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Plasma and Liver Hepatitis C Virus Variability in Patients Coinfected with Human Immunodeficiency Virus

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Liver and plasma hepatitis C virus (HCV) variability was compared by E2 cloning and sequencing in three patients coinfected with HCV and human immunodeficiency virus (HIV) before and after interferon treatment and in three patients solely infected with HCV. The plasma and liver samples contained unique sequences. In the patients coinfected with HIV, accumulated random mutations produced mostly nonsynonymous substitutions in contrast to the reduced HCV genetic variability seen after treatment.

The course of hepatitis C virus (HCV) infection is modified during coinfection with human immunodeficiency virus (HIV), undergoing enhanced viral replication and accelerated progression to cirrhosis (4, 15, 20, 25). In addition, a sustained virological response occurs in only 25% of the patients coinfected with HCV and HIV that are treated with interferon-ribavarin. HCV has a quasispecies distribution (9, 21, 23) that is best studied in the E2 envelope hypervariable region 1 (HVR1) (14) and is occasionally described as predictive of a favorable outcome (5, 7, 8, 14, 17). However, little is yet known about HCV quasispecies in patients coinfected with HIV (2, 15).

Our study focused on liver samples in three nonresponding patients included in a clinical trial (22) in order (i) to describe liver and plasma HCV variability in patients coinfected with HCV and HIV and in patients solely infected with HCV at baseline and (ii) to compare hepatic and plasmatic quasispecies in patients coinfected with HCV and HIV before and 6 months after completion of anti-HCV treatment.

The three patients coinfected with HIV (P1, P2, and P3) had TCD4 lymphocytes at $>250/mm^3$, undetectable HIV RNA, and chronic HCV hepatitis. Patients P1 and P2 were infected with HCV genotype 3, and patient P3 was infected with HCV genotype 1b. The three HIV-negative genotype 1b-infected patients (P4, P5, and P6) had not received any anti-HCV treatment for several months.

After RNA extraction from plasma and liver (22), a 325-bp fragment encompassing HVR1 region was amplified (11) and cloned (pGEM-T Easy Vector System I; Promega). For the three patients coinfected with HIV 222 clones were evaluated (mean, 18.5 per sample), and for the three HIV-negative patients 95 clones were evaluated. The sequences (CEQ2000; Beckman Coulter) were aligned (CLUSTAL W 1.74), and phylogenetic trees were constructed by the neighbor-joining method. GenBank accession numbers for the original nucleotide sequences presented here are recorded as AY793020 to AY793336.

The quasispecies complexity was calculated by using normalized Shannon entropy (*Sn*) (24). Diversity was analyzed for (i) the mean genetic distance (*d*, i.e., the number of nucleotide differences divided by total number of nucleotides) and (ii) synonymous substitutions (*dS*) and nonsynonymous substitutions (*dN*). The data were by using *t* test results (paired *t* tests or Mann-Whitney nonparametric tests), and correlations were investigated by using Prism 2.01 software.

Table 1 shows detailed complexity and diversity results before treatment for the six patients (baseline) and after interferon treatment for the three patients coinfected with HIV (posttreatment). Before treatment, HCV quasispecies displayed no specific complexity or diversity pattern related to sample types (plasma or liver) or patients characteristics. Table 2 presents the statistical parameters used in this study. Complexity and diversity were significantly correlated. In the three patients coinfected with HIV, synonymous substitutions were the most frequent at baseline. Other researchers previously described a higher diversity in severely immunocompromised patients coinfected with HIV (19) or in patients with end-stage liver disease (1), which was not the case in our patients.

In patients solely infected with HCV, the complexity was significantly higher in HVR1 than in flanking regions (Fig. 1), whereas no difference appeared in patients coinfected with HIV. Since HVR1 is known to harbor both neutralizing and cytotoxic T epitopes, this absence of specific complexity pattern in HVR1 suggests a weak immune pressure, if any, which could result from HIV-related immune deficiency. In these patients, HVR1 quasispecies evolution should therefore more likely be due to a high rate of accumulation of random mutations than to a positive selection pressure.

On the phylogenetic trees (data not shown), each patient's

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TABLE 1. Complexity and diversity of the entire sequence encompassing HVR1 for patients P1 to P6

Patient	Value	Complexity		Diversity	
		Sn nt[a]	*Sn* aa[b]	*d*[c]	*dN/dS*[d]
P1	Plasma baseline	0.975	0.514	0.026	0.309
	Liver baseline	0.889	0.337	0.011	0.053
	Plasma posttreatment	0.207	0.139	0.002	0.333
	Liver posttreatment	0.539	0.442	0.004	**1.25**
P2	Plasma baseline	0.657	0.453	0.008	0.375
	Liver baseline	0.739	0.53	0.01	0.273
	Plasma posttreatment	0.783	0.457	0.01	0.392
	Liver posttreatment	0.38	0.233	0.003	0.286
P3	Plasma baseline	0.959	0.821	0.033	0.448
	Liver baseline	0.714	0.679	0.031	0.444
	Plasma posttreatment	1	0.758	0.015	0.229
	Liver posttreatment	0.256	0.168	0.002	**1**
P4	Plasma baseline	0.54	0.127	0.003	**>1**
	Liver baseline	0.722	0.616	0.007	0.555
P5	Plasma baseline	0.958	0.792	0.022	0.576
	Liver baseline	1	0.787	0.021	0.6
P6	Plasma baseline	0.937	0.769	0.022	**2.25**
	Liver baseline	0.622	0.564	0.015	**1.42**

[a] Shannon entropy (*Sn*) at the nucleotide level (nt).
[b] Shannon entropy (*Sn*) at the amino acid level (aa).
[c] Genetic distance.
[d] *dN/dS* ratio is considered as a measure of immune pressure compared to genetic drifts when >1 (18) and appears in boldface.

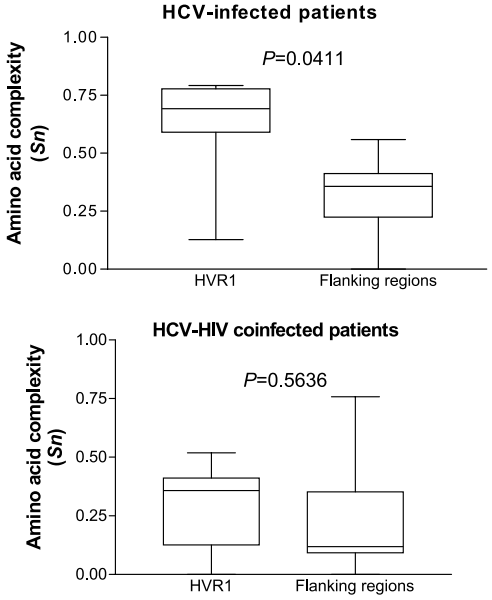

FIG. 1. Comparison of amino acid complexity expressed with Shannon entropy (*Sn*), between HVR1 and in the flanking regions in HCV-solely infected and HCV-HIV coinfected patients. Mann-Whitney test.

sequences clustered independently, thus excluding cross-contamination. The viral variant distribution is presented in Fig. 2. There was not necessarily a dominant variant in each compartment at each time, but each compartment harbored specific variants, as already described (12, 16). For HIV-infected patients P1 and P2, one common dominant variant was present both at baseline and after treatment completion (50 to 89% of the clones). However, in P2, it represented only 5% of the plasma clones after treatment. For patient P3, only one baseline plasma variant was still present after treatment (<50% of the clones), and the liver harbored one predominant but previously undetected variant (89% of the clones). Of the three patients solely infected with HCV, patient P4 displayed a variant distribution similar to that of patients P1 and P2. Patients P5 and P6 had, respectively, 5 and 1 common variants in the plasma and liver, but none predominated. There was one major liver variant in P6 (53% of the clones).

Previous studies of HCV variability over time also showed little evolution in plasma complexity in most instances, both in patients coinfected with HIV and in patients solely infected with HCV (2, 13). Important quasispecies differences between blood and liver, correlated with hepatic fibrosis, have been described (3). Other researchers found no significant variation (12).

After treatment completion, liver complexity was notably reduced in the HIV-infected patients (Table 2). Although unsuccessful, interferon treatment seemed more efficient for eliminating minor liver variants than plasma ones (6). The predominant variant persisting over time in two patients coinfected with HIV appeared to be the closest to the common node connecting all the clones in each patient. This

TABLE 2. Statistical results obtained from comparison of the complexity and diversity data in patients solely infected with HCV or coinfected with HIV and HCV

Parameter	Subset	*P* and/or r^2
HVR1 complexity > flanking regions complexity		$P = 0.04$
Correlation between nucleotide and amino acid complexity	In patients solely infected with HCV	$r^2 = 0.6648$
	In patients coinfected with HIV	$r^2 = 0.7819$
	In both groups of patients	$r^2 = 0.68$
Diversity *d* at baseline > *d* posttreatment in patients coinfected with HIV	In plasma	$P < 0.02$
	In liver	$P < 0.02$
	In both compartments	$P < 0.02$
Correlation between complexity (*Sn*) and diversity (*d*) in patients coinfected with HIV	Nucleotide entropy (*Sn*)	$r^2 = 0.5021$; $P = 0.0099$
	Amino acid entropy (*Sn*)	$r^2 = 0.6506$; $P = 0.0015$

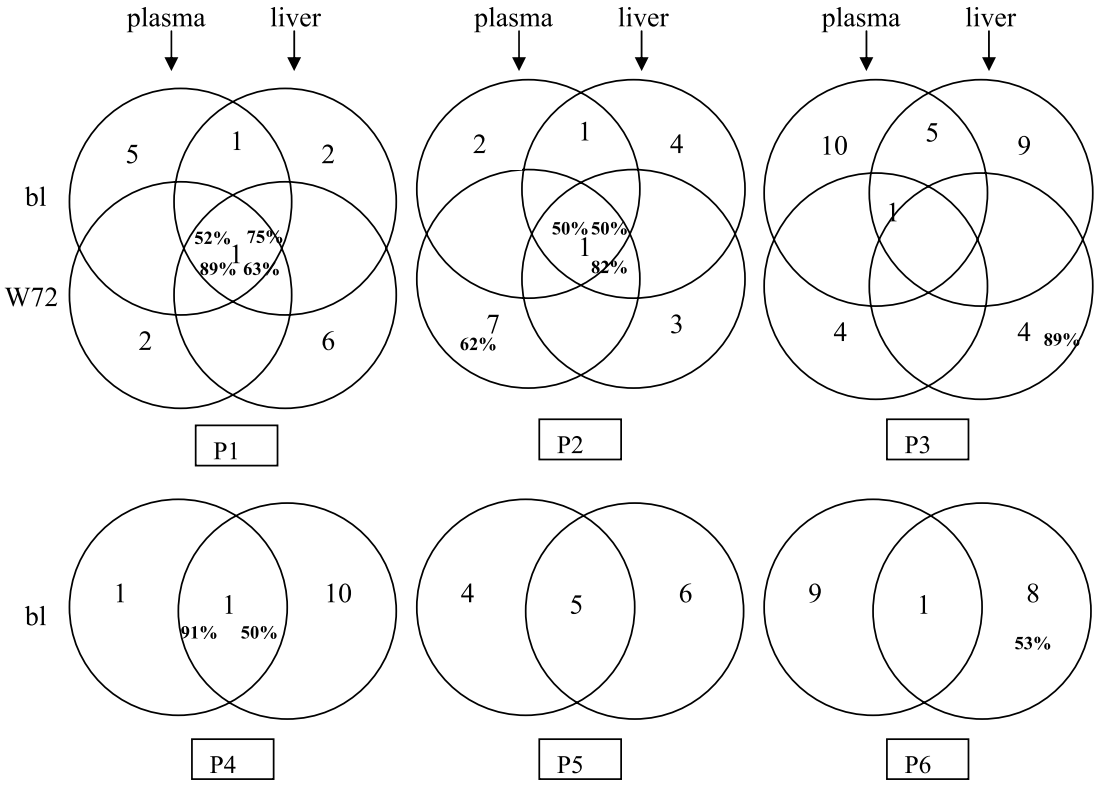

FIG. 2. Schematic representation of variant repartition in plasma and liver compartments and at baseline (bl) and posttreatment (W72). The numbers of the different variants are in black. The percentage of a variant is indicated in boldface only when it predominates (i.e., >50%).

variant could be more stable on an evolutionary level, as if best adapted to its host environment (10, 13) or more pathogenic (6).

In patients P1 and P3, nonsynonymous mutations markedly increased in the liver after treatment (*dN*/*dS* liver ratio of >1): in addition to viral fitness alteration, a possible immunological reaction targeting the liver during interferon treatment and contributing to the selection of major viral variants cannot be excluded.

Finally, diversity decreased significantly after treatment in all patients coinfected with HIV, both in plasma and in liver, as already described (1). Thus, interferon seemed to favor the emergence of more closely related clones, reducing HCV's ability to diversify its genetic repertoire.

In conclusion, the plasma and liver already harbored different HCV quasispecies before treatment, both in patients solely infected with HCV and in patients coinfected with HIV. After unsuccessful interferon treatment, HCV complexity and diversity were both markedly reduced in the HIV-infected patients, but the liver compartment displayed unique evolutionary features.

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