Genetic Variability of Hepatitis C Virus Non-Structural Protein 3 and Virus-Specific CD8+ Response in Patients With Chronic Hepatitis C

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Hepatitis C virus (HCV) variation in specific T-cell epitopes may represent a mechanism of viral persistence in chronic infection. We examined the HCV non-structural protein 3 (NS3), including the immunologically relevant epitopes HCV NS3-2 KLVALGINAV (human leukocyte antigen [HLA]-A2-restricted) and HCV NS3-1391 LIFCHSKKK (HLA-A3-restricted), in 22 HLA-A2+ patients with chronic infection. Significant amino acid variation was found in HCV NS3-2 epitope sequences when compared to the HCV-1 prototype virus. Six of the nine different HCV NS3-2 peptide variants were identified in patients with HCV NS3-2-specific CD8+ cells, detected with an HLA-A2 tetramer made with the HCV-1 prototype peptide. Phylogenetic analysis, including HCV reference sequences other than HCV-1, suggested however that most of the variations in the HCV NS3-2 epitope could be related to genetic heterogeneity between HCV reference subtypes. Variation was less common when comparing HCV NS3-2 epitope sequences from the clinical isolates to the most-closely related HCV reference subtype in each case. Some subtype-independent variations were found in epitopic residues probably important for T-cell receptor interaction. In contrast, no significant variation was found in HLA primary anchor sites, flanking regions, or in the contiguous HLA A3-restricted CD8+ T-cell epitope. Ongoing variation was not evident in two selected patients with follow-up. In conclusion, (i) the HCV NS3-2 epitope is not conserved between different HCV strains/subtypes, and (ii) an HLA-A2 tetramer loaded with the HCV-1 prototype NS3-2 peptide may still detect NS3-specific CD8+ cells in some patients with variant viruses. These data may be useful to improve T-cell assays using HCV NS3 peptides, taking into account the genetic diversity of this virus. *J. Med. Virol. 72:575–585, 2004.*
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INTRODUCTION

A member of the Flaviviridae family, hepatitis C virus (HCV) has been recognised as one of the most important human pathogens, affecting over 170 million individuals world-wide [Heintges and Wands, 1997]. Persistent HCV infection is associated with chronic hepatitis, which is one of the leading causes of cirrhosis, hepatic failure and liver transplantation. No vaccine is currently available, and the efficacy of treatment is limited to date.

The HCV genome is characterised by a high replicational error rate and an extensive genetic heterogeneity. Several genotypes and subtypes have been identified [Simmonds, 1999], HCV circulates in a given individual as a mixture of different, but closely related genomes, so-called quasispecies [Martell et al., 1992; Domingo et al., 1997], with a remarkable antigenic diversity. The degree of HCV heterogeneity is variable through the viral genome. The 5' non-coding region (NCR) is highly conserved, core and non-structural regions share moderate variability, and the envelope includes hypervariable regions (reviewed in Forns and Bukh [1999]).

The fact that viral persistence is observed in the majority of patients together with detectable cellular immune responses is puzzling. Indeed, using two class-I human leukocyte antigen (HLA)-tetramers made with peptides from the HCV non-structural 3 (NS3) protein, NS3-specific CD8+ cells are detected ex vivo in approximately 75% of HCV chronically infected patients [He et al., 1999]. Published data indicate that HCV class I-restricted T-cell epitopes located within the NS3 protein are commonly recognised by liver-infiltrating and circulating cytotoxic T-lymphocytes (CTL) of HCV-infected patients [Cerny et al., 1995; Koziel et al., 1995; Rehermann et al., 1996a,b; Chang et al., 1997, 2001; Lechner et al., 2000a,b]. Mutation on target epitopes for CTL has been proposed as one of the mechanisms contributing to persistent HCV infection, but the number of studies in chimpanzees and humans is limited [Weiner et al., 1995; Chang et al., 1997; Erickson et al., 2001]. Thus, determination of the amino acid variability of HCV in relevant CTL epitopes may be important for the design of both new T-cell assays and broadly cross-reactive prophylactic or therapeutic vaccines.

Therefore, a study was undertaken to determine whether the amino acid sequence of the HCV NS3 protein, including immunologically relevant T-cell epitopes, varies between viruses isolated from different chronically-infected individuals with the same immunogenetic background (HLA-A2+). We examined HCV NS3 variation at two molecular levels: (i) the phylogenetic relationships between nucleotide sequences, and (ii) the amino acid variability in CD8+ T-cell epitopes. In addition, we analysed HCV NS3-specific CD8+ cells in peripheral blood mononuclear cells (PBMCs) from the same patients by using an HLA-A2 tetramer made with the peptide sequence from the HCV-1 prototype virus. We hypothesised that HCV NS3 variants are frequent and that they may play a role in HCV persistence.

PATIENTS AND METHODS

Patient Population

Twenty-two HLA-A2 positive patients seen in the liver clinics of the Palo Alto or San Francisco Veterans Affairs Medical Centers or the Stanford University Medical Center were studied. All patients had HCV antibodies by a second or third generation ELISA (Chiron, Emeryville, CA), positive HCV-RNA by PCR,

negative hepatitis B surface antigen and had well-established chronic HCV infection (elevated serum aminotransferase levels for at least 6 months). One patient was co-infected with human immunodeficiency virus (HIV), and one patient was a liver transplant recipient with recurrent HCV infection. HLA haplotypes were determined by staining PBMCs with the monoclonal antibodies MA2.1 [McMichael et al., 1980] and BB7.2 [Parham and Brodsky, 1981], and FITC-labelled goat anti-mouse IgG, followed by flow cytometry. Double positive samples were considered HLA-A2+. Blood samples from healthy anti-HCV seronegative, and from anti-HCV positive HLA-A2 negative individuals were used as negative controls. Informed consent was obtained in every case, and the institutional Boards at Stanford University, and the University of California San Francisco, approved research protocols following the guidelines of the 1975 Declaration of Helsinki.

Blood Samples and PBMCs

For HCV-RNA analysis and quantitation, blood samples were collected in tubes without additives and processed not later than 4 hr. Serum aliquots were kept at -80°C till use. For HLA typing and tetramer staining, PBMCs (overall 5×10^5 to 5×10^7 cells) were isolated from fresh heparinized blood by density gradient separation (Ficoll-Paque, Amersham-Pharmacia, Upssala, Sweden). PBMCs that were not examined the same day of collection were cryopreserved in 10% dimethylsulfoxide, 90% foetal calf serum in liquid nitrogen till use, without significant loss of viability.

HCV-RNA Detection, Quantitation and Genotyping

HCV-RNA (qualitative) was detected using the Amplicor HCV test (Roche Diagnostics, Branchburg, NJ) or a nested RT-PCR of the 5' NCR basically as described [López-Labrador et al., 1997]. HCV-RNA quantitation was carried out with the Amplicor HCV Monitor test v2.0 (Roche Diagnostics). All samples with values higher than 450,000 copies/ml were re-tested at either 1:10 or 1:100 dilution to prevent saturation [Martinot-Peignoux et al., 2000]. HCV-RNA values obtained after dilution were corrected with the appropriate dilution factor. HCV genotyping was performed by a commercial reverse-hybridisation genotyping assay (Inno-LIPA HCV II; Innogenetics, Zwijndrecht, Belgium). Some selected serum specimens were tested for HCV genotype-specific antibodies (Murex HCV serotyping assay 1-6, Murex, Dartford, UK).

Amplification of the HCV NS3 Region and Epitope Sequencing

A fragment of the HCV NS3 region encompassing nucleotides 4,429–4,719 was amplified by nested PCR, including the HLA Class I-restricted viral epitopes HCV NS3-2 KLVALGINAV (A2-restriced, amino acids 1,406–1,415) and HCV NS3-1391 LIFCHSKKK (A3-restricted, amino acids 1,391–1,399). All the nucleotide

and amino acid positions cited herein are referred to the HCV-1 prototype [Choo et al., 1991]. In brief, total RNA was extracted from 140 μl of serum or plasma specimens with the QiaAmp HCV-RNA column kit (Qiagen, Valencia, CA) following the instructions from the manufacturer. Viral RNA was eluted in 30 μl of diethylpyrocarbonate-treated water and used immediately for PCR or frozen at −80°C. Reverse-transcription of viral RNA and primary PCR were carried out in a single tube containing 5 μl of RNA, 3 U of Avian Myeloblastosis Virus Reverse transcriptase (AMV, Promega, Madison, WI), 2.5 U of Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD) and 25 pmol of primers NS3-5 (5′-ACG TAC TCC ACC TAC GGC AA-3′; positions 4,228–4,248) and NS3-6 (5′-AAG GTA GGG TCA AGG CTG AA-3′; positions 4,745–4,765). One microlitre of the primary PCR product was then re-amplified in a secondary PCR round with primers NS3-7 (5′-CAT CCC AAC ATC GAG GAG GT-3′; positions 4,429–4,449) and NS3-8 (5′-TTG CAG TCT ATC ACC GAG TC-3′; positions 4,699–4,719). Cycling conditions for the primary RT-PCR round were as follows: {42°C 30 min; 95°C 5 min; 35 cycles (95°C, 42°C, 72°C; 45 sec each); 72°C 4 min}. The conditions used for the secondary PCR round were {95°C 5 min; 35 cycles (95°C, 42°C, 72°C; 45 sec each); 72°C 4 min}. A single band of the expected size (289 bp) was observed after agarose gel electrophoresis. Secondary PCR products were then purified using the Qiaquick PCR Clean-up kit (Qiagen). Thirty picogram of purified DNA were used for sequencing with the ABI Prism BigDye Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, CA). DNA sequencing was undertaken in both strands using either primer NS3-7 (sense) and NS3-8 (antisense). Sequencing reactions were run in an Applied Biosystems 377A DNA sequencer at the VA Palo Alto Medical Center DNA Sequencing facility.

Sequence Comparison and Phylogenetic Analysis

Multiple alignments of the HCV NS3 nucleotide and deduced amino acid sequences were obtained using the Clustal V algorithm [Higgins, 1994] of the DNASTAR software package (DNASTAR, Madison, WI). Nucleotide sequences from different HCV reference strains deposited in the GenBank database were also included in the alignments (see below). A phylogenetic tree was then reconstructed under a maximum-likelihood criterion using PAUP* (version4b8) [Swofford, 1998]. Prior to tree reconstruction, alternative models of sequence evolution were evaluated by nested likelihood ratio tests and the Akaike Information Criterion (AIC) [Akaike, 1974] using the program Modeltest 3.0 [Posada and Crandall, 1998]. Once the best evolutionary model was determined, alternative tree topologies were evaluated by heuristic search, using subtree-pruning-regrafting (SPR) for branch swapping after an initial tree was found by neighbour-joining [Saitou and Nei, 1987]. Statistical support for internal branches was evaluated by quartet-puzzling [Strimmer and von Haeseler, 1996]

using TreePuzzle, and by a likelihood ratio test for whether branch lengths were significantly different from zero, as implemented in PAUP*. The HCV-NZL1 reference sequence (subtype 3a) was used as outgroup. Phylogenetic trees were plotted with the program Treeview [Page, 1996].

HCV NS3 HLA Tetramer Staining

PBMCs were stained with the HCV NS3-2 HLA-A2 tetramer [He et al., 1999], loaded with the A2-restricted peptide HCV NS3-2 (KLVALGINAV) from the HCV-1 prototype strain. Tetramers were made essentially as described previously [Altman et al., 1996; He et al., 1999]. For staining, PBMCs (either fresh or cryopreserved) were counted by trypan blue exclusion and at least 5×10^5 to 5×10^6 viable cells were used. Cells were washed in complete RPMI medium and resuspended in 20 μl of fluorescence-activated cell sorter (FACS) buffer (0.5% BSA and 0.05% sodium azide in PBS) supplemented with 1 μg/ml of DNAse I. Three-colour staining was performed by adding a staining mixture containing 1.5 μg of phycoerytrin-labelled tetramer, 0.2 μg of FITC-labelled anti-CD8, and 0.12 μg of each Tri-Colour labelled antibodies to CD4, CD13 or CD19 (Caltag, South San Francisco, CA). Cells were incubated in the dark for 30 min at room temperature, washed twice with FACS buffer, and fixed in 1% of paraformaldehyde. Stained cells were analysed in a FACSCalibur flow cytometer (Beckton Dickinson Biosciences, San Jose, CA), by gating at CD4-CD13-CD19-CD8+ small lymphocytes, and acquiring 50,000–80,000 CD8+ cell events. Negative gating in the FL3 channel (CD4-CD13-CD19-lymphocytes) reduced significantly the background level. Colour compensation controls were included in each round, including single-stained cells with FITC, phycoerytrin or Tri-Colour-labelled anti-CD8 antibodies. Negative controls (both PBMCs from A2+HCV− and A2-HCV+ individuals) were also included in each staining.

The cut-off of the tetramer assay was determined as the level between the tetramer+ CD8+ cell population and the majority of cells in the negative control samples, as previously described [He et al., 1999]. A well-separated population of CD8+ cells with relative fluorescence intensities close to 10^3 or higher were considered tetramer-binding CD8+ cells. Using this cut-off, the lowest percentage at which tetramer+ CD8+ cells can be detected as a separate cell population is 0.01%. To further test the specificity of the assay, tetramer staining was repeated in some selected samples.

Nucleotide Sequences

HCV sequences from different HCV reference subtypes were obtained from GenBank: HCV-1, HCV-H and HCV-J1 (subtype 1a); HCV-N, HCV-UN, HCV-C2, HCV-TA, HCV-TB, HCV-BK, HCV-J, HCV-JK1, HCV-J4/83 and HCV-J4/91 (subtype 1b); HCV-CG9 (subtype 1c); HCV-J6 (subtype 2a); HCV-J8 (subtype 2b); HCV-NZL1 (subtype 3a); and HCV-TR (subtype 3b). Accession

TABLE I. Demographic, Clinical, and Virological Data of the
Patients Studied

Demographics	
Gender (M/F; %M)	19/3 (86.4%)
Age (mean ± SD); range	50 ± 8 (36–66)
Liver histology	
Stage of fibrosis (mean ± SD)	1.41 ± 0.94
Inflammation grade[a] (mean ± SD)	1.71 ± 0.85
Total score	3.12 ± 1.45
Liver biochemistry	
ALT[b] (mean ± SD)	136.8 ± 91.28
AST[b] (mean ± SD)	89.16 ± 60.98
AST/ALT (mean ± SD)	1.5 ± 0.47
HCV genotype	
Genotype 1	20 (90.9%)
Genotype non-1	2 (9.1%)
Viral load (mean $\log_{10}$ copies/ml ± SD)	5.68 ± 0.62
Other	
OLT[b]	1
HIV co-infection	1

[a]Inflammation grade score 0–4.
[b]ALT, serum alanine aminotransferase; AST, serum aspartate amino-
transferase; and OLT, orthotopic liver transplantation.

numbers are as follows: M62321, M67463, D10749,
S62220, M96362, D10934, D11168, D11355, M58335,
D90208, G77093, D13558, D10750, D14853, D00944,
D10988, D14853 and D49374, respectively.

RESULTS
Patient Characteristics, HCV Genotype and Viral Load

The main demographic, clinical and virological char-
acteristics of the patients included in the study are

shown in Table I. HCV viral load ranged from 4.7 to 6.8
$\log_{10}$ copies/ml. Distribution of HCV genotypes was as
follows: 1a n = 17; 1b n = 3; and 2b n = 2. Mixed infection
was not detected in any case using the LIPA genotyping
assay.

Amino Acid Variation in the HCV NS3-2 Epitope

The deduced amino acid sequence of the HCV NS3-2
epitope isolated from each patient was first compared to
that of the HCV-1 prototype (subtype 1a). Overall, the
HCV NS3 amino acid sequence was highly conserved
among the clinical isolates (86–100% homology to HCV-
1). Nine different HCV NS3-2 variants were found in 15
patients: variants NS3-2.1 (KLVAMGLNAV), NS3-2.2
(KLVAMGINAV), NS3-2.3 (KLVALGVNAV), NS3-2.4
(KLLSLGINAV), NS3-2.5 (KLSALGLNAV), NS3-2.6
(ALRGMGVNAV), NS3-2.7 (KLVGLGVNAV), NS3-2.8
(KLVTLGVNAV) and NS3-2.9 (KLSGLGLNAV). Repe-
tition of viral RNA extraction in selected serum samples
from three different patients, followed by reverse trans-
cription, PCR amplification and direct sequencing, did
not show introduction of Taq DNA polymerase mis-
incorporation errors in our PCR-sequencing assay.

Once variation to the HCV-1 prototype was estab-
lished, the nucleotide and deduced amino acid se-
quences of the viral isolates from each patient were
compared to different HCV reference sequences depos-
ited in the GenBank database (Table II). Interestingly,
most of the HCV NS3-2 epitope variants matched with
reference HCV isolates other than HCV-1. In several

TABLE II. HCV Genotype, Amino Acid Variations Found in the HCV NS3-2 Epitope
Compared to the HCV-1 Prototype Sequence (Subtype 1a), and Correspondence to the
Matching HCV Reference Sequence Found in the GenBank Database*

Patient code	HCV genotype (LIPA)	HCV NS3-2 amino acid sequence	Matching HCV subtype in GenBank		
HCV-1		KLVALGINAV			KLVALGINAV
S11	1a	----------	HCV-1	(1a)	----------
S13	1a/b[a]	----------	HCV-1	(1a)	----------
S16	1a	----------	HCV-1	(1a)	----------
S32	1a	----M-L---	HCV-1	(1a)	----------
S8	1a	----M-----	HCV-1	(1a)	----------
S9	1a	------V---	HCV-J1	(1a)	------V---
S33	1a/b[a]	------V---	HCV-J1	(1a)	------V---
S2	1b	--LS------	Not found		
S17	1b	--S---L---	HCV-C2	(1b)	--S---L---
S23	2b	A-RGM-V---	HCV-J8	(2b)	A-RGM-V---
S20	1a	----------	HCV-1	(1a)	----------
S24	1a	----------	HCV-1	(1a)	----------
S30	1a/b[a]	----------	HCV-1	(1a)	----------
S7	1a	----------	HCV-1	(1a)	----------
S15	1a	------V---	HCV-J1	(1a)	------V---
S18	1a	------V---	HCV-J1	(1a)	------V---
S21	1a	------V---	HCV-J1	(1a)	------V---
S25	1a	------V---	HCV-J1	(1a)	------V---
S3	1a	---G--V---	HCV-J1	(1a)	------V---
S19	1a	---T--V---	HCV-J1	(1a)	------V---
S14	1b	--SG--L---	HCV-UN	(1b)	--SG--L---
S1	2b	A-RGM-V---	HCV-J8	(2b)	A-RGM-V---

*Dashes indicate identity to the HCV-1 prototype sequence shown at the top.
[a]1a/b subtypes indeterminate by LIPA.

cases, the HCV NS3-2 epitope was identical (patients S9, S33, S15, S18, S21 and S25) or very similar (patients S3 and S19) to that of the HCV-J1 isolate. In patient S17, the HCV NS3-2 epitope was identical to that of the HCV-C2 isolate, and in patient S14 to that of the HCV-UN isolate. The two HCV NS3-2 sequences from patients infected with HCV subtype 2b (S1 and S23) were identical to that of the HCV-J8 isolate (subtype 2b).

Phylogenetic Analysis

To further investigate the relationships between the HCV clinical isolates and the different HCV reference sequences, a maximum-likelihood phylogenetic analysis of the NS3 region (nucleotides 4,510–4,644) was performed. The best model of sequence evolution for the complete data set was the Hasegawa–Kishino–Yano model with 43.64% of invariable sites and a gamma distribution (shape parameter = 1.197) to account for site variation among the remaining sites. The transi-tion/transversion ratio was 4.089. The optimal phyloge-netic tree obtained with these parameters is shown in Figure 1, panel a. In the phylogenetic tree, there is a clear differentiation among the major HCV types. Within subtypes however, there are only few significant internal branches, pointing towards a very close re-latedness of the variants within a subtype, and preclud-ing the resolution of a well-supported phylogeny at this level. Similar results were obtained when a separate phylogenetic analysis was carried out using only the HCV subtype 1a sequences, but with a somewhat better support for differentiating subtype 1a isolates (Fig. 1, panel b). The same tree topology was obtained when the data set was re-analysed excluding the HCV NS3-2 epitope, indicating that tree topology was not affected by the variability of this viral epitope (data not shown).

Phylogenetic analysis supported the findings of simple sequence comparison. Isolates from patients S3, S7, S16, S20, S21, S24, S30 and S32 grouped close to HCV-1; and isolates from patients S8, S13 and S25
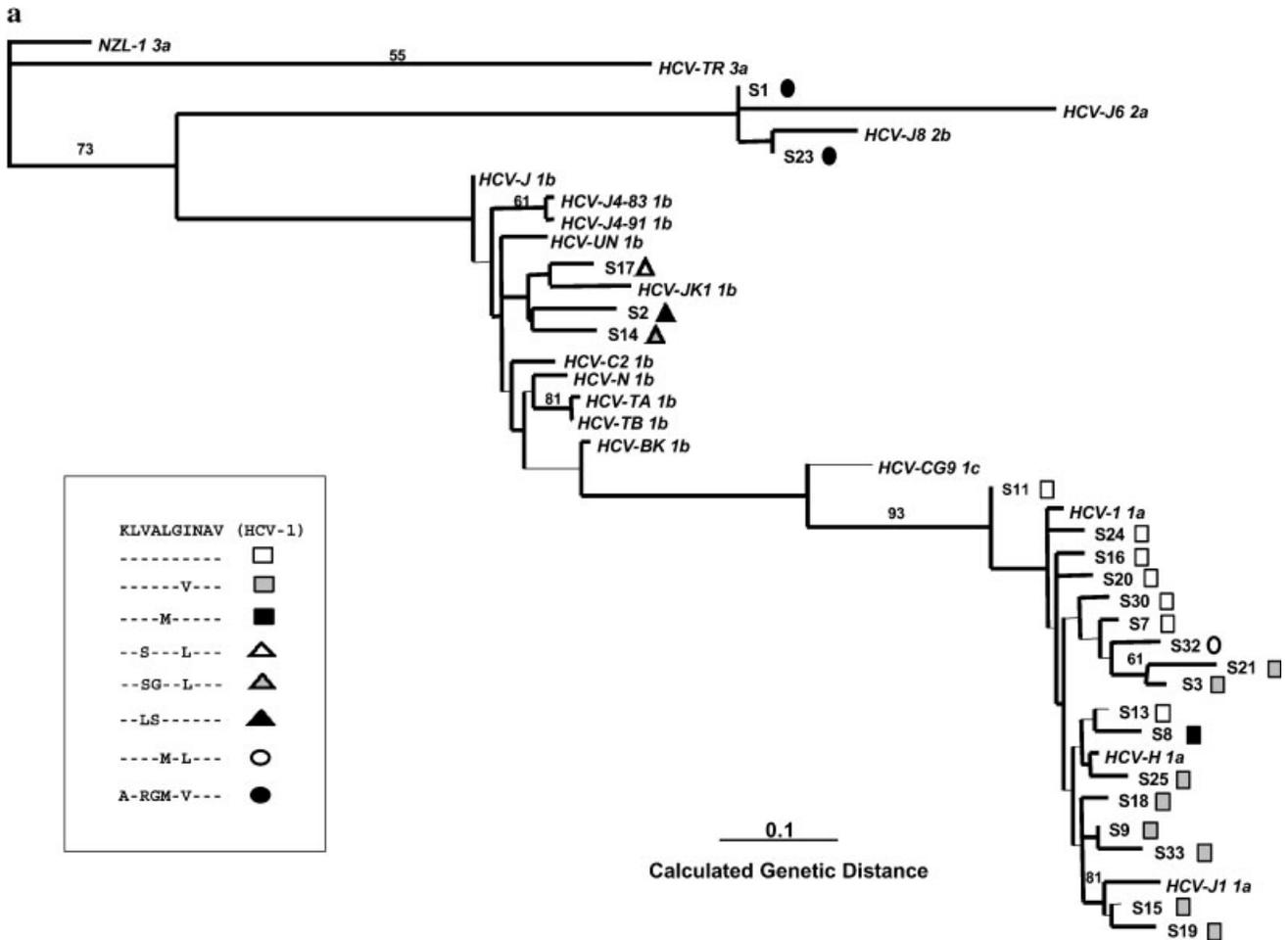
Fig. 1. **a**: Maximum-likelihood phylogenetic tree constructed with the hepatitis C virus (HCV) non-structural 3 (NS3) nucleotide sequences from patients and the reference HCV subtypes found in GenBank. The different HCV NS3-2 peptide variants are indicated by squares, triangles, or circles. The tree was constructed as described in materials and methods. Branches not different from zero ($P > 0.05$) are shown as thin lines. Numbers in internal branches represent support values from the quartet-puzzling analysis. Five different groups of HCV isolates related to HCV-1, HCV-H, HCV-J1, HCV-JK1 and HCV-J8 are differentiated. **b**: Separate phylogenetic analysis for HCV subtype 1a sequences.
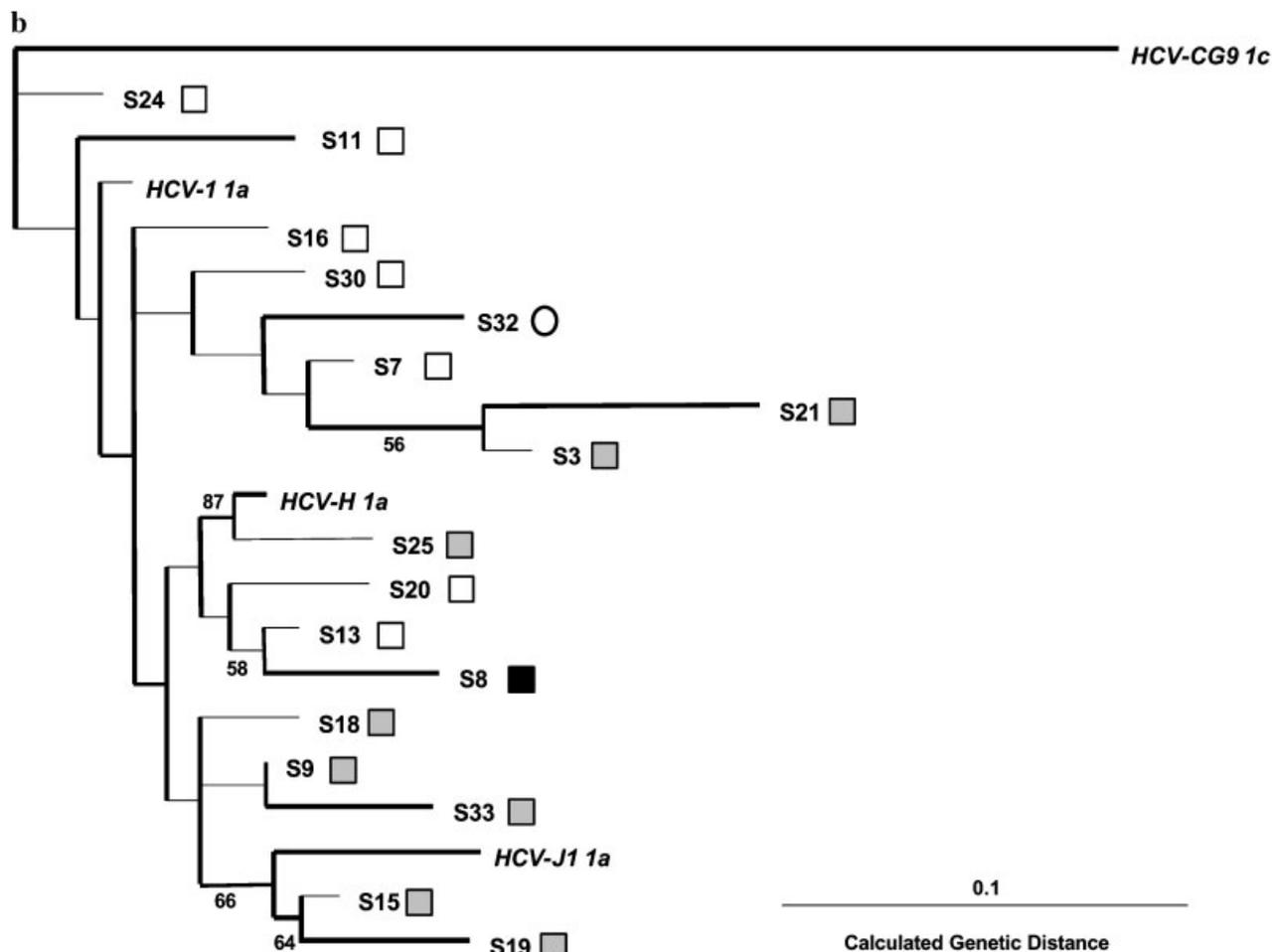
Fig. 1.   (*Continued*)

close to HCV-H. Isolates from patients S9, S15, S18, S19 and S33 (variant KLVALGVNAV) were in the same branch of the tree that HCV-J1 (NS3-2 epitope KLVALGV-NAV). Isolates from patients S2, S14 and S17 grouped with HCV-JK1 (within subtype 1b sequences); and finally, as expected, isolates from patients S1 and S23 clustered together with HCV-J8 (subtype 2b). These results suggested further that amino acid variation in the HCV NS3-2 epitope could be related to the genetic heterogeneity between HCV subtypes.

Detection of HCV NS3-2 Specific CD8+ Cells by Tetramer Staining

To check whether the study patients elicited a CD8+ response to the HCV NS3-2 epitope, isolated PBMCs were stained with the class-I HLA tetramer HCV NS3-2. Figure 2 shows representative examples of HCV NS3-2 tetramer staining in PBMCs. The average background staining of the HCV NS3-2 tetramer in negative controls (both PBMCs from A2+HCV− and A2-HCV+ patients) was lower than 0.002% of peripheral CD8+ cells, as described elsewhere [He et al., 1999]. The lower limit of

detection of our tetramer assay was 0.01% of total CD8+ cells; that is, tetramer+ CD8+ cells at levels 0.01% or higher (at least five-times the background) were considered antigen-specific CD8+ cells.

Ten of the 22 patients (45%) had detectable HCV NS3-2 tetramer-binding CD8+ cells (Table III). The frequency of such cells ranged from 0.01 to 0.9% of the total circulating CD8+ cells, in agreement with previous studies in chronically-infected patients [He et al., 1999; Lechner et al., 2000b]. Repetition of tetramer staining in selected samples gave reproducible results (data not shown). HCV NS3-2 tetramer+ CD8+ cells were not detected in the patient with recurrent HCV infection post-transplantation, who was under regular immuno-supression. In contrast, HCV NS3-2 tetramer+ CD8+ cells were detected in the only patient with HIV antibodies. This patient however, had no signs of AIDS, a history of high CD4+ T-cell counts (>300/ml), low HIV viremia (<1,000 copies/ml) and no treatment for HIV disease.

Tetramer staining was positive in PBMCs from 7 of the 15 patients infected with HCV NS3-2 variants (including 6 different variant sequences). No specific

(a) Negative controls
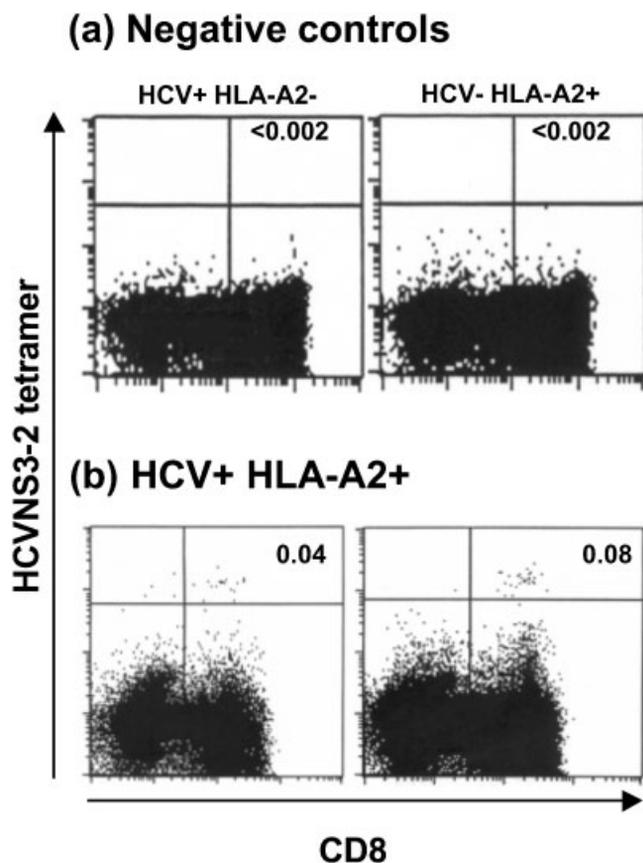
Fig. 2. HCV NS3-2 tetramer staining of peripheral blood mononuclear cells (PBMCs) from representative individuals. **Panel a**: HCV+ HLA-A2− (left) and HCV − HLA-A2+ (right). Negative controls showed background staining levels below 0.002% of total CD8+ cells. **Panel b**: Examples of positive HCV NS3-2 tetramer staining (tetramer+ CD8+ cells in the upper-right quadrant). The cell population shown is gated at small lymphocytes by forward and side scattering and at CD4-CD14-CD19-cells.

HCV NS3-2 variant peptide, or particular amino acid variation, was clearly associated with negative tetramer staining. Among patients infected with HCV subtype 1a (n = 17), tetramer+ CD8+ cells were detected in (i) three out of the seven patients with viral isolates sharing the HCV-1 prototype peptide KLVALGINAV; (ii) two out of the six infected with the variant KLVALGVNAV (S9 and S33); and (iii) two patients infected with variants KLVAMGLNAV (S32) or KLVAMGINAV (S8). In contrast, patients with HCV NS3-2 variants KLVGLGVNAV (S3) or KLVTLGVNAV (S19) had no detectable tetramer+ CD8+ cells. Among patients infected with HCV subtype 1b (n = 3), tetramer+ CD8+ cells were detected in two (S2 and S17, variants KLLSLGINAV or KLSALGLNAV, respectively), but not in the remaining one (S14, variant KLSGLGLNAV). Interestingly, from the two patients infected by HCV subtype 2b, one (S23) had detectable tetramer+ CD8+ cells, despite the high amino acid variation of this subtype in the NS3-2 epitope. To clarify this issue, previous infection with other HCV genotypes in this patient was investigated by

determining HCV NS3 type-specific antibodies in serum by a commercial serotyping assay. Antibodies to HCV genotype 1, and a low titer of antibodies to HCV genotype 2 (at the level of the cut-off value) were evident by serotyping, probably indicating an infection by genotype 1 in the past and a more recent infection with genotype 2 (as suggested by LIPA). Of interest, this patient had a history of intravenous drug use, with a subsequent high risk for multiple exposures.

Amino Acid Variation in the NS3-2 Epitope With Respect to the Most-Closely Related HCV Strain

Given the results of the phylogenetic analysis, the deduced amino acid sequence of HCV NS3 (amino acids 1,388−1,433) isolated from each patient was then compared with that of the most-closely related HCV reference subtype (Fig. 3). As suggested by phylogeny, most of the observed amino acid differences to the HCV-1 prototype seemed related to the genetic heterogeneity between different HCV isolates/subtypes. For instance, variants NS32.3 (KLVALGVNAV), NS3-2.5 (KLSALGLNAV) and NS3-2.6 (ALRGMGVNAV) seem typical of isolates HCV-J1, HCV-C2 and HCV-J8, respectively. In addition, when using the most-closely related HCV reference sequence for comparison, mean amino acid variation was significantly reduced (Table IV). Variant HCV NS3-2 sequences were then evident in only 8/22 patients (arrows in Fig. 3), either in the presence or in absence of HCV NS3-2 tetramer+ CD8+ cells.

No amino acid variation was observed in the HLA primary anchor residues of the NS3-2 peptide, whereas some subtype-specific amino acid variations were located in secondary anchor residues (positions P3 and P7 in patients S2, S14, S17 and S32; Fig. 3). In addition, in six patients (four with detectable tetramer+ CD8+ cells) some HCV NS3-2 amino acid variations were located in epitopic residues in positions P4, P5 or P6. Of interest, in the HCV-1 prototype NS3-2 peptide, the residues potentially involved in TCR contact are all non-polar, hydrophobic, and with an aliphatic side chain, whereas the variations observed in the current study include polar side chains and beta-turns. In turn, flanking regions of the NS3-2 epitope were conserved: variants occurred in only 4/22 patients (Fig. 3), in positions non-contiguous to the epitope. In contrast to the A2-restricted NS3-2 epitope, amino acid variation was almost absent in the proximal A3-restricted T-cell epitope NS3-1391 LIFCHSKKK, with only one amino acid change among the 22 HCV clinical isolates analysed (Fig. 3). Finally, sequencing analysis in two patients with detectable tetramer+ CD8+ cells (S17 and S33), did not show amino acid variations in the HCV NS3-2 or HCV NS3-1391 viral epitopes after 6 or 10 months of follow-up (data not shown).

DISCUSSION

How HCV persist in the face of host immune responses is an unresolved question. Viral variation may represent

TABLE III. Amino Acid Variations Found in the HCV NS3-2 Epitope, Compared to the Most Closely-Related HCV Subtype by Phylogenetic Analysis, and Percentage of Detectable HCV NS3-2-Specific CD8+ Cells*

Patient code	Percentage of HCV NS3-2 tetramer+ CD8+ cells	HCV NS3-2 amino acid sequence	Most-closely related reference isolate/subtype by phylogeny (maximum likelihood)			Number of amino acid variations	
						HCV-1 reference	Phylogeny reference
HCV-1		KLVALGINAV			KLVALGINAV		
S11	0.01	----------	HCV-1	(1a)	----------	0	0
S13	0.04	----------	HCV-H	(1a)	----------	0	0
S16	0.08	----------	HCV-1	(1a)	----------	0	0
S32	0.03	----M-L---	HCV-1	(1a)	----------	2	2
S8	0.01	----M-----	HCV-H	(1a)	----------	1	1
S9	0.01	------V---	[a]HCV-J1	(1a)	------V---	1	0
S33	0.07	------V---	[a]HCV-J1	(1a)	------V---	1	0
S2	0.02	--LS------	HCV-JK1	(1b)	------V---	2	3
S17	0.06	--S---L---	HCV-JK1	(1b)	--S---V---	2	1
S23	0.9	A-RGM-V---	HCV-J8	(2b)	A-RGM-V---	5	0
S20	Negative	----------	HCV-1	(1a)	----------	0	0
S24	Negative	----------	HCV-1	(1a)	----------	0	0
S30	Negative	----------	HCV-1	(1a)	----------	0	0
S7	Negative	----------	HCV-1	(1a)	----------	0	0
S15	Negative	------V---	HCV-J1	(1a)	------V---	1	0
S18	Negative	------V---	[a]HCV-J1	(1a)	------V---	1	0
S21	Negative	------V---	HCV-1	(1a)	----------	1	1
S25	Negative	------V---	HCV-H	(1a)	----------	1	1
S3	Negative	---G--V---	HCV-1	(1a)	----------	2	2
S19	Negative	---T--V---	HCV-J1	(1a)	------V---	2	1
S14	Negative	--SG--L---	HCV-JK1	(1b)	--S---V---	3	2
S1	Negative	A-RGM-V---	HCV-J8	(2b)	A-RGM-V---	5	0

*Dashes indicate amino acid identity to the HCV-1 prototype sequence shown at the top. The numbers of amino acid variations in each case were calculated comparing the autologous viral sequence to either the HCV-1 sequence or to the most closely related HCV reference sequence.
[a]Relatedness to HCV-H or HCV-J1 was not clearly differentiated by phylogenetic analysis.

a mechanism of persistence in several chronic infections. Some of the mechanisms of viral escape by epitope variation include mutation in HLA anchor residues [De Campos-Lima et al., 1996; McMichael and Phillips, 1997]; and in HLA-TCR interaction sites [Franco et al., 1995]. In the case of HCV, studies on chronically infected patients analysing the circulating viral sequence and CTL response to different epitopes using cromium-release assays showed viral epitope variants associated with the presence of CTL responses, suggesting prior immune selection [Chang et al., 1997]. Thus, in the course of chronic HCV infection, selection for viruses bearing variant epitopes may modify the immune response to the virus.

In the current study, we examined amino acid changes occurring in T-cell epitopes of HCV isolated from chronically infected patients with the same immunogenetic background (HLA-A2+). The presence of HCV-specific CD8+ cells was analysed in the same patients by staining PBMCs with HLA-class I tetramers. It was found that, while the frequency of amino acid variations in the HCV NS3-2 epitope was high when using the HCV-1 prototype as reference sequence, it was significantly reduced when using the phylogenetically most closely related HCV reference sequence. These results suggest that most of the variation in HCV NS3-2 corresponds to heterogeneity between HCV strains/subtypes. Interestingly, HCV subtype-independent variations in the NS3-2 epitope were mainly located at epitopic residues, potentially involved in TCR interaction. Finally, detection of HCV NS3-2-specific CD8+ cells (using a tetramer based in the HCV-1 prototype peptide) in PBMCs from patients with variant autologous virus suggested either cross-reactivity or mixed infection.

The main finding of the current study, which may have important practical implications, is that the HCV NS3-2 epitope is heterogeneous between different HCV strains/subtypes. Detailed phylogenetic analysis of the NS3 region allowed the comparison of the HCV NS3 sequence from individual patients to that of the most-closely related HCV reference subtype. Using this approach, few HCV isolates displayed amino acid variations in the NS3-2 epitope. It is concievable that previous studies might have considered subtype-specific variations in this NS3 epitope as CTL escape mutants [Chang et al., 1997], because most of the available data on the HCV CTL response are based on the screening of CTL clones with either peptide or recombinant antigens derived from the HCV-1 prototype (subtype 1a) [Koziel et al., 1995; Rehermann et al., 1996b; Chang et al., 1997; Nelson et al., 1997]. Clearly, future studies will need to take into account HCV viral genotype and subtype sequence heterogeneity to avoid important misinterpre-

```
Isolate      NS3-2 tet+   HCVNS3-1391        HCVNS3-2 1406
(Genotype)   CD8+ cells   (A3-restricted)    (A2-restricted)
                                                *        *
HCV-1 (1a)                GRH LIFCHSKKK CDELAA KLVALGINAV AYYRGLDVSVIPTSGDVV
  S11 (1a)      0.01      --- ---------- ------ ---------- ------------------
  S16 (1a)      0.08      --- ---------- ------ ---------- ------N-------D---
→ S32 (1a)      0.03      --- ---------- ------ ---M-L---- ------------------
  S20 (1a)      Neg       --- ---------- ------ ---------- ------------------
  S24 (1a)      Neg       --- ---------- ------ ---------- --F---------A----
  S30 (1a)      Neg       --- ---------- ------ ---------- ------------------
  S7  (1a)      Neg       --- ---------- ------ ---------- ------------------
→ S3  (1a)      Neg       --- ---------- ------ ---G--V--- ------------------

HCV-H (1a)                --- ---------- ------ ---------- ------------------
  S13 (1a)      0.04      --- ---------- ------ ---------- ------------------
→ S8  (1a)      0.01      --- ---------- ------ ---M------ ------------------
→ S25 (1a)      Neg       --- -------R-- ------ ----V----- ------------------

HCV-J1 (1a)               --- ---------- ------ ----V----- ------------------
  S9  (1a)      0.01      --- ---------- ------ ----V----- ------------------
  S33 (1a)      0.07      --- ---------- ------ ----V----- ------------------
  S18 (1a)      Neg       --- ---------- ------ ----V----- ------------------
  S15 (1a)      Neg       --- ---------- ------ ----V----- ------------------
  S21 (1a)      Neg       --- ---------- ------ ----V----- ------------------
→ S19 (1a)      Neg       --- ---------- ------ ---T--V--- ------------------

HCV-JK1 (1b)              --- ---------- ------ --S---V--- ------------------
→ S2  (1b)      0.02      --- ---------- ------ --LS------ P-----------------
→ S17 (1b)      0.06      --- ---------- ------ --S---L--- -------------N--
→ S14 (1b)      Neg       --- ---------- ------ --SG--L--- ------------------

HCV-J8 (2b)               --- ---------- ------ A-RGM-V--- -------------Q----
  S23 (2b)      0.9       --- ---------- ------ A-RGM-V--- ------------------
  S1  (2b)      Neg       --- ---------- ------ A-RGM-V--- ------------------
```

Fig. 3. Alignment of the deduced HCV NS3 amino acid sequence obtained from the 22 patients. The HCV sequences from the clinical isolates are aligned with those of the most closely related HCV subtype in each case (bold). The HCV NS3-2 (NS3-1406) and NS3-1391 epitopes are framed. Positions 1–10 of the viral peptide are numbered. TCR interaction sites of the viral peptide are indicated (shadowed areas). Amino acid variations with respect to the HCV-1 prototype sequence are indicated in capital letters. Amino acid variations in the HCV NS3-2 epitope unrelated to the most closely related subtype (small boxes) were found in eight patients (arrows). NS3-2 tet+ CD8+ cells: Percentage of HCV NS3-2 tetramer+ CD8+ T-cells in PBMCs. Asterisks: Primary HLA anchor sites of the HCV NS3-2 (NS3-1406) peptide.

tations when identifying the appropriate peptides for T-cell assays.

The data suggest that variation in residues implicated in HLA anchoring or processing of this HCV NS3-2 epitope is not common. In contrast, subtype-independent variations seem mainly located in positions likely implicated in TCR interaction. It is interesting to speculate that these variant peptides may induce an impaired T-cell signalling and/or activation of the effector function, as shown in Hepatitis B virus infection [Bertoletti et al., 1994]. Along this line, mutation in HCV NS3 or in multiple epitopes that impair CTL recognition

TABLE IV. Amino Acid Variation in the HCV NS3-2 Epitope in Patients From our Study, Compared to the HCV-1 Prototype or to the Most-Closely Related HCV Reference Sequence by Phylogenetic Analysis

| | Amino acid variations | | |
	From HCV-1 (1a) prototype	From most closely related sequence	P-value
Total number of variations	30	14	
Mean ± SD	1.36 ± 1.47	0.59 ± 0.85	<0.05[a]
Patients with variant virus	15/22 (68.2%)	9/22 (40.9%)	<0.05[b]

[a]Wilcoxon test.
[b]Chi-square test.

were associated with persistent infection in the chimpanzee model [Weiner et al., 1995; Erickson et al., 2001]. Development of functional T-cell assays to check this hypothesis in our patients was beyond the scope of the current study. Besides, determination of whether HCV NS3 amino acid variations found in the current study were transmitted or mutations which arise in the individual patients remains elusive, because data about the sequence of the original infecting virus was not available. Future longitudinal studies on special cohorts (i.e., acute infection, or well-characterised single-source outbreaks with available samples of the source virus) will be needed to clarify this point. Nonetheless, and consistent with previous reports [Chang et al., 1997], we could not detect emergence of mutations over time in two selected patients after a follow-up period of 6–10 months.

We also analysed the presence of HCV NS3-2 specific CD8+ cells in PBMCs by using a validated, very sensitive and specific HLA-tetramer staining technique, which allows direct quantitation of HCV-specific T-cells ex vivo without the need for in vitro amplification and peptide stimulation [He et al., 1999]. Using the HCV NS3-2 Class I HLA tetramer, we detected NS3-2-specific CD8+ cells in six patients despite the autologous viral epitope was different from the HCV-1 peptide sequence loaded in the tetramer. Surprisingly, HCV NS3-2 tetramer+ CD8+ cells were also detected in one of the two patients infected with HCV subtype 2b (five amino acid variations in the NS3-2 epitope). Presumably, and in the light of epidemiological and serotyping data, tetramer+ CD8+ cells in this patient had been elicited by a previous infection with HCV genotype 1, and remained detectable after reinfection with subtype 2b virus. The reasons because tetramer+ CD8+ cells were detected in some of the remaining patients infected with viruses different from HCV-1 may be: (i) non-specific binding (especially when the percentage of tetramer-binding cells is very low); (ii) cross-reactivity; and (iii) mixed infection. In our hands, the average background staining of the HCV NS3-2 tetramer in negative controls (both A2+HCV− and A2-HCV+) was consistently lower than 0.002% of peripheral CD8+ cells, at least five-times below the lower limit of detection of our tetramer assay [He et al., 1999]. Furthermore, the tetramer did not stain PBMCs from all the patients with NS3-2 epitope variants, as we would expect for non-specific staining. Consistent with our results, cross-subtype CTL reactivity against core and NS3 peptides derived from HCV-1 sequences has been reported elsewhere using traditional CTL assays [Koziel et al., 1995; Rehermann et al., 1996b; Nelson et al., 1997]. However, further specific studies, including functional T-cell assays, are needed to clarify if the T-cell response is cross-reactive between HCV genotypes and subtypes. Finally, mixed infection was not evident in our patients using LIPA, a method which detects subtype mixes representing low levels (1–2%) of the whole viral population [Qian et al., 2000]. Unfortunately, identification of previous infection by different subtypes of HCV genotype 1 was not possible,

because the limitations of commercially available serotyping tests.

The main drawbacks of the current study are the analysis of two CTL epitopes and the lack of data about the sequence of the original infecting virus. Obviously, since the CD8+ T-cell response against HCV is multi-specific, we cannot exclude the role of mutation in other epitopes. This important issue awaits studies based in recently developed comprehensive methods for determination of multispecific HCV T-cell responses, such as the use of peptide libraries spanning the complete HCV polyprotein [Lauer et al., 2002]. The fact that HCV amino acid variation in the HLA-A2+ patients from the current study was almost absent in the contiguous A3-restricted NS3-1391 epitope might suggest that variation in the A2-restricted NS3-2 epitope is not a random event. However, no specific HCV NS3-2 epitope sequence was clearly associated with the presence or absence of HCV NS3-2 tetramer+ CD8+ cells. As mentioned above, assumption that the HCV NS3-2 epitope variants identified may represent or not CTL mutants cannot be made without data from the original infecting virus. Because of the difficulty in obtaining such information in chronically infected patients, identification of CTL mutants needs for prospective studies analysing HCV variation in CTL epitopes over-time, especially after acute HCV infection or in single source outbreaks.

In summary, this is the first study reporting that naturally occurring HCV NS3-2 epitope variants can be related to sequence heterogeneity between HCV strains/subtypes. Mutation in this epitope was not evident over time in two patients, and HCV epitope-specific CD8+ cells were detectable in some patients with HLA-class I tetramers loaded with an HCV-1 prototype peptide, despite significant amino acid difference to the autologous virus. Future characterisation of the variant HCV NS3-2 peptides described above may be useful for the design of more accurate T-cell stimulation assays, for the synthesis of novel HCV HLA-tetramers, and for the development of vaccines and therapeutic strategies aimed at boosting the HCV-specific CD8+ T-cell response.

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