Correspondence

Methods Used to Examine Compartmentalization of Viral Populations between the Genital Tract and Peripheral Blood

To the Editor—In a recent Journal publication, hepatitis C virus (HCV) from the genital tract of persons coinfected with HIV-1 were reported to differ, or appear compartmentalized, from viruses within the peripheral blood [1]. Phylogenetic analyses showed that certain individuals had multiple identical E2 sequences within the genital tract or blood that were distinct from sequences in their other tissue. Furthermore, the genetic diversity of the highly variable region-1 (HVR-1) of HCV in some participants’ genital tract or blood was as low as <0.001 nucleotide substitutions per site (see table 3 in [1]), considerably lower than previously reported for acute [2] or chronic HCV infections [3].

These observations, we suspect, are likely to be an artifact of resampling the same viral templates, resulting in the erroneous reporting of multiple identical sequences and very low viral diversity. Knowledge of the amount of viral nucleic acid template used for amplification and cloning of sequences is crucial for assessing this likelihood [4]. Minosse et al. report neither the amount of RNA that was subjected to reverse transcription nor the efficiency of this polymerase chain reaction (PCR) assay, compared with that of the Roche Amplicor assay used to quantify viral RNA. Thus, it is not possible to ascertain the number of viral templates used to derive the stated number of clones and whether these laboratory protocols may have resulted in resampling of viral templates. If resampling occurred, this would lead mistakenly to the conclusions that HCV populations had low levels of viral diversity and were compartmentalized between tissues.

Several strategies have been used by others to reduce or eliminate the chance of resampling of the same virus after PCR, including generation of one clone from each PCR [5], dilution of specimens to end point followed by multiple PCRs with direct sequencing [5, 6], and amplification of sufficient templates relative to the number of clones generated to make resampling unlikely [2, 4, 6]. Contamination of PCR amplicons between tubes is another cause of identical or similar genotypes that can be minimized by extraction of nucleic acids and PCR from each tissue being performed on different days or in different laboratories [7].

The sequence data in the Minosse et al. article appear to have been evaluated for viral compartmentalization using a subjective assessment of neighbor-joining trees and by differences in genetic diversity between tissues. Statistical methods based on those developed by Slatkin and Maddison [8], as well as a parsimony-based technique [9], provide objective methods to evaluate the distribution of viral sequences between tissue compartments [9, 10].

Furthermore, the HCV genetic diversity was analyzed by Minnose et al. using Student’s t test. Because viral sequences derived from each individual are inherently linked, viral diversity should be assessed using statistical analyses that account for nonindependent data.

Despite an appeal that careful and appropriate methods be used to evaluate viral populations over a decade ago [4], numerous subsequent publications report sequence data that appear to include resampling of nucleic acid templates, which has led to a misrepresentation of the biology that they aimed to describe. We assert that it is essential for reviewers and editors to be aware of methods necessary for appropriate characterization of genotypes within a population and to require that laboratory procedures be sufficiently detailed within manuscripts to allow readers to evaluate how the viral population was sampled. Last, we propose that readers participate in an open dialogue when clarification of methods is required.

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References


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Reply to Bull et al.

To the Editor—Bull et al. [1] pointed out a number of possible artifacts in our study [2], leading to possible erroneous underestimation of highly variable region–1 (HVR–1) diversity of hepatitis C virus (HCV) in clinical samples derived from 2 different body sites (i.e., blood and cytobrush). Actually, diversity values in the analyzed samples fell within the range reported by several other authors [3–5] for many of the study subjects, whereas they were remarkably low (<0.001 nucleotide substitutions per site) only for 1 subject in the blood and cytobrush and for 2 additional subjects in the cytobrush. As pointed by Bull et al., as well as by others, resampling could be a problem, because of the low amount of template in the analyzed samples. However, we are reasonably confident that this problem was overcome in our study, for a number of reasons. First, as shown in table 2 in [2], all the subjects, had HCV viremia exceeding 4.5 log IU/mL. In particular, the woman with blood diversity <0.001 (patient F) had HCV viremia of 4.9 log IU/mL. Because, in the quantitative assay used (Cobas Amplicor Monitor), 1 IU corresponds to 2.2–2.5 copies, and the sensitivity of the polymerase chain reaction (PCR) used for cloning is ~10 copies, the resampling risk in blood is negligible.

Concerning cytobrush, we were unable to measure the concentration of HCV RNA in this type of sample, because of the scarcity of the available material, as stated in the article. However, to ensure adequate sampling of the viral populations being studied, the minimum number of amplifiable cDNA target molecules in these samples was determined by performing 3-

Figure 1. Quasispecies analysis of hepatitis C virus (HCV) from cytobrush and blood specimens of 8 women with HIV-HCV coinfection. The phylogenetic tree of HCV nucleotide sequences was obtained using the maximum parsimony method, by applying close neighbor interchange (MEGA package; version 2.1). Nos. at nodes indicate the frequency (%) of their occurrence in bootstrap analysis based on 500 replicates. Values ≥80% are shown. Patients are identified by different colors. Circles, plasma clones; triangles, cytobrush clones.