Differences in hepatitis C virus quasispecies composition between liver, peripheral blood mononuclear cells and plasma

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Hepatitis C virus (HCV) exists in vivo as a highly variable mixture of closely related genomes (quasispecies), but the pathogenetic significance of such heterogeneity is still largely unknown. To investigate this issue, we compared the composition of HCV quasispecies found in the liver, peripheral blood mononuclear cells (PBMC) and plasma of ten patients by single-strand conformation polymorphism analysis of the E2/NS1 region and sequencing of the variants detected. We found considerable quasispecies differences between the liver and PBMC in all the patients, involving variant numbers, relative quantities and relative electrophoretic mobilities, but no apparent tissue-specific trend. Genome variants present in the liver and/or PBMC were not detected in the corresponding plasma samples, while certain HCV variants were present only in plasma. No dominant amino acids or amino acid pattern characteristic of variants present solely in the PBMC were detected in the E2/NS1 region sequenced.

Hepatitis C virus (HCV) exists within infected hosts as a variably complex system of related genomes (quasispecies) generated by the limited fidelity of RNA replication. Recent evidence has shown that quasispecies composition exhibits extensive variation within individual isolates (Okada et al., 1992) and can vary spontaneously both over time (Kao et al., 1995) and with liver disease progression (Koizumi et al., 1995). In addition, the extent of HCV quasispecies diversity has been reported to be predictive of responsiveness to interferon treatment (Moribe et al., 1995) and to decrease markedly following interferon therapy (Gonzales-Peralta et al., 1996; Yun et al., 1996). However, direct information on the pathogenetic implications of HCV quasispecies heterogeneity is still limited.

In this study we compared the composition of HCV quasispecies found in the liver, peripheral blood mononuclear cells (PBMC) and plasma of ten persistently infected patients, by using single-strand conformation polymorphism (SSCP) analysis of the E2/NS1 region and nucleotide sequencing of variants obtained by this method. The patients selected were all positive for anti-HCV antibody by third-generation tests (Ortho, Milan, Italy), negative for human immunodeficiency virus antibody (Behring, Marburg, Germany) and viraemic by nested RT–PCR with primers covering the 5′ untranslated region (5′-UTR) of the HCV genome (Vatteroni et al., 1994). None of the patients had received antiviral treatments. Plasma samples and PBMC were collected at the time of liver biopsy. First, a 5′-UTR nested RT–PCR was carried out and viraemic samples were then reamplified with primers covering the E2/NS1 region to obtain a 350 bp fragment spanning nt 1284 to 1634 of the HCV genome (Enomoto et al., 1994). Briefly, viral RNA was reverse-transcribed using the antisense primer (GCTCCGGGCACCCGGAGTTGAA, position 1668 to 1692) and then amplified for 35 cycles (denaturation at 94 °C for 45 s, annealing at 60 °C for 30 s and primer extension at 72 °C for 60 s) using the sense primer (GCCATTATTACGTCACCGCATGCG, position 1261 to 1285). One-tenth of the volume of the first PCR product was reamplified for 25 cycles with internal primers (GCTTGGGATATGATGAGTTGTC, position 1284 to 1309; and GGTGGAGGGAGTATTGAGTT, position 1611 to 1634) using an annealing temperature of 58 °C. The amplified product was subsequently extracted from the gel and purified using the QIAquick gel extraction kit (Qiagen). Asymmetric PCR was carried out on the purified product under the same conditions as for the second-round PCR described above, except only the inner sense oligonucleotide was used as primer. For SSCP analysis, 8 µg of the single-strand DNA

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products was mixed with two volumes of formamide loading dye (95% deionized formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and electrophoresed in a 10% non-denaturing polyacrylamide gel. Electrophoresis was carried out at 23 °C for 5 to 6 h and then the gel was visualized by silver staining. Electrophoretic mobility of DNA products was recorded using a GDS 7500 gel documentation system (UVP) and SSCP bands were quantified by the GelBase Pro software program (UVP). Bands were detected as peaks and the height and y-axis position of each peak, corresponding to the intensity of silver staining and electrophoretic mobility, respectively, were expressed as number of pixels by computer analysis. For subsequent computations, bands were considered distinct when their y-axis positions differed by at least five pixels.

To validate our SSCP approach we first examined its reproducibility by testing at least four samples from one liver biopsy, one PBMC and one plasma sample obtained from an individual whose HCV in preliminary tests had shown a very complex SSCP pattern (patient A78). As discussed more extensively below, the SSCP gel patterns differed depending on the tissue origin of the samples examined, but the migration patterns obtained by repeatedly assaying the same sample were similar in the number, appearance, intensity and positions of bands (data not shown), thus demonstrating the intra-assay reproducibility of the method. The reliability of our method was further confirmed by sequencing four bands obtained by SSCP analysis of patient A78 (Fig. 1). One band each was selected from plasma and liver because they occupied similar positions (bands i, iv) while the other two bands were in independent positions and derived from plasma (band ii) or PBMC (band iii). Sequence reactions of PCR products were performed with an automatic DNA sequencer (Pharmacia). Sequence data were aligned using the CLUSTAL V program of the PC-GENE software package (IntelliGenetics). As shown in Fig. 1, bands (i) and (iv) exhibited identical nucleotide sequences while bands (ii) and (iii) differed from them in 43 and 10 nt positions, respectively. As expected, most of the differences detected were located in the HVR1 region. The high sensitivity of the method used was confirmed by subsequent results.
showing that sequences differing at a single nucleotide position were detected as separate bands (data not shown). As a further control, we examined four sets of plasma and PBMC samples obtained from two HCV patients at weekly intervals. The results showed that SSCP patterns remained essentially unchanged over the observation period (data not shown). This was important because the occurrence of rapid HCV quasi-species fluctuations would have rendered the subsequent study less meaningful.

Table 1 summarizes the results obtained from the ten patients studied. HCV was present in the form of variably complex quasispecies in all the samples examined, except two (PBMC of patient A6 and plasma of patient A55), which exhibited single bands. The numbers of sequence species detected in the PBMC and plasma were more variable than in the liver samples as they ranged between one and nine (mean 5.3) vs two and six (mean 4.1). Relative quantitative proportions of the bands present in each tissue also varied considerably in individual HCV isolates. We arbitrarily scored as predominant those variants whose band intensity was at least 20% higher than that of any other band within the SSCP pattern under scrutiny. A clearly predominant variant was detected only in three liver samples and in the PBMC and plasma of five of the ten patients. The tissues of the other patients showed two or more high-intensity bands, indicating that the corresponding sequence species were similarly represented in the virus population of these tissues.

SSCP patterns in the three tissues of individual patients were also quite divergent in the numbers of bands as well as in their intensities, without an apparent tissue-specific trend. Thus, for example, isolate A55 exhibited five and two virus variants in PBMC and liver, respectively, and only one in plasma. In contrast, isolate A24 yielded five variants in the liver, three in the PBMC and as many as eight in plasma. Predominant bands were the same in the three tissues in one patient only (patient B1). Interestingly, certain species were present in all three tissues (ubiquitous variants), others were present in two tissues and still others were present in one tissue only (unique variants). The latter variants were found in all the tissues examined and often represented a large proportion of the spectrum of variants found in each tissue. Several unique bands were of high intensity and, in certain samples, represented the predominant variant. Finally, the total number of distinct bands found in the three tissues of each patient ranged between four and 14, thus demonstrating a considerable variability in the amount of HCV heterogeneity observed in individual patients.

Previous studies on HCV quasispecies composition in...
Fig. 2. Amino acid sequence alignment in the E2/NS1 region of unique and ubiquitous variants obtained by SSCP analysis of four patients. The variants sequenced are numbered according to electrophoretic mobility. Amino acid residues are indicated by the standard single-letter code and numbered according to HCV-J (Kato et al., 1990). Asterisks indicate amino acid identity. Bar above alignment indicates the HVR1 region.

different body tissues had given conflicting results. Sakamoto et al. (1995) detected no differences between HCV quasispecies in the liver and plasma of eight subjects with different clinical situations. In contrast, differences were observed by Fujii et al. (1996) in the liver and plasma of two out of 11 patients and by Saito et al. (1996) in the liver, plasma and PBMC of one patient. The latter authors and Horie et al. (1996) also detected differences in the HCV quasispecies found in cancerous and non-cancerous liver tissue of patients with hepatocarcinoma. The reasons for these discrepant findings are not clear but most likely they reflect differences in the sensitivity of the techniques used rather than variables relative to the HCV isolates examined because our group of patients included subjects infected with different subtypes, at various stages of disease and with varying plasma viraemia loads (Table 1).

Hepatocytes are considered the principle site of HCV replication. PBMC are also believed to support HCV growth, though their degree of permissiveness is still uncertain (Lerat et al., 1996). Thus, the differences in quasispecies composition between these tissues may arise from factors in the liver and PBMC that favour the growth of certain variants over others. Such factors might include the availability of receptors and the degree of permissiveness of the respective cell types. Interestingly, HCV diversity tended to be greater in PBMC than in the liver. This was somewhat surprising because one would expect an increased number of variants where there is a high rate of virus replication and so this may be a consequence of the heterogeneous cell populations present in PBMC. On the other hand, the genetic region of HCV investigated is known to represent an important target for human immune responses (Weiner et al., 1992; Kumar et al., 1994). Thus, heterogeneity in this region might also be a consequence of tissue-specific differences in immune effectors and subsequent selection of escape mutants. Yet another explanation is that PBMC serve as a sort of archive for HCV variants that have already disappeared from the liver as a result of immune pressure.

In an attempt to understand the mechanisms responsible for the differences in liver and PBMC quasispecies, we sequenced one ubiquitous and two unique PBMC variants for each of four isolates and compared their deduced amino acid sequences. As shown in Fig. 2, the two unique variants of each isolate showed variable degrees of divergence between themselves. Thus, in isolates A28 and A37 the unique variants sequenced differed from each other by one and three amino acids, respectively, and differences were all located outside the HVR1 region. In the other two isolates the unique variants showed a greater degree of heterogeneity and most substitutions were located within the HVR1 region. More importantly, the divergences between the unique and the corresponding ubiquitous variants were highly variable in extent and showed no dominant amino acid or amino acid pattern characteristic for the PBMC unique variants. This, however, does not exclude the existence of tissue-tropic HCV variants because the quasispecies nature of HCV in infected hosts is reflected also by changes in more conserved regions such as the core region (Horie et al., 1996).

The hypothesis that single amino acid substitutions in the envelope surface glycoprotein may correlate with the selection of tissue-tropic variants has recently been documented for lymphocytic choriomeningitis virus (Dockter et al., 1996).
At least theoretically, plasma should contain all the HCV variants produced in the whole body. However, we found that several variants detected in the liver and/or PBMC were not present in the corresponding plasma samples. SSCP analysis can fail to detect minor variants, i.e. sequences representing 5% or less of the genomic population (Kurosaki et al., 1993). Failure to demonstrate a variant in plasma may therefore reflect a quantitative or a qualitative difference. It should be noted, however, that certain variants present in the liver and PBMC but not in plasma were of high intensity, indicating that they represented a significant proportion of the quasispecies or even the predominant variants. Thus, the variants undetected in plasma might be genetic sequences that are not released from infected cells (Saito et al., 1996) or are processed before reaching the bloodstream, or they might be newly emerged virus mutants that have yet to enter the circulation.

As noted in the introduction, the implications of virus quasispecies structure and variations in HCV pathogenesis are currently the focus of intensive investigation. The present data are compatible with the idea that different HCV variants may have different life histories within the infected host, and encourage further studies aimed at investigating this important issue.

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References


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