HIV Infection and Antiretroviral Therapy: Effect on Hepatitis C Virus Quasispecies Variability

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Background. Hepatitis C virus (HCV) quasispecies variability has been associated with liver disease progression. The effects of human immunodeficiency virus (HIV) coinfection and highly active antiretroviral therapy (HAART) on HCV quasispecies variability have not been firmly established.

Methods. We determined HCV quasispecies complexity and diversity in 69 subjects, 28 of whom were HIV infected, using clonal frequency analysis via heteroduplex mobility analysis of the second envelope gene hyper-variable region. Nucleotide sequencing was performed for a small subset of subjects.

Results. HIV-positive, HAART-naive subjects had significantly lower HCV quasispecies complexity and diversity than did both HIV-negative and HIV-positive HAART-treated subjects. In multivariate analysis, HIV infection predicted decreased complexity ($P < .0001$) and diversity ($P = .001$) of HCV quasispecies, whereas HAART predicted increased complexity ($P = .013$) and diversity ($P = .026$). For 4 of 6 patients, sequence analysis yielded data supporting the model that positive host pressure drives HCV quasispecies heterogeneity, although data favoring the hypothesis of selective outgrowth of the most fit variants were also observed.

Conclusion. HIV coinfection is associated with decreased HCV quasispecies variability, which appears to be reversed by effective HAART. Although HIV- and HAART-related effects on host immune pressure are likely to play a role in the observed differences in HCV genetic heterogeneity, other mechanisms may be operative.

Of HIV-infected individuals in the Western world, 20%–30% are coinfected with hepatitis C virus (HCV) [2–4]. The rates of HCV-associated liver disease progression and liver failure are significantly increased in HCV/HIV-coinfected persons, compared with persons with HCV infection alone [5], and, among persons with HIV infection, disease progression appears to be inversely correlated with the peripheral CD4 cell count [6–9]. These observations suggest that HIV-related immune suppression or immune dysregulation is responsible for the accelerated liver disease seen in individuals with HIV coinfection. This hypothesis is further supported by studies reporting that highly active antiretroviral therapy (HAART) is associated with slower progression of HCV disease [10] and decreased HCV-related mortality [11].

In a given individual, HCV exists as several closely related yet genetically distinct populations termed “quasispecies” [12]. We previously defined 2 distinct components of HCV quasispecies variability—namely, complexity (the average number of unique quasispecies variants within a quasispecies sample) and diversity (the average genetic distance within a quasispecies sample) [13]. Although the mechanisms driving HCV quasispecies diversification are not completely defined, humoral pressure on the second envelope gene hypervariable (E2-HVR) region has been associated with quasispecies diversification through immune escape mechanisms [12, 14]. The clinical relevance of this genetic variation is also uncertain, but decreased variability in HCV quasispecies over time has been associated with more-advanced liver disease [15–20]. Certain immunosuppressed states are associated with reduced sequence variation and quasispecies heterogeneity [15, 21, 22]. However, studies of HCV/HIV-coinfected subjects have reported both increased
[23] and a trend toward decreased [24] quasispecies heterogeneity, compared with that seen in subjects with HCV infection alone. Consistent with the hypothesis that immune pressure induces HCV sequence variation, it has been reported by some investigators that HIV-infected individuals with higher CD4 cell counts [24, 25] or delayed HIV disease progression [26] have greater quasispecies variability. A recent longitudinal study found an increase in HCV quasispecies diversity over time in coinfected subjects treated with HAART [27], whereas a second study found no changes in genetic variability after initiation of HAART [28].

The primary objectives of the present study were as follows: (1) to compare HCV quasispecies heterogeneity (complexity and diversity) in subjects with or without HIV coinfection, (2) to examine the association between HCV sequence variation and CD4 cell count, and (3) to determine the effect of HAART on HCV quasispecies variability in HCV/HIV-coinfected individuals. To our knowledge, the present study is the first to concurrently evaluate the effects of HIV infection and HAART on HCV quasispecies complexity and diversity.

PATIENTS, MATERIALS, AND METHODS

Patients. Patients who were infected with HCV genotype 1 and who had a parenteral risk factor for HCV infection (i.e., injection drug use [IDU] or blood transfusion) were recruited from clinics at Harborview Medical Center (Seattle, WA). Patients were excluded from the study if they had an acute infection of any type, were positive for hepatitis B surface antigen, or had any history of receiving treatment for HCV infection. Written, informed consent was obtained from each patient prior to study participation, and the study protocol was approved by the University of Washington Human Subjects Committee.

HIV-infected subjects were included in the study if they were antiretroviral therapy (ART) naive and had persistently detectable levels of HIV in plasma, or if they had been receiving HAART for at least 6 months and had sustained suppression of HIV RNA levels to <400 copies/mL. Subjects were questioned regarding the year of blood transfusion or onset of IDU, and they completed questionnaires regarding recent and lifetime use of alcohol [29]. The Skinner questionnaire was adapted by the Hepatitis C Antibody negative.

Laboratory testing. Clinical laboratory data were collected or determined within 30 days of enrollment of subjects in the study. Serum samples were stored at −80°C for HCV quasispecies analysis. To avoid misclassification, confirmatory HIV antibody testing of HIV-negative subjects was performed, but it was initiated after enrollment had begun. Previously enrolled subjects were recontacted and were asked to consent to HIV testing; all but 4 of these subjects could be reached for testing, and these 4 had all been HIV negative in the past and had denied engaging in risk behaviors in the interim. All tested subjects (37 of 41) in the HIV-negative group were confirmed to be HIV antibody negative.

Serum HCV RNA levels were determined at the University of Washington Clinical Virology Laboratory. HCV RNA was detectable and quantifiable in all samples. Through July 2002, HCV RNA levels were determined using a third-generation branched DNA assay (Versant HCV RNA 3.0; Bayer Diagnostics) (lower limit of detection, 600 IU/mL), after which time levels were determined using an in-house real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assay (lower limit of detection, 50 IU/mL). These 2 assays were well-correlated (R² = .81) [31]. HCV genotype was assigned using restriction fragment–length polymorphism analysis of the 5′ noncoding region [32]. Through July 2003, HIV–1 RNA quantitation was done using a branched DNA assay (lower limit of detection, 50 [i.e., 1.70 log₁₀] copies/mL). Later determinations were performed using real-time RT-PCR technology (lower limit of detection, 30 [i.e., 1.48 log₁₀] copies/mL).

Quasispecies analysis. HCV quasispecies analysis of E2-HVR was performed using the clonal frequency analysis (CFA) technique described elsewhere [13, 15, 33]. In brief, 20 clones per patient were amplified by RT-PCR performed with the use of AmpliTaq polymerase (ABI), were hybridized to the 32P-labeled major variant probe, and were fractionated by gel electrophoresis performed using a polyacrylamide mutation detection enhancement gel (BioWhittaker Molecular Applications), which allows for highly efficient resolution of genetic distance within quasispecies populations and the number of unique variants within a population. The distance of a heteroduplex band migration relative to the distance of a homoduplex control migration is proportional to the number of nucleotide mismatches between probe and target [15, 33]. Quasispecies complexity is the normalized number of unique quasispecies variants within a sample, whereas quasispecies diversity is the average genetic distance between individual clones within the same sample. A heteroduplex mobility ratio (HMR) is calculated by dividing the distance (in millimeters) from the origin of the gel to the heteroduplex by the distance (in millimeters) from the origin to the homoduplex control. The HMRs for all variants in the population are averaged to provide the final HMR. An HMR value closer to 1.0 indicates a less diverse population.

The nucleotide sequences of a subset of clones were deter-
Table 1. Demographic and clinical characteristics of study subjects, according to HIV status and highly active antiretroviral therapy (HAART) status.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1 (n = 41)</th>
<th>Group 2 (n = 11)</th>
<th>Group 3 (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, years</td>
<td>45.5 ± 7.3</td>
<td>40.5 ± 7.7</td>
<td>41.4 ± 6.6</td>
</tr>
<tr>
<td>Male sex, % of subjects</td>
<td>63.4</td>
<td>54.5</td>
<td>76.4</td>
</tr>
<tr>
<td>Race, % of subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>82.9</td>
<td>72.7</td>
<td>64.7</td>
</tr>
<tr>
<td>Black</td>
<td>14.6</td>
<td>18.2</td>
<td>29.4</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0</td>
<td>9.1</td>
<td>5.9</td>
</tr>
<tr>
<td>Native American</td>
<td>2.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HCV infection duration, mean ± SD, years</td>
<td>22.0 ± 8.4</td>
<td>15.3 ± 9.9</td>
<td>14.5 ± 7.5</td>
</tr>
<tr>
<td>Alcohol use, g/day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In lifetime</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>47.5 ± 72.2</td>
<td>73.6 ± 96.7</td>
<td>46.6 ± 41.4</td>
</tr>
<tr>
<td>Median (range)</td>
<td>17.1 (0–329.2)</td>
<td>39 (9–333.2)</td>
<td>42.3 (0–144.9)</td>
</tr>
<tr>
<td>In past 6 months</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>7.8 ± 22.1</td>
<td>26.0 ± 72.1</td>
<td>1.3 ± 2.9</td>
</tr>
<tr>
<td>Median (range)</td>
<td>0 (0–120.7)</td>
<td>0 (0–241.4)</td>
<td>0 (0–8.9)</td>
</tr>
<tr>
<td>IDU in past 6 months, % of subjects</td>
<td>15</td>
<td>27</td>
<td>17</td>
</tr>
<tr>
<td>ALT level, mean ± SD, IU/L</td>
<td>98 ± 83</td>
<td>66 ± 33</td>
<td>107 ± 59</td>
</tr>
<tr>
<td>Infected with HCV genotype 1a, % of subjects</td>
<td>82.9</td>
<td>100</td>
<td>88.2</td>
</tr>
<tr>
<td>HCV RNA level, log_{10} mean ± SD, IU/mL</td>
<td>6.0 ± 0.7</td>
<td>6.1 ± 0.7</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>CD4 cell count, mean ± SD, cells/μL</td>
<td>977 ± 316</td>
<td>333 ± 309</td>
<td>408 ± 237</td>
</tr>
<tr>
<td>CD4 cell count nadir, mean ± SD, cells/μL</td>
<td>NA</td>
<td>263 ± 205</td>
<td>165 ± 133</td>
</tr>
<tr>
<td>HCV disease grade, mean ± SD</td>
<td>2.1 ± 0.7</td>
<td>2.2 ± 0.8</td>
<td>2.4 ± 0.8</td>
</tr>
<tr>
<td>HCV disease stage, mean ± SD</td>
<td>2.0 ± 0.9</td>
<td>2.3 ± 1.0</td>
<td>2.3 ± 1.0</td>
</tr>
<tr>
<td>Cirrhosis noted on liver biopsy, % of subjects</td>
<td>11</td>
<td>14</td>
<td>12</td>
</tr>
</tbody>
</table>

NOTE. Groups were compared using the χ² test (for categorical variables) or Student’s t test for unequal variances (for continuous variables). P > 1.0, for between-group comparisons, unless indicated otherwise. ALT, alanine aminotransferase; group 1, HIV-negative subjects; group 2, HIV-positive, HAART-naive subjects; group 3, HIV-positive subjects receiving HAART; HCV, hepatitis C virus; IDU, injection drug use; NA, not applicable.

a Group 1 vs. group 2, P = .07; group 1 vs. group 3, P = .04.
b Group 1 vs. group 2, P = .06; group 1 vs. group 3, P = .002.
c Median (range) is provided because data are skewed by extreme values.
d Group 1 vs. group 2, P = .06; group 2 vs. group 3, P = .03.
e Group 1 vs. group 2, P = .0001; group 1 vs. group 3, P < .0001.
f Data were available for 38 HIV-negative subjects, 7 HIV-positive, HAART-naive subjects, and 17 HIV-positive subjects who were receiving HAART.
g Grade range, 0–4.
h Stage range, 0–4.

In past 6 months

- Alcohol use: g/day
- ALT level: mean ± SD, IU/L
- Infected with HCV genotype: % of subjects
- HCV RNA level: log_{10} mean ± SD, IU/mL
- CD4 cell count: mean ± SD, cells/μL
- CD4 cell count nadir: mean ± SD, cells/μL
- HCV disease grade: mean ± SD
- HCV disease stage: mean ± SD
- Cirrhosis noted on liver biopsy: % of subjects

Patient characteristics. The demographic and clinical characteristics of patients, according to HIV serostatus and HAART status, are detailed in table 1. When HIV-positive subjects were compared with HIV-negative subjects overall, HIV-positive subjects were younger (mean age ± SD, 41.1 ± 6.9 vs. 45.5 ± 7.3 years; P = .01), had a significantly shorter duration of HCV infection (mean ± SD, 14.8 ± 8.5 vs. 22.0 ± 8.4 years; P = .0009), and lower CD4 cell counts (mean ± SD, 389 ± 279 vs. 977 ± 316 cells/μL; P < .0001). Liver biopsy data were available for 24 HIV-positive subjects and for 38 HIV-negative sub-

mined using the second-round PCR primers and the Applied Biosystems model 3730XL DNA analyzer (ABI). Nucleotide sequences were optimally aligned using the Clustal W program [34]. Synonymous (ds) and nonsynonymous (dN) substitution rates were calculated using the Synonymous/Non-synonymous Analysis Program (SNAP) [35–37]. Technologists performing quasispecies analyses were blinded to all clinical data, including HIV status.

**Statistical methods.** Groups were compared using the χ² test or Student’s t test for unequal variances, where appropriate. Correlations were assessed using the Spearman correlation coefficient. No adjustment was made for multiple comparisons. The independent effect of a predictor on quasispecies complexity and diversity was determined using multiple linear regression with robust variance estimates. P < .05 was considered to denote statistical significance. Statistical analyses were conducted using Stata software (version 8.0; Stata).

**RESULTS**

**Patient characteristics.** The demographic and clinical characteristics of patients, according to HIV serostatus and HAART status, are detailed in table 1. When HIV-positive subjects were compared with HIV-negative subjects overall, HIV-positive subjects were younger (mean age ± SD, 41.1 ± 6.9 vs. 45.5 ± 7.3 years; P = .01), had a significantly shorter duration of HCV infection (mean ± SD, 14.8 ± 8.5 vs. 22.0 ± 8.4 years; P = .0009), and lower CD4 cell counts (mean ± SD, 389 ± 279 vs. 977 ± 316 cells/μL; P < .0001). Liver biopsy data were available for 24 HIV-positive subjects and for 38 HIV-negative sub-

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jests. There was no difference between the HIV-positive and HIV-negative groups with respect to liver disease stage (mean ± SD, 2.3 ± 1.0 vs. 2.0 ± 0.9; \( P = .35 \)) or grade (mean ± SD, 2.3 ± .8 vs. 2.0 ± .7; \( P = .20 \)). Only 1 subject who had not undergone liver biopsy had clinical evidence of cirrhosis (decreased liver function). All other patients in the cohort had normal liver function.

**Association between HCV genetic heterogeneity and host and virus factors.** A total of 1344 clones from 69 subjects were analyzed (median number of clones analyzed, 20 and 19 in the HIV-negative and HIV-positive groups, respectively). When compared with HIV-negative subjects, HIV-positive subjects had lower quasispecies complexity (mean ± SD, 5.5 ± 2.8 vs. 4.1 ± 3.1 clones/sample) and diversity (mean HMR ± SD, .968 ± .037 vs. .978 ± .032), but these differences did not reach statistical significance (\( P = .06 \) and \( P = .21 \), respectively). There was a trend toward an association between duration of HCV infection and complexity (\( P = .06 \)), but adjustment for the duration of HCV infection in multivariate analyses did not influence the effect of HIV infection on complexity or diversity. Neither IDU nor alcohol use (whether expressed as a continuous or categorical variable) was associated with complexity or diversity (data not shown). There also was no association between quasispecies complexity or diversity and serum HCV RNA levels (\( P = .86 \) and \( P = .62 \); by Spearman’s correlation, respectively). Finally, neither quasispecies complexity nor diversity predicted liver disease stage (\( P = .74 \) and \( P = .67 \), respectively).

In univariate analysis, CD4 cell count (log transformed) predicted both complexity and diversity. On average, doubling the CD4 cell count yielded an expected increase in complexity of .54 (95% confidence interval [CI], .21–.88; \( P = .002 \)), and an expected increase in diversity (decrease in HMR) of .005 (95% CI, .001–.008; \( P = .015 \)).

**Association of HCV genetic heterogeneity with HAART.** All of the HAART-naive subjects had detectable levels of HIV in plasma samples (median HIV RNA level, 23,356 [i.e., 4.37 log10] copies/mL; mean HIV RNA level, 165,167 [i.e., 5.22 log10] copies/mL; range, 1060 to >1,000,000 HIV RNA copies/mL). All of the HIV-positive subjects who were receiving HAART had sustained suppression of HIV levels to <400 HIV RNA copies/mL. In one subject who was receiving HAART, an HIV RNA level of 58 copies/mL was detected, whereas all other subjects had undetectable levels according to the results of a highly sensitive assay (see Patients, Materials, and Methods). The median duration of HAART was 46 months (mean ± SD, 45.1 ± 24.1 months; range, 6–76 months).

Representative results of clonal frequency analyses are illustrated in figure 1. The HIV-negative subject (figure 1A) and the HIV-positive subject receiving HAART (figure 1B) had appreciably greater HCV quasispecies heterogeneity, compared with the HAART-naive, HCV/HIV-coinfected subject (figure 1C). This is readily apparent by visual comparison of the gel autoradiograms, with greater shifts in radioactive bands, compared with baseline, indicating higher diversity within the sample, and with an increased number of unique banding patterns within the sample indicating greater complexity (see Patients, Materials, and Methods). Diversity and complexity scores for the 3 representative subjects are provided in the figure 1 legend. Overall results of quasispecies analysis for the 3 groups are shown in figure 2. HIV-positive, HAART-naive subjects had significantly lower quasispecies complexity than did HIV-negative subjects (mean ± SD, 2.6 ± 1.5 vs. 5.5 ± 2.8 clones/sample; \( P = .001 \)), and they also had lower complexity than did HIV-positive subjects receiving HAART (mean ± SD, 5.1 ± 3.5 clones/sample; \( P = .02 \)). Untreated HIV-positive subjects also had significantly lower diversity than did both HIV-negative subjects (mean HMR ± SD, .992 ± .015 vs. .968 ± .037, respectively; \( P = .002 \)) and treated HIV-positive subjects (mean HMR ± SD, .969 ± .038; \( P = .03 \)). There was no difference in complexity and diversity...
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Figure 2. Hepatitis C virus (HCV) quasispecies complexity (A) and diversity (B), according to subject group. Complexity is expressed as the number of unique HCV quasispecies identified, and diversity is expressed as the heteroduplex mobility ratio. Horizontal lines within the boxes denote median values. Lower and upper limits of boxes denote the 25th and 75th percentiles, and vertical bars denote lower and upper adjacent values. Dots denote values outside the adjacent values. Groups were compared using Student’s t test for unequal variances. HAART, highly active antiretroviral therapy.

when HIV-negative subjects were compared with HIV-positive subjects receiving HAART.

To investigate the types of pressures driving HCV quasispecies diversification in the HCV/HIV-coinfected population, nucleotide sequence analysis was performed on 57 unique variants from 6 patients (3 patients from the HAART-naive group and 3 patients from the group receiving HAART) (GenBank accessions numbers DQ294034 to DQ294091). The specimens were selected based on maximum divergence from the quasispecies major variant in the CFA experiment, to best determine intrapopulation dS and dN nucleotide substitutions per site. The dN:dS ratios for the HAART-naive patients were 0.14, 0.98, and 1.16 (mean, 0.76), compared with 0.68, 1.16, and 1.72 (mean, 1.26) for the HAART-treated subjects. Importantly, the 3 patients with dS:dS ratios of <1 had no evidence of being under positive selective pressure, as discussed below.

Independent effects of HIV coinfection and HAART on HCV genetic heterogeneity. In multiple linear regression analysis, HIV infection was associated with decreased quasispecies complexity and diversity (P<.0001 and P = .001, respectively), whereas HAART was associated with increased complexity and diversity (P = .013 and P = .026, respectively). The log-transformed CD4 cell count was not associated with quasispecies complexity or diversity in the multivariate analysis (P = .28, for both). Furthermore, after adjustment for the CD4 cell count, the magnitude of the effects of HIV infection and HAART on complexity and diversity were only partially diminished (the coefficients for HIV infection changed by 22% and 30%, respectively, and the coefficients for HAART changed by 10% and 11%, respectively). Finally, adjustment for possible confounding factors, such as alcohol use or IDU, hepatitis C duration, and disease stage, did not affect the associations between HIV or HAART and complexity or diversity (data not shown).

DISCUSSION

Hepatitis C liver disease appears to progress more rapidly in individuals who are coinfected with HIV [5], and disease progression in those who are HIV infected has been associated with the degree of immune suppression, as measured by the peripheral CD4 cell count [6–9]. Because HCV quasispecies variability is thought to be immune driven, we sought to determine whether genetic variability is influenced by coinfection with HIV and/or by treatment of HIV infection. After adjustment for HAART and other possible confounding factors in our analyses, the results confirm our hypotheses that HIV coinfection is associated with decreased HCV quasispecies variability, whereas HAART is associated with increased variability, to a level observed in HCV-monoinfected subjects.

The few investigators who have studied the effect of HIV on HCV genetic variability have reported conflicting results. Two earlier, small studies reported that HIV coinfection was associated with increased HCV quasispecies variability [23, 38]. These findings are counter to findings noted for other immunocompromised subjects, for whom immunosuppression was associated with decreased, rather than increased, quasispecies variability [15, 21, 22, 39]. A third study reported a trend toward decreased complexity in 27 HCV/HIV-coinfected subjects, compared with 10 subjects infected with HCV alone, although it found no difference in diversity between the 2 groups [24]. The largest study to date compared 52 HCV/HIV-coinfected patients who were entering an HCV treatment trial with 32 HIV-negative untreated control subjects, and it found that quasispecies complexity was identical in the 2 groups [40]. However, HIV treatment status was not well defined, and quasispecies analysis was performed using the single-strand conformational polymorphism technique,
which is the least accurate method for analyzing complex genetic populations, because genetically different variants frequently comigrate during SSCP and are impossible to resolve.

In a recent longitudinal study, Blackard et al. [27] found that quasispecies complexity and diversity in the E2-HVR, as well as immune selection pressure on this region, increased over time in 11 HCV/HIV-coinfected subjects who were followed for up to 48 weeks after initiation of HAART. However, in a second study by Babik et al. [28], there was no change in E2-HVR complexity or diversity in 7 HCV/HIV-coinfected patients after they had received HAART for an average of 7 months, and there was no evidence for immune selection pressure. These disparate findings are quite possibly related to the inclusion of different HCV genotypes and shorter duration of HAART in the latter study. In summary, both the influence of HIV infection on HCV quasispecies variability, as well as the subsequent effects of HAART therapy on HCV quasispecies diversity over time in the coinfected population, remain controversial.

In light of the aforementioned controversy, we studied a relatively large number of well-characterized subjects and carefully controlled for variables that have been associated with quasispecies variability, including duration of HCV infection [41], genotype [25, 28, 40], HCV load [28], alcohol use [42, 43], and ART [27, 28]. Although the present study is limited by its cross-sectional design, we decided a priori to exclude subjects who might be in various stages of immune reconstitution, to have immunologically and clinically distinct groups. By design, the HIV-infected subjects in the present study were either HAART naive or had been receiving HAART for a median duration of 46 months (only 1 subject had been treated for <12 months) and had sustained suppression of HIV. In addition, we analyzed an average of 20 clones from each subject, an amount required to prevent undersampling errors. We used the well-established CFA technique for quasispecies analysis, and we have previously demonstrated highly significant correlation between this method and the reference standard, nucleotide sequencing, in clinical studies of hepatitis C [13, 15, 17, 33, 44, 45]. Our choice to use the CFA technique was based on superior throughput, which allowed us to analyze a total of 1344 individual quasispecies variants in the present study. Thus, differences in both study design and methodological approach are reasons why our results differ from the results of previous reports.

In exploring how HIV infection and HAART might influence HCV quasispecies heterogeneity, we observed that peripheral CD4 cell counts were associated with increased quasispecies complexity and diversity on univariate analysis. However, when the CD4 cell count was added to a multivariable model that included HIV status and HAART, it was no longer associated with quasispecies diversity or complexity. This is not altogether surprising, given that the CD4 cell count is influenced by, and therefore highly correlated with, both HIV infection and HAART. More importantly, adjusting for the CD4 cell count did not nullify the effects of HIV and HAART on HCV quasispecies complexity or diversity. This finding suggests that HIV- and HAART-related changes in T cell function, and not just changes in T cell number, play a role in driving HCV quasispecies variability, which may explain the inconsistent findings of previous cross-sectional and longitudinal studies of this topic [19, 24–28, 40].

The long-term consequences of HAART-related changes in HCV quasispecies variability are not known. However, we and other investigators have observed greater quasispecies heterogeneity in subjects with a more favorable liver disease outcome during natural HCV infection (D.G.S., unpublished data) [18, 19] and in HCV-infected liver transplant recipients [15–17, 20]. One possible mechanism is that protective host pressures drive HCV quasispecies diversification toward a less pathogenic virus-host relationship, even though such pressures are incapable of clearing the virus in the majority of cases. On the other hand, immune suppression is clearly associated with hepatitis C acceleration, both in the HCV/HIV-coinfected population [6–9], and in the liver transplant population [46, 47].

Nucleotide sequencing was performed on the HCV E2-HVR for 6 patients, to better assess the genetic evidence for the type of pressures driving quasispecies diversification. On the basis of the study by Blackard et al. [27], we expected to find additional evidence that positive pressure (i.e., the host immune response) drives viral quasispecies diversification. On average, quasispecies from HAART-naive subjects had lower dN:dS ratios, compared with quasispecies from HAART-treated subjects (mean, 0.76 vs. 1.26, respectively), which is consistent with previously proposed models of virus-host dynamics driving HCV quasispecies evolution. However, only 3 of the 6 quasispecies populations that we sequenced showed convincing evidence of positive selection (dN:dS ratios >1); for 1 population, results were equivocal (dN:dS ratio, 0.98), and, for 2 populations, the dN:dS ratios were considerably <1 (0.14 and 0.68). Importantly, the 0.68 ratio was noted for a patient who was receiving HAART, who presumably should have restored anti-HCV immune responses.

The preferential incorporation of synonymous mutations into quasispecies populations argues against immune selection and in favor of the selective outgrowth hypothesis as the mechanism driving viral diversification. In the latter model, quasispecies evolution is driven by the selective growth advantage inherent to variants that develop mutations conserving or optimizing structure and/or function (i.e., synonymous mutations), as opposed to evolution driven by positive pressure against specific variants (i.e., immune selection). The unfortunate limitation of most in vivo experimentation is that the mechanisms driving viral diversification can only be inferred. Efficient tissue culture systems that are capable of supporting the growth of HCV clinical
isolates are necessary to allow precise evaluation and comparisons of variant fitness. However, even these systems may have inherent selection biases, so the combination of in vitro and in vivo experimentation is essential before meaningful conclusions can be drawn regarding the role of HCV quasispecies diversification in hepatitis C pathogenesis. The association between host immunity and viral evolution should be studied on a case-by-case basis, because specific disease mechanisms may play a more important role in one infected individual, compared with another, or within the same individual at different time intervals.

In conclusion, we found that HIV coinfection was associated with decreased HCV quasispecies complexity and diversity only after adjusting for HAART. In addition, effective HIV treatment was associated with increased complexity and diversity, and the genetic heterogeneity of HCV in treated HIV-infected subjects did not differ from that noted in subjects infected with HCV alone. Interestingly, whereas a higher CD4 cell count was associated with greater complexity and diversity, multiple regression analysis suggested that HIV infection and HAART influence HCV quasispecies variability through additional mechanisms. Our ongoing longitudinal studies are testing the hypothesis that HIV infection and HAART induce changes in the HCV-directed immune response that relate directly with changes in HCV genetic variability. Such prospective, integrative studies are likely to shed light on the influence of HIV coinfection and HIV treatment on HCV quasispecies and the HCV immune response and, eventually, on their effect on HCV-associated liver disease.

References

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