Frequent Compartmentalization of Hepatitis C Virus Variants in Circulating B Cells and Monocytes

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Differences in the composition of the hepatitis C virus (HCV) quasispecies between plasma and blood mononuclear cells (BMC) strongly suggest that BMCs support viral replication. We examined the frequency of such compartmentalization, the cell types involved, the constraints exerted on the different variants, and the role of immunoglobulin-complexed variants. We screened the hypervariable region (HVR1) of HCV isolates from 14 *HBsAg*- and HIV-seronegative patients with chronic HCV infection. HCV RNA was amplified and cloned from plasma, the immunoglobulin G (IgG)-bound fraction, and total and sorted BMCs (CD19+, CD8+, CD4+, and CD14+ cells). Compartmentalization was estimated using a matrix correlation test. The ratio of nonsynonymous/synonymous substitutions (d_N/d_S ratio) was calculated for each compartment. HCV RNA was detected in 3/3 BMC, 11/11 CD19+, 10/11 CD14+, 4/11 CD8+ and 0/11 CD4+ cell samples. HVR1 sequences were significantly different between plasma and at least one cellular compartment in all nine cases analyzed, and between B cells (CD19+) and monocytes (CD14+) in all five available cases. IgG-bound variants were distinct from cellular variants. D_N/d_S ratios were similar (n = 3) or lower (n = 6) in cellular compartments compared with plasma and the IgG-bound fraction. In conclusion, HCV compartmentalization is a common phenomenon. B cells and monocytes harbor HCV variants showing a low rate of nonsynonymous mutations, a feature that might contribute to the persistence of HCV infection. (HEPATOLOGY 2004;39:817–825.)

Hepatitis C virus (HCV) is an enveloped, positive-stranded RNA virus that circulates *in vivo* as a complex population of closely related variants referred to as a *quasispecies*.[1] Hepatitis C infection is frequently chronic, and its quasispecies nature probably plays a major role in viral persistence.[2,3] HCV genetic heterogeneity is particularly strong within a small hypervariable region (HVR1) that encodes a 27–amino acid peptide located at the N-terminus of the second envelope glycoprotein (E2) and is subjected to selective pressures by humoral and cellular immune responses.[4]

One potential viral strategy explaining the persistence of HCV is infection of immune cells. Convincing evidence of HCV lymphotropism has been obtained *in vitro* in experiments showing that minor quasispecies components of strain H77 are selected in lymphoblastoid cell lines.[5] Subsequently, it was found that blood mononuclear cells (BMC) from chimpanzees that had been inoculated with this strain became infected by the same lymphotropic quasispecies components as those selected *in vitro*.[6] *In vivo*, HCV RNA has been detected in BMC by many teams.[7–14] Viral replication in these cells, shown by the detection of negatively stranded HCV RNA, an obligatory replication intermediate, generally occurs at a low level.[9,15–18] Mutations in the 5′ untranslated region—the viral internal ribosomal entry site—have been described in HCV strains isolated from BMCs. These mutations could influence the translational efficiency of the internal ribosomal entry site structure in lymphoblastoid cell lines[19] and point to HCV adaptation to BMCs.[6,20,21] Infection of these cells by adapted strains could have functional consequences and constitute a reservoir for HCV.[22]

Abbreviations: HCV, hepatitis C virus; BMC, blood mononuclear cell; HVR1, hypervariable region; IgG, immunoglobulin G; E2, second envelope glycoprotein; IgD, immunoglobulin D; RT-PCR, reverse-transcriptase polymerase chain reaction; E1, first envelope glycoprotein.

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Significant differences in the HVR1 quasispecies composition between plasma and BMCs (*i.e.*, compartmentalization), although so far studied only in small series of patients, appear to be frequent.[11,23] Data on the distribution of viral variants among the different blood cell types (B cells, T cells, and monocytes) are scarce, and the constraints exerted on plasma and cellular variants have never been studied. Furthermore, variants that appear to be BMC-specific may in fact be complexed with circulating antibodies and may be bound to these cells via their Fc receptors. Immunoglobulin G (IgG)-bound HCV variants cluster differently from plasma quasispecies,[24,25] but IgG-bound and BMC variants have never been compared. We therefore examined the frequency of HCV quasispecies compartmentalization, and the cell types involved, to compare the constraints exerted on BMC-associated, IgG-bound, and plasma variants.

Patients

Fourteen patients (nine women and five men, all between 25 and 68 years of age) chronically infected with HCV for between 10 and 35 years were included with their informed consent and local ethics committee approval. The patients were sampled on the day of pretreatment liver biopsy. They had various stages of chronic hepatitis, ranging from very mild fibrosis (F0) to cirrhosis (F4). They harbored HCV genotype 1b (n = 11) or 3 (n = 3). The route of infection was either transfusion (n = 10) or intravenous drug use (n = 4). All were *HBsAg*- and HIV-seronegative, and none had received antiviral therapy.

Methods

Cell Sorting

Mononuclear cells were isolated from 10 mL of ethylenediaminetetraacetic acid–treated blood by centrifugation through a Ficoll (Lymphoprep, Nycomed, Oslo, Norway) density gradient. Recovered cells were washed three times, counted, and immediately separated by immunomagnetic positive selection with antibodies directed against subset-specific surface molecules. Between 2 and 10×10^6 BMC were suspended in 1 mL of RPMI (Invitrogen, Cergy-Pontoise, France) culture medium. Twenty-five microliters of goat antimouse IgG magnetic beads (Dynabeads, Dynal, Oslo, Norway) were coated with 1.5 g of anti–immunoglobulin D (IgD) mAb (IADB6, SBA) and incubated with total BMCs for 20 minutes with gentle rotation. After magnetic separation, the remaining cells were subjected to sequential positive sorting with anti–CD19-, anti–CD14-, anti–CD8-, and anti–CD4-coated beads (Dynal) as described previously.[11] All sorted cells were washed five times in RPMI medium. Cell subsets were stored at −80° C until use. The purity of each fraction (>95%) was checked by flow cytometry as described previously.[11]

Purification of IgG Molecules

IgG was isolated from plasma by using recombinant protein A-sepharose (InterbioTech, Montlucon, France). Fifty microliters of protein A sepharose, washed three times in PBS, was added to 140 microL of plasma and incubated for 1 hour at 4°C with gentle rotation. Protein A–coupled immunoglobulin was washed five times in PBS (to remove unattached virions) and stored at −80°C until used.

Nucleic Acid Extraction, Reverse-Transcriptase Polymerase Chain Reaction, and Quantification of HCV RNA

RNA was extracted from 140 μL of plasma using the QIAmp viral RNA kit (Qiagen, Courtaboeuf, France), and from cell subsets using the RNeasy minikit (Qiagen). To detect the HCV genome, one fifth of the plasma or cellular RNA extract was subjected to reverse-transcriptase polymerase chain reaction (RT-PCR) with Ready-To-Go RT-PCR beads (Pharmacia Biotech, Uppsala, Sweden). The PCR procedure and the outer and inner primers for HVR1-nested PCR have been described elsewhere.[11] The HVR1 primers generate a 307-bp product (nucleotides +956 to +1262 according to the numbering system of Choo) encompassing the C-terminal transmembrane region of the first envelope glycoprotein (E1) and the N-terminal hypervariable region of E2. Positive-strand HCV RNA was quantified by using real-time PCR (Light Cycler RNA Master Hybridization Probe, Roche Diagnostics, Idaho Falls, ID) as described previously.[26]

Cloning and Sequencing of PCR Products

HVR1 PCR products were inserted into the pGEM-T Easy Vector system (Promega Corporation, Madison, WI) and transfected into *Escherichia coli* JM109 competent cells (Promega). After overnight incubation at 37° C, insertion was checked by PCR using the 5′ untranslated region or HVR1 inner primer pair on white colonies. Each PCR product with the correct molecular weight was sequenced, bidirectionally and automatically, using the inner primer pair on an ABI377 sequencer with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and Amplitaq DNA Polymerase (Perkin Elmer/Applied Biosystems Division, Foster City, CA).

Sequence Analysis and Statistical Methods

Nucleotide and deduced amino acid sequences of cloned products were aligned using the CLUSTAL W program version 1.5.[27] Pairwise nucleotide distances were calculated using the Kimura two-parameter method with a transition-to-transversion ratio of 2. This matrix was used to determine both evolutionary relationships among sequences and correlations with the compartmental distribution. Phylogenetic trees were constructed using the neighbor-joining algorithm—a cluster analysis method that fits sequences, such as those of HCV quasispecies, that have high similarity scores. Statistical evaluation of the obtained topology was based on 500 replications of bootstrap sampling.

Mantel's test was used to determine if sequences from a given compartment were genetically closer to each other than to sequences from other compartments.[11,28] This test compares the Kimura two-parameter distance matrix with a compartment distribution matrix (Mc) of the same dimensions, where Mc(i, j) = 0 if sequences I and j are from the same compartment, and Mc(i, j) = 1 in other cases. The Pearson correlation coefficient r2 was computed for all pairs, excluding the diagonals of both matrices (observed r2). The null distribution was constructed by permuting the rows and columns of the Mc matrix 1,000 times. The number of times that the observed r2 was exceeded during the 1,000 permutations gave the exact *P* value of the observed correlation. Software written by Philippe Casgrain was used for this purpose (http://www.fas.umontreal.ca/biol/casgrain/fr/labo/R/index.html).

As an index of HVR1 genetic complexity within a given compartment, normalized Shannon entropy was calculated as: $Sn = -\mathrm{i}(pi \ln pi)/\ln N$, where *pi* is the frequency of each amino acid sequence and *N* the total number of sequences analyzed in each compartment. *Sn* theoretically ranges from 0 (no complexity) to 1 (maximum complexity). The mean Kimura distance, reflecting genetic diversity, was calculated between all pairs of variants from each compartment.

Synonymous (d_S) and nonsynonymous (d_N) distances were calculated by using the Jukes-Cantor correction in the Molecular Evolutionary Genetics Analysis software package version 1.01 (http://megasoftware.net/text/downloads.sht). The mean ratio between d_N and d_S was established, for each compartment, from the pairwise distances of HVR1 sequences. The ratios were compared among compartments. Comparison of these d_N/d_S ratios gives an estimate of the difference in constraints exerted on the HVR1 in the different compartments. Positive constraint is inferred if d_N/d_S is greater than 1; conservative constraint is inferred if it is less than 1.[29]

Antigenic Analysis

Peptide sequences were analyzed according to their estimated antigenicity, hydrophobicity, and polarity. Antigenic grouping was performed both manually and using Parker's algorithm.

Results

Detection of HCV RNA in Plasma, Plasma IgG, and BMCs. The amount of total cellular RNA extracted varied according to the cell type. Unsorted BMC yielded 2 to 5 μg of total RNA, while CD19+ and CD14+ fractions yielded 0.5 to 1.5 μg. HVR1 sequences were detected in the plasma and BMCs of all 14 patients. Among the 11 patients whose BMCs were sorted, HVR1 was amplified from CD19+ cells in all cases, CD14+ cells in 8/9 cases, CD8+ cells in 4/11 cases, and CD4+ cells in 0/? cases (Table 1). All four patients with detectable HCV RNA in CD8+ cells had severe liver fibrosis (precirrhosis in one and cirrhosis in three). HVR1 was also detected in the IgG-bound fraction of all four patients tested. No relation was found between plasma viral load and successful HVR1 amplification in the different compartments. Sufficient cellular RNA (500 ng or more) was available for quantitative PCR in 8/14 patients (see Table 1). Mean viral load (log), normalized to 1 μg of total cellular RNA, was 4.6 in unsorted BMC, 4.6 in CD19+ IgD cells, 4.7 in CD19+ IgD+ cells, and 3.9 in CD14+ cells. HVR was subcloned by using PCR products from at least 500 ng of cellular RNA to avoid a sampling bias. In three cases (cases 7, 8, and 9), HVR subcloning PCR was thus performed with more than 10,000 HCV RNA copies. For plasma samples, HVR subcloning PCR was performed with an estimated average of 40,000 copies. Light cycler PCR confirmed the negative results obtained with nested HVR PCR.

Entropy and d_N/d_S Ratio According to the Compartment. A total of 32 compartments were analyzed by cloning in nine patients. Between 10 and 24 clones were available for each compartment (total 450). Only clones with matching sequences obtained with sense and antisense primers were taken into account, yielding a total of 373 clones (mean 12 per patient; range 6–20). No particular differences in entropy or genetic diversity were observed between plasma, IgG-bound, or cellular compartments. These parameters were not related to the quantity of HCV RNA measured in the different compartments and did not correlate with each other (paired *t* test). No correlation between entropy, genetic diversity, and the dN/dS ratio was found. Entropy and d_N/d_S values were independent of the number of clones studied (data not shown).

Table 1. Detection Through HVR Nested-PCR and Quantification of Positive-Strand HCVRNA in Different Compartments in 14 Patients

Number of cells	Plasma	IgG	BMC 2×10^6	CD19+ 2×10^5	CD19+, IgD+ 1×10^5	CD19+, IgD− 1×10^5	Cd14+ 4×10^5	Cd8+ 6×10^5	Cd4+ 8×10^5	Cloning
#1	^{10}5.4	^{15}P	–	–	^{9}P	^{6}P	^{8}P	N	N	yes
#2	^{16}4.9	^{7}P	–	–	N	^{14}P	^{6}P	N	N	yes
#3	^{20}5.1	^{7}P	–	–	^{13}P	^{9}P	^{13}P	N	N	yes
#4	^{20}4.8	^{20}P	–	–	^{20}P	^{20}P	N	N	N	yes
#5	^{13}4.7	–	–	^{16}P	–	–	^{6}P	^{16}P	N	yes
#6	^{19}5.8	–	–	^{17}P	–	–	^{11}P	^{6}P	N	yes
#7*	^{10}6.2	–	^{11}P(5.1)	–	–	–	–	–	–	yes
#8	^{12}5.7	–	^{11}P(4.2)	–	–	–	–	–	–	yes
#9	^{11}5.8	–	^{10}P(4.0)	–	–	–	–	–	–	yes
#10	6.0	–	P(5.1)		P(4.8)	P(4.8)	P(4.1)	P(4.2)	N(<2)	No
#11	6.1	–	P(4.5)		P(5.1)	–	–	N(<2)	N(<2)	No
#12	5.2	–	P(5.1)		P(3.1)	P(3.5)	P(4.2)	N(<2)	N(<2)	No
#13	5.1	–	–		P(5.0)	P(5.8)	–	N(<2)	N(<2)	No
#14	6.1	–	–		P(5.1)	P(<2)	P(3.5)	P(3.3)	N(<2)	No

Abbreviations: P, positive; N, negative; ^{xx}, number of clones for this compartment.

NOTE. Logarithmic expression of HCV RNA copies normalized for 1 μg of total RNA (in parenthesis for cellular compartments) or for 1 ml of plasma.

*For all patients except one, BMC amplification or cell sorting was performed from 10×10^6 cells. For patient #7, PCR was performed on RNA corresponding to 4×10^5 cells.

The d_N/d_S ratios were not significantly different among the available compartments in 3/9 patients. In each of the other six patients, the d_N/d_S ratios for plasma sequences were significantly higher than the d_N/d_S ratios for total BMCs or for at least one cell subset. In two cases, d_N/d_S ratios were significantly lower for sequences in both CD14+ and CD19+ cells than in plasma. The d_N/d_S ratios were not different between plasma and IgG-bound quasispecies in the four cases tested (Fig. 1).

Phylogenetic and Mantel Analysis of Quasispecies Distribution. Bootstrapped phylogenetic trees suggested clustering of clones according to their compartmental origin in the three patients from whom plasma and unsorted BMC variants were available (Fig. 2A). Statistical significance was demonstrated by Mantel's test in these three patients (Table 2). In four of the six patients whose BMCs were sorted and from whom more than two cellular compartments were available, analysis of phylogenetic trees showed a unique cluster of variants with bootstrap values greater than 80, in CD14+ cells from case 2, CD19+ cells from cases 4 and 6, CD8+ cells from cases 5 and 6, and IgG-bound plasma variants from case 3. The genetically different compartments contained more than one cluster of variants in some cases (CD19+IgD+ cells in cases 1 and 4, CD14+ cells in case 3, and IgG-bound plasma variants in case 2). Mantel's test, being based on correlations between pairwise genetic and phenotypic distances, overcomes the difficulties posed by the interpretation of phylogenetic trees with multiple clusters. For each patient, we thus performed pairwise analysis of variants according to the compartment. By using this test in the six patients whose BMCs were sorted, we found that plasma variants differed from CD19+ cell variants but not from CD14+ cell variants in two patients; from CD14+ cell variants but not from CD19+ cell variants in two patients; from CD19+, CD14+, and CD8+ cell variants

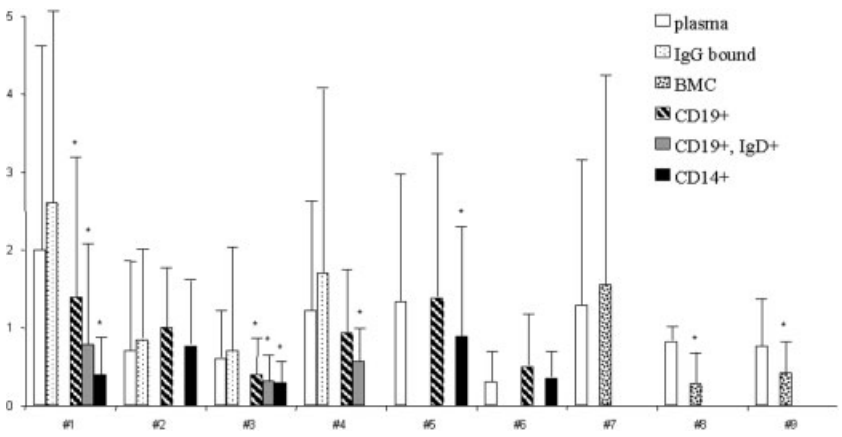


Fig. 1. The d_N/d_S ratios ($\pm$ standard deviation) for the HCV variants found in the different compartments in nine patients (CD8+ compartments are not shown). *Significant difference with the plasma compartment ($P<.05$).

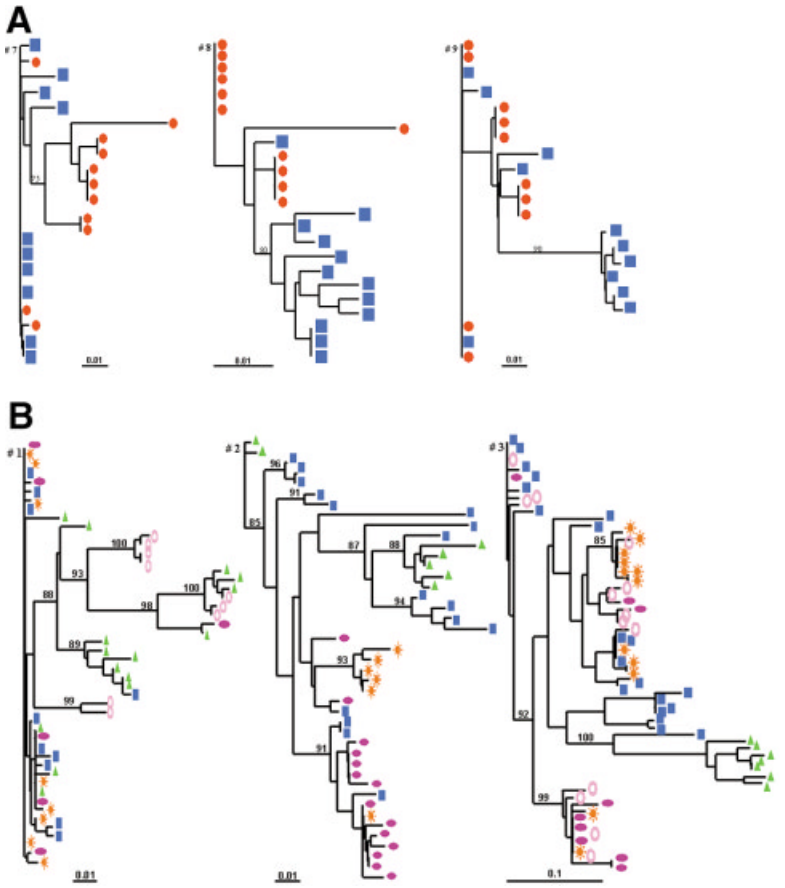


Fig. 2. Phylogenetic trees of plasma, nonsorted BMC, IgG-bound, CD14+, CD19+IgD+, and CD19+IgD− cells of HVR1 HCV variants in six patients. Some bootstrap values are indicated. Blue: plasma; red: nonsorted BMC; green: IgG-bound; orange: CD14+; pink: CD19+IgD+; and purple: CD19+IgD−.

in one patient; and only from CD8+ cell variants in one patient. CD14+ cell variants were significantly different from CD19+ cell variants in all five available cases. We were able to compare the quasispecies distribution in memory (IgD−) and naïve (IgD+) B cells from three patients and found that it was significantly different in two cases. IgG-bound variants differed significantly from plasma variants in three of the four available cases. IgG-bound variants were always distant from cellular variants in Mantel's test. Thus, in all nine patients, BMCs contained specific variants that were nonrandomly distributed between CD14+, CD19+, and CD8+ cells.

Amino Acid Sequence Analysis. Alignment of deduced amino acid sequences revealed clearly distinct peptide motifs between plasma and at least one cellular compartment, confirming the results of Mantel's test. Most nonsynonymous mutations occurred in the HVR1. Despite these mutations, no noteworthy change in hydrophobicity or transmembrane parameters was found from one compartment to another. Application of Parker's algorithm showed that these mutations led to changes in antigenicity. Among the six patients in whom HVR was cloned in different cell subtypes, we identified mutations between cell subtypes—at both functional and structural positions—in every case. Figure 3 shows the alignment of amino acid sequences from patients 1, 2, and 3 and the consensus pattern defined by Penin.[30] Finally, we detected no hot spot for mutations between compartments.

Discussion

This study of immunocompetent subjects with chronic hepatitis C demonstrates that HCV quasispecies compartmentalization among plasma and BMC subsets, based on analysis of the HVR1 region, is a common phenomenon. Previous studies had already pointed to HCV HVR1 compartmentalization, but appropriate statistical methods were not used and the nature of the cellular subsets was not investigated.[10,23] By using the Mantel's test, which does not depend only on the interpretation of phylogenetic trees, we found that B cells and monocytes were the main BMC subsets involved in HCV compartmentalization, and that variants harbored by B cells and monocytes were always different from each other. These variants were also genetically distant from circulating IgG-bound variants that, in three of the four patients studied here, clustered differently from plasma variants, as reported previously.[24,25] Our data show that only a minor fraction of plasma variants are immunocomplexed and thus rule out the possibility that compartmentalization is

Table 2. Mantel's Test Applied to 9 Subjects for Whom Subcloning of HVR Was Performed in Different Compartments

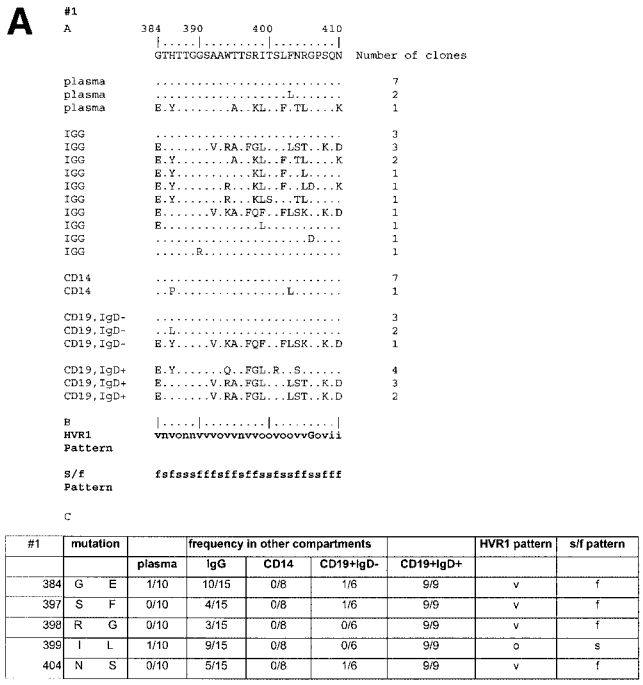
No.	Fractions	Plasma	IgG-Bound	CD19+		CD14+
				IgD−	IgD+	
#1	IgG-bound	< 0.001	–	–	–	–
	CD19+.IgD−	0.044	< 0.001	–	–	–
	CD19+. IgD+	< 0.000	< 0.001	< 0.001	–	–
	CD14+	NS	< 0.001	NS	< 0.001	–
#2	IgG-bound	0.014	–	–	–	–
	CD19+. IgD−	< 0.001	< 0.001	–	–	–
	CD14+	NS	< 0.001	0.021	–	–
#3	IgG-bound	< 0.001	–	–	–	–
	CD19+. IgD−	NS	0.030	–	–	–
	CD19+. IgD+	NS	0.010	NS	–	–
	CD14+	0.005	0.001	< 0.001	0.001	–
#4	IgG-bound	NS	–	–	–	–
	CD19+. IgD−	< 0.001	0.001	–	–	–
	CD19+. IgD+	0.019	0.010	< 0.001	–	–
#5	CD19+	NS	–	–		–
	CD14+	NS	–	0.047		–
	CD8+	< 0.001		< 0.001		< 0.001
#6	CD19+	< 0.001	–	–		–
	CD14+	< 0.001	–	0.001		–
	CD8+	< 0.001	–	< 0.001		< 0.001
#7	BMC	< 0.001	–	–		–
#8	BMC	< 0.001	–	–		–
#9	BMC	0.001	–	–		–

P values are indicated for each pairwise comparison of compartments for each of the 9 patients. A significant difference in quasispecies composition between two compartments existed for $P < 0.05$. NS = non significant result.

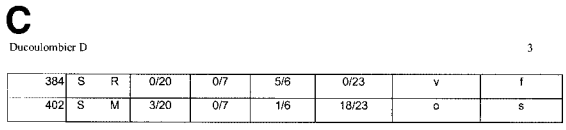
due simply to cellular association of IgG-bound variants through Fc receptors.

We did not observe a complete clustering of variants in each compartment for each patient, which raises the possibility of nonspecific binding of plasma variants to cells or suggests that the cell sorting was not totally specific. Intercompartmental contamination was probably negligible, as suggested by the absence of HCV RNA in the CD4+ cell subset and in most CD8+ cell subsets. Another possible explanation is that some variants are adapted to both lympho-mononuclear cells and to the plasma/liver environment. Conversely, compartmentalization could be related to a sampling bias. As in previous studies, we found relatively low viral load values in the different cellular subsets (approximately 10,000 HCV RNA copies/500,000 cells). For this reason, and because sample volumes were limited, we could not both quantify and subclone HVR RNA in all patients and in all compartments. In three cases (cases 7, 8, and 9), the quantities of HCV RNA used for PCR and HVR cloning were within the same range (between 10^4 and 10^5 copies) in plasma and cellular compartments. In the five patients in whom viral load was measured in cellular compartments, the quantity of HCV RNA amplified in PCR experiments was approximately 10,000 copies per 500 ng of total RNA. These data strongly suggest that PCR cloning was obtained in plasma and cellular compartments from 10,000 to 50,000 HCV RNA molecules, inferring that our results were not due to a sampling bias owing to extremely different viral loads. The similar entropies in plasma and cellular compartments indirectly confirm the absence of a sampling bias.

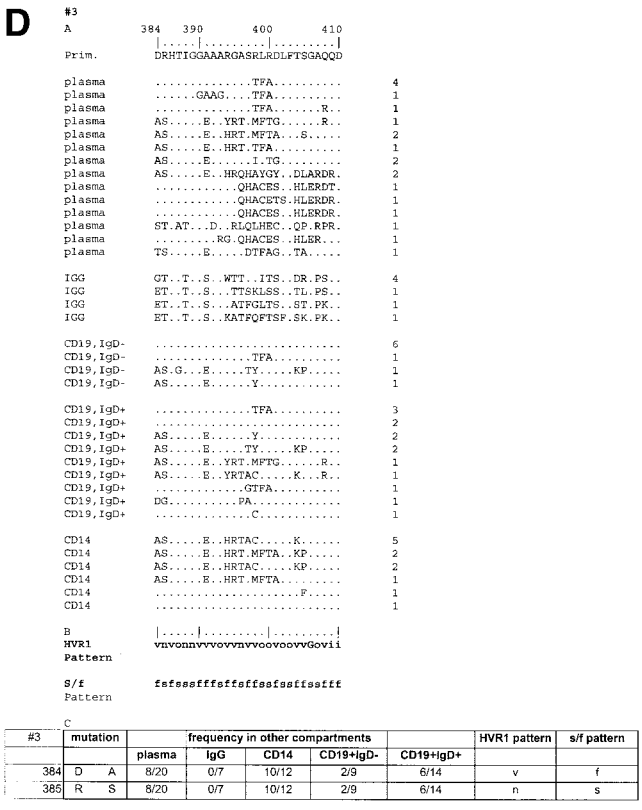
It is generally agreed that both positive and negative constraints influence the quasispecies structure of HCV and lead to the selection of the best-adapted variants. These constraints are partially reflected by the d_N/d_S ratios. D_N/d_S ratios found on cellular subsets variants were significantly lower (n = 6) or equivalent (n = 3) to those calculated for plasma or IgG-bound plasma variants. This suggests a trend for a weaker constraint on variants harbored by B cells or monocytes than on variants circulating in the plasma. Although the nature of these constraints remains to be determined, they could favor the persistence of some strains in cellular compartments. The d_N/d_S ratio was not related to the quantity of HCV RNA, to the entropy of variants, or to the number of clones within a compartment. Absence of relation between d_N/d_S ratios and entropy was reported previously for the HVR.[31] Longitudinal studies are clearly required to observe for each compartment, the relations between constraints as reflected by d_N/d_S ratio and subsequent changes of entropy or genetic diversity. The differences in d_N/d_S ratios between compartments, together with the statistically significant compartmentalization of HCV variants, may



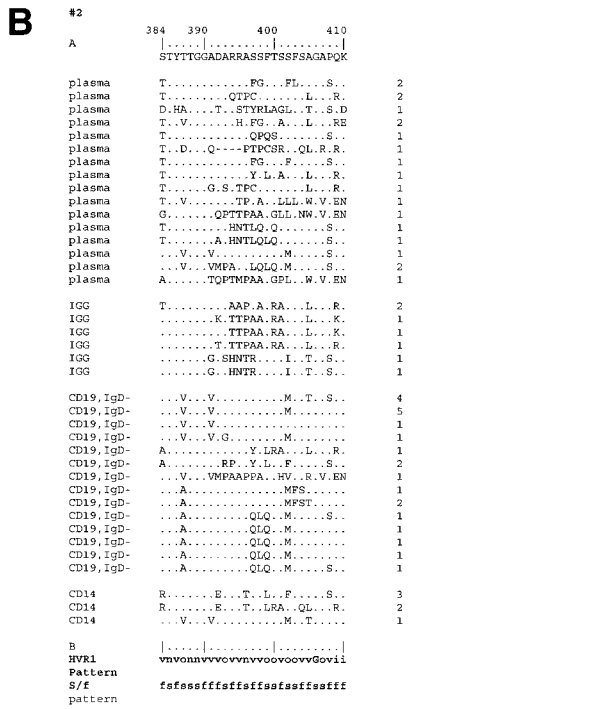
#1	mutation		frequency in other compartments					HVR1 pattern	s/f pattern
			plasma	IgG	CD14	CD19+IgD-	CD19+IgD+		
384	G	E	1/10	10/15	0/8	1/6	9/9	v	f
397	S	F	0/10	4/15	0/8	1/6	9/9	v	f
398	R	G	0/10	3/15	0/8	0/6	9/9	v	f
399	I	L	1/10	9/15	0/8	0/6	9/9	o	s
404	N	S	0/10	5/15	0/8	1/6	9/9	v	f



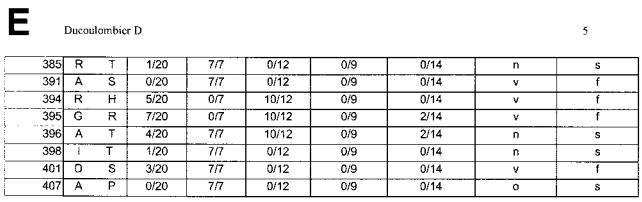
384	S	R	0/20	0/7	5/6	0/23	v	f
402	S	M	3/20	0/7	1/6	18/23	o	s



#3	mutation		frequency in other compartments					HVR1 pattern	s/f pattern
			plasma	IgG	CD14	CD19+IgD-	CD19+IgD+		
384	D	A	8/20	0/7	10/12	2/9	6/14	v	f
385	R	S	8/20	0/7	10/12	2/9	6/14	n	s



#2	mutation			frequency in other compartments			HVR1 pattern	s/f pattern
			plasma	IgG	CD14	CD19+IgD-		
384	S	T	15/20	2/7	0/6	0/23	v	f



385	R	T	1/20	7/7	0/12	0/9	0/14	n	s
391	A	S	0/20	7/7	0/12	0/9	0/14	v	f
394	R	H	5/20	0/7	10/12	0/9	0/14	v	f
395	G	R	7/20	0/7	10/12	0/9	2/14	v	f
396	A	T	4/20	7/7	10/12	0/9	2/14	n	s
398	I	T	1/20	7/7	0/12	0/9	0/14	n	s
401	D	S	3/20	7/7	0/12	0/9	0/14	v	f
407	A	P	0/20	7/7	0/12	0/9	0/14	o	s

Fig. 3. Amino acid alignment in the hypervariable region 1 (HVR1) of three patients (1, 2, and 3) for whom variants from plasma, IgG-bound plasma, CD19+IgD−, CD19+,IgD+, and CD14+ compartments were available. (A) The left-hand column indicates the origin of the HCV variants; the right-hand column, the number of clones exhibiting the mutations. Consensus corresponds to the amino acid sequence most frequently derived from alignment of all clones. Amino acids identical to consensus sequence are represented by a dot (·). Main mutations are squared. (B) HVR1 consensus hydropathy pattern established by Penin and colleagues.[30] This consensus was derived from analysis of 1,382 HVR1 sequences reported in the European Molecular Biology Laboratory database. Hydrophobic (o), neutral (n), hydrophilic (i), and variable (v) positions are presented. G is a fully conserved Gly residue. The s/f pattern summarizes the putative main role of each HVR1 position (s, structural; f, functional). (C) The table shows the frequency of mutations per compartment.

point to autonomous HCV replication in blood B lymphocytes and/or monocytes. Our data are consistent with other studies that concluded that variants found in peripheral blood mononuclear cells probably replicate slowly and contribute little to plasma viral load.[17,32,33] However, we cannot completely rule out the possibility that HCV could infect BMCs because of altered envelope sequence, but not replicate, or that variants found in

monocytes have been endocytosed. In one patient studied, additional sequencing of core and E1 demonstrated that memory B cells were infected by HCV subtype 1a, whereas subtype 1b was found in all the other compartments, including the liver (data not shown). Similar HCV genotypic compartmentalization has also been reported in brain and cerebrospinal fluid.[18,34] This genotypic compartmentalization strongly supports the existence of autonomous HCV replication in lymphomononuclear cells.

We found that B cells and monocytes were the two main cell types involved in HCV compartmentalization. The functions of these two cell types are clearly abnormal during chronic HCV infection. B cell dysfunction is reflected by IgG1 restriction, low-titer and delayed-onset antibody responses,[35] an increased frequency of naïve B cells[36] and t(14;18) translocation[37] Impaired allostimulatory function of dendritic cells derived from blood monocytes has been repeatedly demonstrated.[14,38] Our data are compatible with the possibility that some of the functional modifications may be due directly to infection of these cell types by HCV variants**.** We also found that compartmentalization differed among the patients studied. For instance, HCV RNA was only detected in CD8+ T cells from the four patients with severe fibrosis. Although these data are preliminary, they suggest that variants with different cellular tropism might emerge during the course of chronic infection. This phenomenon also occurs during the course of HIV-1 infection, with variants switching from macrophage tropism to T cell tropism. Longitudinal studies of compartmentalization in larger groups of patients (possibly in the liver transplant setting) are required to confirm this hypothesis. Like other viruses,[39–41] HCV variants may be selected on the basis of a specific amino acid sequence involved in host cell binding and entry. We found no cell-specific signature or preferential position for mutations in the small region analyzed here, contrary to suggestions by Goutagny and colleagues.[42] Analysis of complete E1 and E2 sequences will be required to identify protein motifs possibly related to the cellular tropism of HCV variants.

In conclusion, our data demonstrate that, during chronic HCV infection, B cells and/or monocytes frequently harbor specific HCV variants. The data also suggest that these variants are subjected to lesser constraints. This phenomenon could be involved in the persistence of HCV and might be an important determinant of the natural history and therapeutic outcome of the infection.

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