HCV epitopes presented by Patr-A0901, -B1601, and -B1701 (Figure 6A).

CBO609

Viruses from this animal contained an alanine (A1942) to glycine (G) substitution in the Patr-B1701 restricted $NS4_{1939}$ epitope (i.e., DAAGRVTAIL), which reduced class I MHC binding by almost 60-fold (Figure 6B). CBO609 did not express Patr-A0901 or -B1601 and the $NS4_{1963}$ and $NS5_{1989}$ epitopes that they present were not mutated (Figure 6A).

CH-503

Mutations fixed in the HCV quasispecies from this animal were located exclusively in the Patr-B1601 and -B1701 restricted epitopes (Figure 6A). The Patr-B1701 restricted $NS4_{1939}$ epitope contained an alanine (A1941) to serine (S) mutation (DASARVTAIL) that resulted in a 14-fold reduction in class I MHC binding (Figure 6B). This substitution was unique amongst those observed in this study because target cells pulsed with the variant peptide were efficiently recognized by CTL-specific for the HCV-1/910 index peptide (Figure 6C). The K1992R substitution observed in the Patr-B1601 restricted $NS5_{1989}$ epitope (SDFRTWLKA) was more typical in that it prevented recognition by CTL generated from the liver of CH-503 after 7 years of chronic infection (Figure 6D). This animal did not express Patr-A0901 and predictably the $NS4_{1963}$ epitope was intact (Figure 6A).

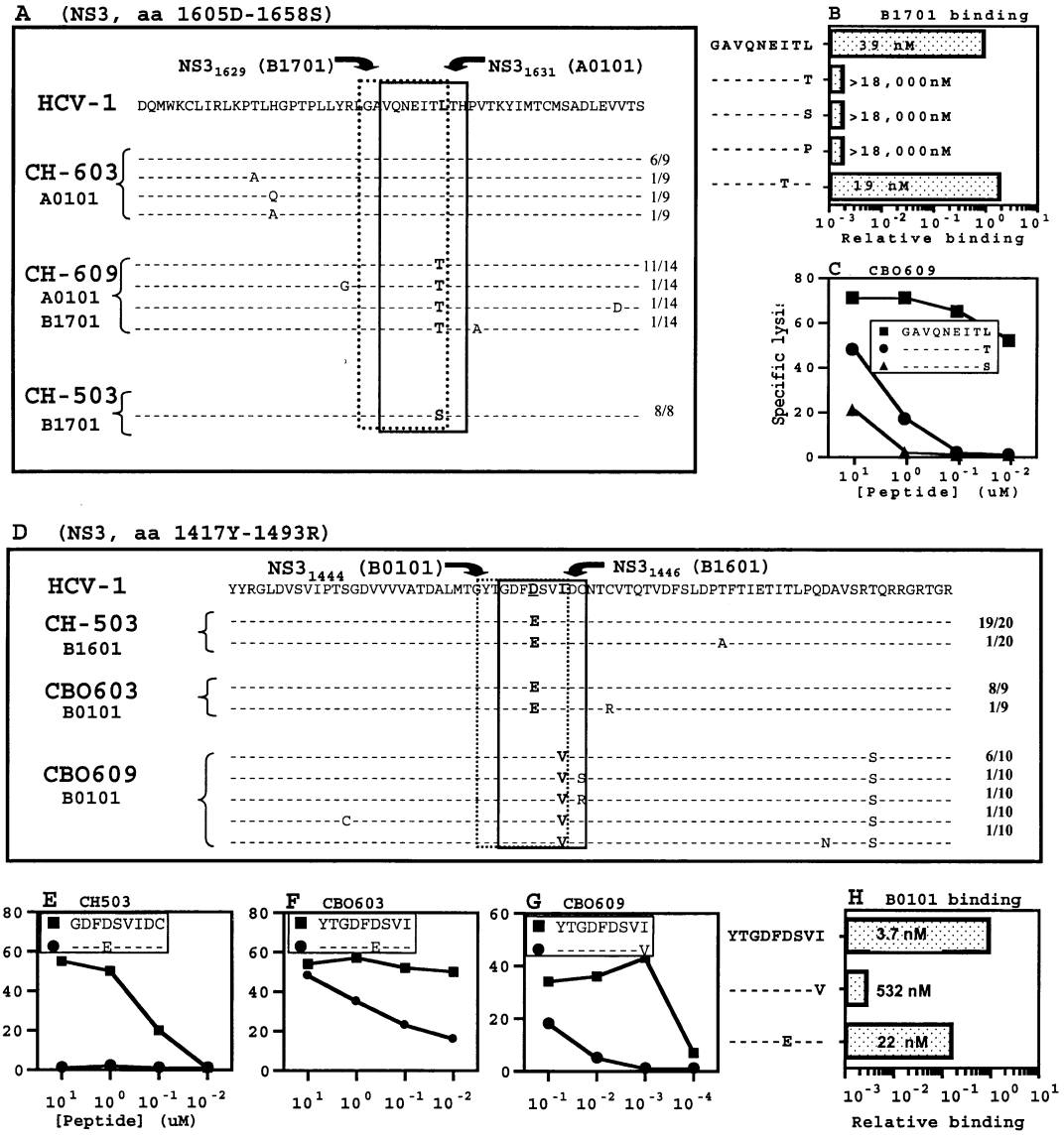

Figure 5. Sequence of Overlapping HCV-1 NS3 Epitopes in Chronic Hepatitis C

A segment of the NS3 gene encoding amino acids 1605D–1658S was amplified from the plasma of three chronically infected chimpanzees as described in the legend to Figure 3.

(A) Amino acid sequences of overlapping epitopes $NS3_{1629}$ (Patr-B1701 restricted) and $NS3_{1631}$ (Patr-B0101 restricted) are outlined.

(B) Relative Patr-B1701 binding capacity of the $NS3_{1629}$ parental peptide and variants found in animals with chronic and resolved infections. The inhibitory concentration 50 (IC50) for each peptide is shown.

(C) A $NS3_{1629}$-specific CTL line generated from the blood of chimpanzee CBO609 was tested for lysis of target cells pulsed with the L1637S and L1637T variants.

(D) A segment of the NS3 gene encoding amino acids Y1417–R1493 was amplified from the plasma of three chronically infected chimpanzees as described in the legend to Figure 3. Amino acid sequences of overlapping epitopes $NS3_{1444}$ (Patr-B0101 restricted) and $NS3_{1446}$ (Patr- B1601 restricted) are outlined.

(E) A CTL line established from the liver of CH-503 at 7 years postinfection was tested for lytic activity against autologous target cells pulsed with the HCV-1 $NS3_{1446}$ index epitope or the $D_{1449}E$ variant (GDFESIVDC) circulating in plasma.

(F) A CTL line generated from the blood of CBO603 was tested for recognition of the $NS3_{1444}$ index and a D1449E substituted peptides.

(G) BLCL target cells sensitized with $NS4_{1444}$ or the I1964V substituted peptide were tested for lysis by an $NS4_{1444}$-specific CTL line generated from the blood of CBO609.

(H) Relative binding capacity to Patr-B0101 binding of the I1452V or D1449E variants and the $NS3_{1444}$ parental peptide. The inhibitory concentration 50 (IC50) for each peptide is shown.

CBO603

Chimpanzee CB0603 is Patr-A0901 positive and approximately 50% of the HCV genomes in his plasma at year 5 postinfection contained a P3 isoleucine (I1964) to leucine (L) substitution in the $NS4_{1963}$ epitope (QWLSSECTTPC) (Figure 6A). CTL lines generated from the liver of this animal failed to recognize the P3-substituted peptide (Figure 6E). CBO603 did not express Patr-B1601 so the

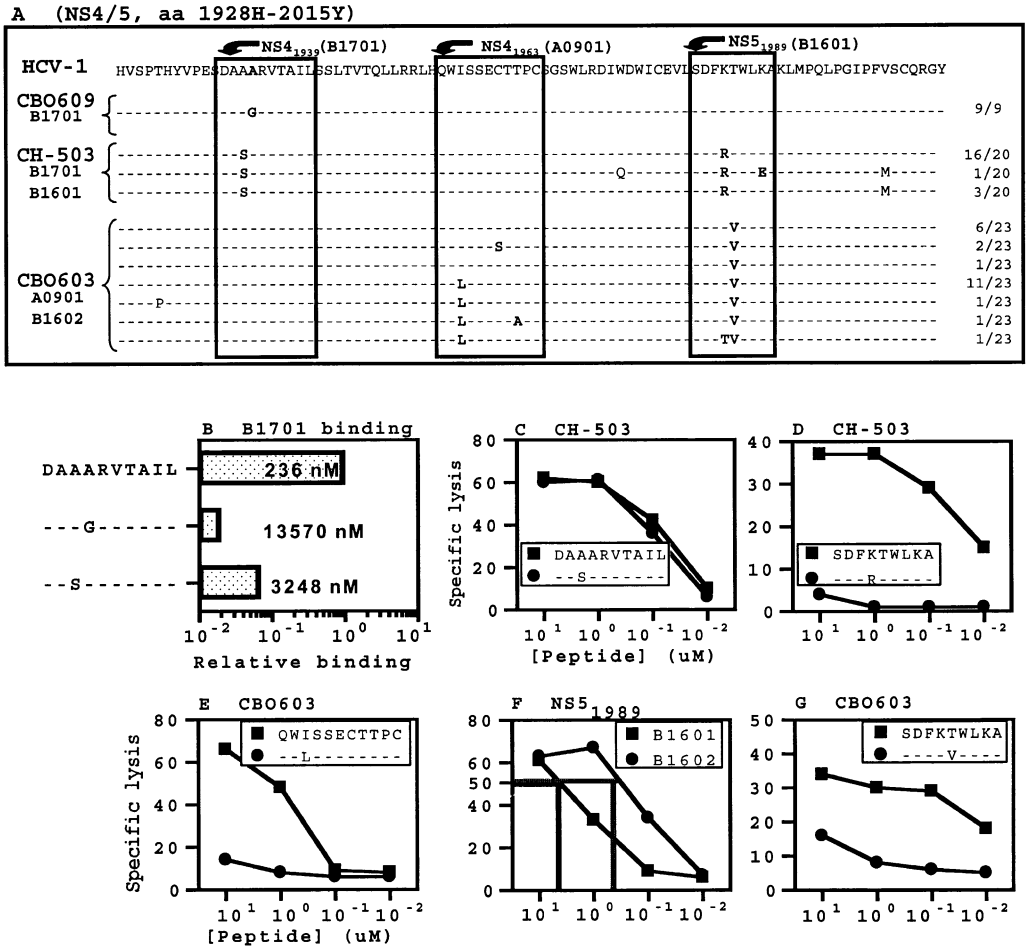

Figure 6. Sequence of NS4b and NS5a Epitopes in Chronic Hepatitis C

(A) Amino acid sequence frequency for three epitopes including $NS4_{1939}$ (Patr-B1701 restricted), $NS4_{1963}$ (Patr-A0901 restricted), and $NS5_{1989}$ (Patr-B1601 restricted).

(B) Reduction in binding to Patr-B1701 by A1942G and A1941S variants detected in chimpanzees CBO603 and CH-503, respectively. Inhibitory concentration 50 (IC50) values for the parental peptide and two variants are shown.

(C) A CTL line derived from the blood of CH-503 was tested for recognition of the A1941S variant peptide on autologous target cells.

(D) A CTL line derived from the liver of CH-503 was tested for recognition of the K1992R variant peptide on autologous target cells.

(E) A CTL line from the liver of CBO603 was tested for recognition of target cells pulsed with the $NS5_{1962}$ epitope and its I1964L variant.

(F) Target cells transfected with the Patr-B1601 or B1602 class I molecules were pulsed with varying concentrations of the parental $NS5_{1989}$ peptide and tested for lysis by a Patr-B1601 restricted CTL line derived from the liver of chimpanzee CH-503. The concentration of peptide required to achieve 50 percent specific lysis is indicated.

(G) An $NS5_{1989}$-specific CTL line derived from the blood of CBO603 was tested for lysis of autologous target cells pulsed with the index peptide or the T1993V variant.

threonine (T1993) to valine (V) mutation in epitope $NS5_{1989}$ (SDFKVWLKA) (Figure 6A) was unexpected. However, he did express Patr-B1602, which differs from Patr-B1601 by a single amino acid at position 76 of the class I MHC heavy chain α1 domain (data not shown). Consistent with this close similarity, we noted that Patr-B1602 could also present antigen to Patr-B1601 restricted T cells. Indeed, presentation of $NS5_{1989}$ was remarkably efficient as 10-fold less peptide was required to achieve 50% lysis of Patr-B1602 versus Patr-B1601 positive targets by a Patr-B1601 restricted CTL line from CH-503 (Figure 6F). Most importantly, Patr-B1602 restricted CTL were expanded from the blood of CBO603 with the $NS5_{1989}$ peptide and they failed to recognize target cells sensitized with the T1993V substituted variant (Figure 6G).

Comparison of Mutation Rates in Epitope Versus Flanking Regions

If $CD8^+$ CTL exert selection pressure then the frequency of amino acid replacement substitutions should be higher in class I MHC restricted epitopes than in flanking regions or nonrestricted epitopes (i.e., epitopic sequences in animals that do not express the appropriate class I MHC molecule). Therefore, we compared the consensus sequence of the HCV-1/910 challenge virus (Choo et al., 1992) with RNA genomes circulating in the three persistently infected animals in order to calculate the rate of synonymous (dS) and nonsynonymous (dN) nucleotide substitutions in epitope and flanking regions. Within flanking regions, dS was approximately 7-fold higher than dN (0.0272 versus 0.0043) (Table 1). An

Table 1. Mean Numbers of Synonymous Substitutions per Synonymous Site (dS) and Nonsynonymous Substitutions per Site (dN) in HCV Epitope and Nonepitope (Remainder) Regions: Comparison with HCV-1/910 Inoculum Consensus Sequence

	ds (± SEM)	dN (± SEM)	p (dS = dN)[a]
Remainder	0.0272 (0.0350)	0.0043 (0.0013)	< 0.001
Epitopes:			
restricted	0.0808 (0.0349)	0.0639 (0.0154)	n.s.
nonrestricted	0.0655 (0.0337)	0.0083 (0.0070)	n.s.
P (restricted=remainder)[a]	n.s.	0.002	
P (restricted=nonrestricted)[a]	n.s.	0.004	

[a] Paired sample T test.

equally high dS:dN ratio was estimated for nonrestricted epitopes (Table 1). These observations are consistent with the principal that synonymous mutations are neutral, but amino acid replacement substitutions are usually deleterious and effectively eliminated by selection pressure (Nei and Gojobori, 1986). One important exception to this pattern was noted. dN was approximately one order of magnitude greater in restricted epitopes (0.0639) than in nonrestricted epitopes (0.0083, $p = 0.004$) or flanking regions (0.0043, $p = 0.002$) (Table 1). This statistically significant increase in dN for class I MHC restricted epitopes is consistent with the hypothesis that mutations described in Figures 3–6 resulted from positive selection pressure and not chance.

The pattern of mutation within class I MHC restricted epitopes is striking, because with few exceptions, the same nonsynonymous nucleotide substitution was fixed in the quasispecies mastercopy for years. Substitutions at secondary sites within the epitope were rarely observed (Figures 3–6). For instance, the Patr-B1601 restricted epitope $NS3_{1446}$ contained a single substitution (D1449E) in all 20 genomic clones established from the plasma of CH503 after 7 years of infection (Figure 5D). A pairwise comparison of sequences from all three chronically infected animals confirmed that dN was not statistically different in class I MHC restricted epitopes (0.0028), nonrestricted epitopes (0.0031), and flanking (remainder) regions (0.0034) (Table 2). This indicated that selection pressure was directional and did not contribute to ongoing diversification of the virus during several years of chronic infection.

Discussion

We previously reported a temporal kinetic relationship between intrahepatic CTL populations and spontaneous resolution of HCV infection in chimpanzees (Cooper et al., 1999). This study provides evidence for mutation of multiple class I MHC restricted epitopes early in the course of chronic HCV infection. The role of $CD8^+$ CTL in control of HCV replication is further reinforced by the statistically significant increase in the nonsynonymous mutation rate in class I MHC restricted epitopes but not unrestricted epitopes or flanking sequences of the viral genome. These data indicate that at least in chimpanzees, amino acid substitutions in class I MHC restricted epitopes are selected and possibly maintained by HCV-specific $CD8^+$ CTL populations that exert positive Darwinian selection pressure.

In animal CBO609 (Hulk) mutation of multiple class I MHC restricted epitopes in the acute phase of infection (Figure 3A) occurred coincident with a strong but narrowly focused intrahepatic CTL response (Cooper et al., 1999). We previously reported NS3- and NS4-specific CTL in the liver of this animal between 14 and 36 weeks postinfection (Cooper et al., 1999), and cytolytic activity was restricted predominantly by the Patr-B1701 class I molecule (data not shown). Mutation of two Patr-B1701 restricted epitopes ($NS3_{1629}$ and $NS4_{1939}$) at week 16 postinfection (Figure 3A) was temporally associated with this acute phase CTL activity, and is consistent with early selection of virus variants that are stable in the liver for several years. A total of 4 epitopes in nonstructural proteins mutated in the first 16 weeks of infection, but exactly how the virus escaped from an apparently multispecific CTL response is not clear. Multiple codominant CTL populations might not be uniformly distributed in an organ as large as the liver. Under this scenario, the virus is required to escape only a limited set of CTL populations found in immediate proximity to an infected hepatocyte or inflamed lobule. Progeny virions could accumulate mutations in different epitopes through successive cycles of infection and replication. If CTL populations infiltrated the liver in a staggered fashion, it is also possible that mutations accumulated serially over the first 4 months of infection in CBO609. In support of

Table 2. Mean Numbers of Synonymous Substitutions per Synonymous Site (dS) and Nonsynonymous Substitutions per Site (dN) in HCV Epitope and Nonepitope (Remainder) Regions: Pairwise Comparison of All HCV Sequence

	ds (± SEM)	dN (± SEM)	p (dS = dN)[a]
Remainder	0.0170 (0.0034)	0.0034 (0.0008)	0.001
Epitopes:			
restricted	0.0237 (0.0094)	0.0028 (0.0002)	0.045
nonrestricted	0.0295 (0.0295)	0.0031 (0.0017)	n.s.
P (restricted=remainder)[a]	n.s.	n.s.	
P (restricted=nonrestricted)[a]	n.s.	n.s.	

[a] Two sample T test.

this contention, escape mutations appeared sequentially over a period of several weeks to months in the other two chronically infected animals. For instance, amino acid substitutions were observed in five of six epitopes targeted by CH-503, but they appeared in the quasispecies anywhere from 3 to 21 months postinfection (Figure 3B). A similar pattern of escape was observed in CBO603 (Figure 3C). The gradual accumulation of escape mutations probably reflects CTL selection pressure that is focused on a narrow set of the most dominant epitopes at any given point in time. Narrowly focused CTL populations and oscillating patterns of CTL dominance have been implicated in generation of HIV escape variants (McMichael, 1998) and may also contribute to persistence of HCV.

It is noteworthy that mutation of all class I MHC restricted epitopes was not an absolute requirement for HCV persistence. For instance, two Patr-A0101 restricted epitopes were intact after 5 years of virus replication in CBO603 (Figures 4D and 5A). In addition, one Patr-B1601 restricted E1 epitope ($E1_{233}$) was not changed in chimpanzee CH503 after 7 years of infection (Figure 3B). Lack of selection pressure against these epitopes might be expected if they are subdominant or interact weakly with class I MHC or TcR ligands. It is also possible that CTL populations targeting these epitopes do not reach a threshold frequency required for clearance of HCV (Lechner et al., 2000a) or that they are defective in antiviral function (i.e. cytolysis or cytokine production) (Lechner et al., 2000b; Sobao et al., 2001). These invariant epitopes could be useful targets for therapeutic vaccination to control HCV replication.

Almost all escape mutations detected in the chronically infected animals were fixed early in the infection. Consistent with observations in HCV-infected humans (Chang et al., 1997), they did not diversify further during several years of follow-up (Table 2). Using conditions that reproducibly expanded CTL-specific for index HCV-1/910 peptides, we attempted unsuccessfully to expand variant-specific CTL in the liver and blood of these animals. Generation of new variant-specific CTL populations might be prevented by a lack of adequate HCV-specific T cell help observed in these chimpanzees and also in chronically infected humans. Another possibility is that mutant epitopes selectively stimulate expansion of index (wild-type)-specific CTL, a phenomenon known as original antigenic sin, which was first described for antibodies but recently extended to class I MHC restricted epitopes (Klenerman and Zinkernagel, 1998).

In the present study, we observed multiple, sequential changes in some epitopes during the first few months of infection. In chimpanzee CH-503, the L1637P substitution in epitope $NS3_{1629}$ appeared first, but was rapidly replaced by a second variant, L1637S. Two substitutions in the $NS4_{1939}$ epitope (A1942V at month 3 and A1946T at month 10) were also transiently present in the quasispecies before fixation of the A1941S variant at month 16 postinfection (Figure 3B). Interestingly, the A1946T substitution conferred resistance to lysis by $NS4_{1939}$-specific CTL (data not shown) but did not guarantee survival of this virus population in chimpanzee CH-503. Conditions in the first few months of infection may be favorable for generation of variant-specific CTL that could select against these mutants. However, the L1637P substituted $NS4_{1629}$ peptide failed to bind Patr-B1701 so it is unlikely it was targeted by variant-specific CTL. We predict that some early substitutions, such as L1637P, provided escape from the CTL response but were unstable because they interfered with the function of HCV proteins and virus replication.

The relevance of our observations to human HCV infection remains to be established, but key features of persistent infection are similar in both species. Most importantly, $CD8^+$ T cells required for selection and perhaps maintenance of mutant viruses are present in many chronically infected chimpanzees and humans. Multispecific CTL circulate in the peripheral blood of both species, but frequencies are usually very low, and unconventional methods of antigen-specific expansion are required for their detection (Wong et al., 2001). CTL do appear to be enriched in the liver, the site of active virus replication. Two recent studies demonstrated that HLA-A2 restricted, HCV-specific CTL are not detected in the blood of chronically infected humans using soluble, tetrameric class I MHC molecules, but some are present in the liver at frequencies as high as 4% (Grabowska et al., 2001; He et al., 1999). Enrichment in the liver is consistent with successful cloning of intrahepatic HCV-specific CTL lines from many chronically infected humans and chimpanzees. In this study, the number of unique CTL populations cloned from the liver of the chronically infected animals ranged from 2 (CBO609) to 6 (CH-503). Multispecific CTL populations have also been established from the liver of a few chronically infected humans, including one individual who targeted 5 different HCV epitopes (Wong et al., 1998). Detection of HCV-specific CTL may be more efficient in chimpanzees than humans because antigens used in screening assays are matched exactly with the virus used to infect the animal. It should also be noted that chronic hepatitis C is a progressive disease in humans so the presence or absence of HCV-specific CTL may be influenced by the duration of infection, genotype of the virus, virus load, extent of fibrosis or cirrhosis, or other factors. Animals in this study were infected for 5–7 years, a relatively short period of time in the natural history of human HCV infection. Liver histology was usually normal or displayed mild periportal inflammation, liver transaminases were normal or elevated only slightly above baseline values, and virus loads were fairly stable at 10^5 (CBO603, CBO609) to 10^6 (CH-503) genome equivalents per ml of plasma. This rather benign course of infection is also observed in some infected humans, and we speculate that liver disease might be attenuated by early mutation of multiple class I MHC restricted epitopes, which would otherwise be targeted by immunopathogenic CTL.

Mutations in class I MHC restricted HCV epitopes have been observed in human subjects and many do impair CTL recognition (Chang et al., 1997; Kaneko et al., 1997; Tsai et al., 1998). Still, it is uncertain whether these mutations result from $CD8^+$ T cell selection pressure. Design of human studies to address this issue is complex for two important reasons. First, acute hepatitis C is often clinically silent so early virus isolates and CTL populations are difficult to obtain. This hinders direct comparison of epitopes encoded by the infecting virus

and variants that emerge in the first few weeks or months of infection. Second, no consistent pattern of HCV epitope dominance has emerged in humans or chimpanzees. In this study, CTL lines established from the liver of individual animals were used to identify dominant epitopes regardless of class I MHC restriction. Access to liver tissue is restricted in human subjects so HCV peptides presented by the HLA-A2 molecule are often used as a surrogate for the entire CTL response. Even with these limitations, evidence consistent with immune selection pressure was obtained in one study of chronically infected humans who expressed HLA-A2 (Chang et al., 1997). Acute phase virus isolates were not available from these human subjects so epitope sequences encoded by the chronic phase viruses were compared with those of a single prototype HCV virus, the genotype 1a Hutchinson (H77) isolate. HLA-A2 epitopes encoded by the chronic phase viruses were more likely to contain amino acid substitutions if prototype (H77)-specific CTL were present in blood (Chang et al., 1997). Variants often displayed reduced binding to HLA-A2 and/or impaired recognition by prototype-specific CTL. On the other hand, many prototype HLA-A2 epitopes were unchanged in these subjects, but this does not necessarily mean that escape mutations are less important for HCV persistence in humans versus chimpanzees. Our findings in this animal model suggest that information on the sequence of the infecting virus and dominant epitopes targeted by intrahepatic CTL populations will be critical for future studies of escape mutations in humans.

Experimental Procedures

Animals

Infection of the four chimpanzees enrolled in this study with the HCV-1/910 virus stock has been described in detail (Cooper et al., 1999; Erickson et al., 1993; Kowalski et al., 1996). Three animals were chronically infected for 5 (CBO603, CBO609) or 7 years (CH-503) years when enrolled in this study. CBO609 (Hulk) was immunized with recombinant HCV envelope glycoproteins before virus challenge, and CBO603 (Briggsie) was passively immunized with 640 mg of a polyvalent immunoglobulin preparation that contained anti-E1 and E2 antibodies 24 hr prior to infection (Cooper et al., 1999). CH-503 was immunized by dermal scarification with a recombinant vaccinia virus expressing HCV E1 and E2 and then boosted with purified envelope glycoproteins before infection (Erickson et al., 1993). Class I MHC genotyping of these animals and generation of Patr class I transfected 721.221 cell lines was reported elsewhere (Cooper et al., 1999; Kowalski et al., 1996). Chimpanzees were housed at the New Iberia Research Center (NIRC), New Iberia, Louisiana, in accordance with "The Guide for Care and Use of Laboratory Animals" published by the National Research Council (Anonymous, 1996). The Association accredits NIRC for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals were cared for under an experimental protocol approved by the New Iberia Research Center Animal Care and Use Committee.

Peptides

Peptides were produced by either Chiron Mimotopes (San Diego, California) or Research Genetics (Huntsville, Alabama) using fMoc solid phase chemistry with free amine N termini and free acid C termini. Lyophilized peptides were resuspended at a concentration of 10–20 mg/ml in 10% DMSO and water.

Generation of CTL Lines

CTL lines were derived from liver biopsy tissue (Erickson et al., 1993). Briefly, T cells recovered from liver homogenates with anti-CD8 paramagnetic beads were cultured at 10 or 50 cells per well in a 96-well tissue culture plate with 5×10^4 irradiated human PBMC feeder cells, 50 U/ml of IL 2 and anti-CD3 monoclonal antibodies. At least 300 independently derived $CD8^+$ T cell lines were tested for recognition of autologous BLCL target cells infected with recombinant vaccinia viruses expressing different regions of the HCV-/910 polyprotein. 721.221 cells transfected with individual Patr-A, -B, or -C class I MHC molecules of each animal were used to determine class I MHC restriction of the CTL lines. Mapping minimum optimal epitopes with synthetic peptides has been described (refs and CMW unpublished data). CTL lines were derived from peripheral blood by culturing 5×10^6 PBMC with the HCV peptide (10 μM) and tetanus toxoid (1 μg/ml) as a source of T cell help (Cerny et al., 1995). Cells were restimulated on days 7 and 14 with 2×10^6 irradiated autologous PBMC pulsed with a 10 μM concentration of the peptide. IL 2 (20 U/ml) was added to cultures 3 days after each peptide stimulation. CTL activity was assessed on day 21.

CTL Assays

B lymphoblastoid cell lines (BLCL) or 721.221 cells transfected with cloned Patr class I MHC molecules were used as targets. Approximately 1×10^6 cells were incubated for 1 hr with 50 μCi of ^{51}Cr and varying concentrations of peptide epitopes. After washing, 5×10^3 target (T) cells were cocultured with effector (E) CTL lines at an E:T ratio of 20:1 for 4 hr. Supernatants (50 μl) were harvested into Packard Lumaplates and radioactivity was counted in a Wallac Microbeta 1420 scintillation instrument.

Cloning and Sequencing of HCV Genomes

Chimpanzee blood was collected in EDTA and plasma was frozen within 20 min. RNA was extracted from 100 μl of thawed plasma using the RNAEasy protocol and reagents (Qiagen) and cDNA was made as previously described (Weiner et al., 1995). Each region of the HCV genome that contained class I MHC restricted epitopes was amplified with nested sets of PCR primers designed with Primer Express software (ABI). First round PCR with outside primers was performed for 35 cycles (94°C for 10 s; 55°C for 30 s, and 72°C for 30 s). Inside nested primers were added to the product of the first PCR reaction and amplified for an additional 35 cycles as described above. PCR products were immediately subcloned by thymidine/adenosine (T/A) ligation into a DNA plasmid (Invitrogen) and sequenced in both directions using dye terminator chemistry and an ABI 3100 automated DNA sequencer.

Class I MHC Molecule Purification and Binding Assay

Purification of class I molecules and HCV peptide binding assays were performed as described (Allen et al., 1998). 721.221 cells transfected with Patr-B0101 and Patr-B1701 were lysed in 50mM Tris-HCL, pH 8.5, containing 1% NP-40, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF. After filtration supernatants were clarified by centrifugation at 10,000g and then passed two to four times over a column containing protein A sepharose beads conjugated with the anti-HLA (A, B, C) antibody W6/32 (Allen et al., 1998). Patr molecules were eluted with 50 mM diethylamine in 0.15 M NaCl containing 0.4% n-octylglucoside, pH 11.5. 2.0 M Tris, pH 6.8, was added to the eluate to reduce the pH to 8.0. The eluate was concentrated by centrifugation in Centriprep 30 concentrators.

Binding of HCV peptides to soluble Patr molecules was quantified by a competitive inhibition assay. Briefly, varying concentrations of HCV peptides and radiolabeled probe peptides (YTGDFDSVI for Patr-B0101 and YAVQNEITL for Patr-B1701) were coincubated at room temperature for 48 hr with purified Patr-B0101 (1μM) or Patr-B1701 (1 nM), human β2-microglobulin (1 μM), and protease inhibitors. The percent of MHC-bound radioactivity was then determined by size exclusion gel filtration chromatography on a TSK2000 column, or by capturing MHC/peptide complexes on Optiplates (Packard) coated with W6/32 mAb, and determining bound cpm in a microscintillation counter. The inhibitory concentration 50 (IC50) is defined as the nM concentration of competitor HCV peptide yielding 50% inhibition of binding by the radiolabeled probe peptide. Each HCV peptide was tested in 2–4 independent experiments. Relative binding capacity of the HCV peptides was calculated by dividing the IC50 of the test peptide by the IC50 of the index HCV-1/910 peptide. The IC50 of the index peptide was normalized to a value of 1.

Statistical Analyses

Published methods (Nei and Gojobori, 1986) were used to compute dS and dN, the number of synonymous nucleotide substitutions per synonymous site and nonsynonymous nucleotide substitutions per site, respectively, in regions of the HCV genome containing class I MHC restricted epitopes. We performed two separate analyses. First, mean dS and mean dN were estimated by comparing the consensus sequence of the HCV-1/910 virus used to infect the animals with multiple HCV genomes subcloned from their plasma at later time points (5 years postinfection for CBO603 and CBO609, 7 years for CH-503). Second, mean dS and mean dN were calculated for all pairwise comparisons within samples to estimate ongoing diversification of epitopes in the chronic phase of infection. The hypotheses that mean dS was equivalent to mean dN in epitopes restricted by the host, epitopes not restricted by the host, and the flanking (remainder) regions were examined using Student's t test.

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