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Isabelle Vuillermoz, Essam Khattab, Erwin Sablon, Ingrid Ottevaere, David Durantel, et al.. Genetic variability of hepatitis C virus in chronically infected patients with viral breakthrough during interferon-ribavirin therapy.. Journal of Medical Virology, 2004, 74 (1), pp.41-53. 10.1002/jmv.20144 . inserm-00137148

HAL Id: inserm-00137148

<https://inserm.hal.science/inserm-00137148>

Submitted on 4 Sep 2009

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Revised manuscript **JMVir/2003/5692**

Genetic variability of hepatitis C virus in chronically infected patients with viral breakthrough during interferon-ribavirin therapy

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Running title: HCV breakthrough during antiviral therapy

Key words: hepatitis C virus - chronic hepatitis - antiviral therapy - drug resistance - genetic variability

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SUMMARY

Little is known about hepatitis C virus (HCV) breakthrough during antiviral therapy, although it would help in understanding HCV resistance to current antiviral treatments. To analyse the implication of virological factors and the vigour of humoral immune responses in this phenomenon, we studied 9 chronic hepatitis C patients with a viral breakthrough during IFN/ribavirin combination therapy, as well as 5 responders and 5 non responders. The IRES and regions coding for the capsid protein, the PePHD domain of envelope glycoprotein E2 and the NS5A and 5B proteins were amplified by RT-PCR before treatment, before and during breakthrough, and after treatment. The major variant sequence was obtained by direct sequencing. The heterogeneity of quasispecies was studied by SSCP in all patients and sequencing after cloning in seven genotype 1b-infected patients. Humoral responses against HCV epitopes were also analysed. The major sequences of IRES, PePHD and NS5B remained stable during treatment, regardless of the treatment response. However, the capsid protein and the regions flanking PePHD showed sequence variations in breakthrough patients, although no specific mutation was identified. The variable V3 region of NS5A, but not the PKR-binding domain and the ISDR, seemed to be associated with differences in response to treatment. The analysis of HCV quasispecies revealed no characteristic pattern during treatment in breakthrough patients, whose HCV genome profiles looked most similar to that of non responders. The humoral response was similar between groups. In conclusion, viral breakthrough does not seem to be due to selection of resistant strains with signature mutations.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of severe liver disease worldwide. More than 80% of infected individuals develop chronic hepatitis that may progress to cirrhosis and hepatocellular carcinoma. Antiviral treatment associating interferon (IFN) α , or more recently pegylated IFN α , and ribavirin lead to a sustained virological response in more than 50% of patients (Poynard et al., 1998; Manns et al., 2001; Fried et al., 2002).

Non response is currently defined by the detection of HCV RNA in serum at the end of treatment. Schematically, two types of profiles can be observed: the absence of response during treatment or an initial decrease of HCV RNA levels followed by an increase or reappearance of viraemia, also called viral breakthrough. About 10% of patients treated with IFN alone and 5% treated with IFN in combination with ribavirin experience a viral breakthrough during treatment (Lebovics et al., 1995). In the absence of an efficient culture system able to fully replicate HCV, HCV breakthrough represents a unique model for *in vivo* study of resistance factors. Three main hypothesis have been proposed to explain viral breakthrough during IFN treatment: i) the development of anti-IFN antibodies, ii) the down-regulation of IFN receptors, iii) the emergence of resistant viral strains. The first hypothesis can be partially ruled out as anti-IFN antibodies are not consistently found in association with viral breakthrough (Heathcote et al., 1999; Hoffmann et al., 1999; Roffi et al., 1995). Second, the down-regulation of IFN receptors during therapy, as observed for hepatitis B, has not been clearly correlated to treatment response (Nakajima et al., 1990). The third hypothesis, which is the emergence of resistant strains during treatment, has been recently suggested. Indeed, a recent study showed that the evolution of hypervariable region 1 (HVR1) quasispecies during IFN monotherapy was different between sustained responders and patients who experienced a viral breakthrough (Farci et al., 2002). However, no longitudinal study has yet been

performed on other regions within HCV genomes isolated from patients with a viral breakthrough, particularly in regions potentially implicated in IFN resistance. Furthermore, little is known with respect to viral breakthrough during IFN-ribavirin combination therapy.

The causes and mechanisms of HCV resistance to IFN treatment are not yet understood, but several viral genomic regions or viral proteins may affect the antiviral process induced by IFN (Pawlotsky et al, 2003). Changes in the nucleotide sequence and structure of the Internal Ribosome Entry Site (IRES), which regulates the cap-independent translation of the HCV genome, may interfere with the efficiency of protein translation (Soler et al., 2002). Moreover, a recent study showed that IFN α inhibits HCV IRES-directed translation by reducing the expression of La protein, which is necessary for IRES function (Shimazaki et al., 2002). The highly basic Core protein, which encapsidates the viral genome to form a nucleocapsid, acts in multiple cellular processes (McLauchlan, 2000). In particular, it may enhance the expression of the IFN-induced 2'-5' Oligoadenylate Synthetase (OAS) system, leading to degradation of viral RNA (Naganuma et al., 2000). However, other *in vitro* studies showed that it may have anti-IFN activities, as do other HCV structural proteins (Keskinen et al., 2002), by blocking the Jak-Stat transduction pathway (Heim et al., 1999; Hosui et al., 2003). The envelope glycoprotein E2 may interact with the IFN-induced cellular protein kinase PKR and block its inhibitory activity on cellular and viral protein synthesis *in vitro*. This property may rely on a small region of E2 protein that shows high sequence homology with the phosphorylation sites of PKR and eIF2 α and is consequently named PKR and eIF2 α Phosphorylation Homology Domain (PePHD) (Taylor et al., 1999). NS5A, a non structural protein, may also interact with PKR and inhibit its activity (Gale et al., 1997; Polyak et al., 1999). The Interferon Sensitivity Determining Region (ISDR), a part of the PKR binding domain of NS5A, or other domains, such as the V3 region, may be responsible for the anti-IFN activities of NS5A. However, the results of clinical studies performed in Japan and

Western countries are controversial regarding the correlation between the number of mutations in ISDR and the treatment response (Enomoto et al., 1996; Kurosaki et al., 1997; Chayama et al., 1997; Watanabe et al., 2001; Noursbbaum et al., 2000; Pawlotsky et al., 1998; Murphy et al., 2002; Zeuzem et al., 1997; Polyak et al., 1998). Furthermore, the role of NS5A in IFN resistance is still debated, as detailed analysis using HCV replicons showed that HCV susceptibility to IFN was independent of the NS5A sequence (Guo et al., 2001). The RNA-dependent RNA polymerase activity, which is essential for HCV replication, is carried by the NS5B protein (Lohmann et al., 2000). As baseline viral load is a predictor of response to IFN therapy, mutations in NS5B that may affect the viral replication capacity may also influence response to treatment.

The object of this study was to identify factors involved in viral breakthrough during IFN-ribavirin bitherapy. In order to test the hypothesis that resistant strains harbouring relevant mutations may emerge, we performed a detailed analysis of the IRES, capsid, E2, NS5A and NS5B sequences as well as of the quasispecies evolution in patients presenting a viral breakthrough during combination therapy. In parallel we studied the anti-HCV humoral response in these patients and investigated its potential correlation with treatment failure.

PATIENTS AND METHODS

Patients. Nineteen patients chronically infected by hepatitis C virus, negative for HBV and HIV markers, and treated with a combination of interferon α -2b and ribavirin were retrospectively included in the study. Five patients were sustained responders to treatment, 5 patients were non responders and 9 experienced a viral breakthrough during treatment. Sustained response was defined as a decrease, during treatment, of HCV RNA to titres below the detection limit with a normalisation of ALT levels, which were maintained after the end of treatment. In this study, non response was defined as no decrease in HCV RNA titres or a decrease of less than one \log_{10} during and after treatment without a drop of ALT levels down to the normal range. Breakthrough was defined arbitrarily as an initial biochemical and virological response, characterised by a normalisation of ALT levels and a significant decrease of HCV RNA titre of more than one \log_{10} up to no detectable HCV RNA, and a subsequent significant increase of more than one \log_{10} or reappearance of HCV RNA in the serum during therapy, followed by return of ALT levels to abnormal values.

For all groups of patient, at least one serum sample before, at month 6, at the end and after treatment (2 to 12 months after the end of treatment) were studied. For patients with viral breakthrough, it corresponded to one sample before breakthrough and one after. For responders, a sample in the first month of treatment, before disappearance of HCV RNA was also included.

Quantitative measurement of HCV RNA. Quantification of serum HCV RNA was performed by RT-PCR with an internal RNA standard derived from the 5' non coding region (5' NCR) of HCV (COBAS Amplicor HCV Monitor 2.0; Roche Diagnostic Systems). The threshold of detection of this assay was 600 HCV IU/ml.

HCV Genotyping. HCV genotypes were determined with the INNO-LIPA assay (Innogenetics), based on analysis of genotype-specific 5'NCR polymorphisms by hybridisation of PCR products with probes on nitrocellulose strips.

RNA extraction and amplification of HCV genomic regions by RT-PCR. HCV RNA was extracted from 140 µl of serum according to the manufacturer's instructions (QIAmp viral RNA kit, QIAGEN). For the amplification of the IRES and regions coding for the Core, a C-terminal part of E2, NS5A and NS5B proteins, the extracted RNA was initially amplified by RT-PCR (Onestep RT-PCR, QIAGEN) followed by a nested or semi-nested PCR using Taq polymerase (PROMEGA). Conditions of amplification were optimised for each region and all steps were performed in a thermocycler (i-cycler, BIORAD). E2 sequences were RT-amplified with outer primers located at nucleotides 2070-2086 (or 2079-2098 for genotype 1a) and 2580-2562 while nested PCR was performed with inner primers located at nucleotides 2157-2178 and 2440-2418. A primer pair at nucleotide positions 22-49 and 938-910 was used for RT-PCR of IRES plus Core sequences while semi-nested PCR for the IRES was performed with a primer pair at nucleotide positions 22-49 and 419-389 and semi-nested PCR for Core with a primer pair at positions 342-376 and 938-910. The entire NS5 region was obtained through amplification of 3 fragments: the first was RT-amplified with outer primers located at nucleotides 6179-6198 and 7222-7200, the second with outer primers at positions 6859-6877 and 8826-8803 and the third with outer primers at positions 8482-8501 and 9390-9373. Nested PCR was then performed with inner primers at nucleotide positions 6187-6205 and 7171-7155 for the first fragment, at positions 6927-6942 and 8635-8612 for the second one and at positions 8613-8633 and 9343-9323 for the last one. Nucleotide positions correspond to the HCV-J sequence (Genbank accession number: D90208). To check that no single base substitution was introduced by the Taq DNA polymerase during amplification, a

plasmid containing a known E2 sequence was submitted to the 2 rounds of PCR. Sequencing of these PCR products revealed that they were identical to the original E2 sequence.

Analysis of genetic variability by direct sequencing and by sequencing of clones.

PCR products were directly sequenced using the appropriate inner primers (373 A automated DNA sequencer, Applied Biosystems). Final amplification products for each region were extracted from agarose gels, purified on silica columns (Qiaquick Gel Extraction kit, QIAGEN) and quantified by ethidium bromide staining with DNA markers as quantity standards. Purified PCR products for 2 responders, 2 non responders and 3 breakthroughs were cloned into pGEM-T vector (PROMEGA). Transformation of recombinant plasmid DNA into *Escherichia coli* competent cells was performed as specified by the manufacturer and transformants were grown on LB/Ampicillin/IPTG/X-gal plates. For each sample, 10 to 22 clones were amplified with the region-specific primers used for the second round PCRs described above. The PCR products obtained were purified, quantified and analysed by Single Strand Conformation Polymorphism (SSCP) as described thereafter. Clones were grouped according to their SSCP pattern. Plasmids from one or two clones of each pattern were purified (Plasmid Mini Kit, QIAGEN) and sequenced using the dye termination method (ABI PRISM 377 automated DNA sequencer, Applied Biosystems). Sequence analysis, including alignment, translation and phylogeny was performed using the CLUSTALW program. The genetic complexity of viral quasispecies at both the nucleotide and amino acid levels was determined by measuring the Shannon entropy (Wolinsky et al., 1996), defined as $S = -\sum_i (p_i \ln p_i)$, where p_i is the frequency of each sequence in the quasispecies. The normalised entropy, S_n , was calculated as $S_n = S/\ln N$, where N is the total number of sequences analysed. The MEGA 2.1 program was used to calculate distances between pairs of sequences, based on the Kimura two-parameter distance matrix with a transition-to-transversion ratio of 2.0, and the

number of synonymous and non synonymous substitutions per synonymous and non synonymous sites, respectively, based on the Jukes-Cantor correction for multiple substitution.

SSCP analysis. The pattern of quasispecies for each isolate was analysed by Single Strand Conformation Polymorphism (SSCP) of PCR products (Querenghi et al., 2001). The clones obtained for each sample were also submitted to SSCP analysis. Twenty to thirty nanograms of the purified PCR products were mixed with 4 μ l of denaturing solution containing 98% formamide, heated for 10 to 15 minutes at 95°C and chilled immediately on ice. Six microlitres of denatured PCR products were loaded on a 12,5% non denaturing polyacrylamide mini-gel (GenegelExcel, Pharmacia Biotech) and electrophoresis was performed using the Genephor system (Pharmacia Biotech, Orsay, France) at 5° and 20°C under optimised conditions for each region. The DNA bands were subsequently visualised by silver staining (PlusOne DNA silver staining kit, Pharmacia Biotech).

Analysis of humoral immune responses. Selected sera were submitted to INNO-LIA HCV Ab IV (Innogenetics). This Line Immunoassay using synthetic peptides and recombinant proteins coated as discrete lines on a nylon strip detects antibodies directed against seven HCV proteins: capsid, E1, E2, NS3, NS4A, NS4B, NS5A. Detection of HCV-specific antibodies captured on lines was performed using alkaline phosphatase-labelled goat anti-human IgG conjugate and the appropriate enzyme substrate. Because the intensity of staining is proportional to the amount of HCV specific antibodies captured, semiquantitative determination of HCV antibody levels against each epitope tested was obtained by scanning the LIA strips.

Statistical analysis. Data were compared between the 3 groups using the non parametric Kruskal-Wallis test and between the 2 profiles of breakthrough patients using the non parametric Mann-Whitney test. Sex ratios were compared with a Chi-2 test. Results were considered statistically significant when the p value was ≤ 0.05 .

RESULTS

Selection of patients. To test the hypothesis of emergence of resistant strains as a factor of HCV resistance to IFN-ribavirin combination therapy, we analysed nine patients who experienced a viral breakthrough during treatment. They showed an initial virological response characterised by a mean decrease of viral load of $2.5 \log_{10}$, leading to undetectable HCV RNA levels by quantitative PCR in 3 of them (patients #5, 6, 7), followed, in the first 12 months of treatment, by an increased titre or reappearance of HCV RNA (Figure 1). At the time of reduction in viral RNA titres, all but one of the breakthrough patients showed a biochemical response characterised by a normalisation of ALT levels (Figure 1). Patient #5 still had abnormal ALT values during treatment but showed a 2.5-fold decrease at the time of HCV RNA disappearance. There was no difference in the breakthrough patients in whom HCV RNA dropped to undetectable levels versus those in whom HCV RNA decreased but did not become undetectable (Table I). These nine breakthrough patients were compared to responders and non responders, who showed characteristic profiles, i.e. an early and sustained virological and biochemical response and no decrease of viral load or a slight decrease of less than $0.4 \log_{10}$ during and after treatment without any biochemical response, respectively (Figure 1). Only one non responder showed normal ALT levels during treatment but his ALT levels never exceeded the upper normal limit value before, during and after treatment. Table I summarises the clinical, histological and virological features of HCV infection in the 3 groups of patients.

Study of the major variant within viral quasispecies. We first analysed the genetic variability of the IRES (nucleotides 22-419), the capsid protein, the PePHD domain of E2 and the NS5 region (amino acids 1-160, 659-670 and 1973-3011 of the polyprotein, respectively)

by direct sequencing of PCR products before, during and after therapy. Because of a rapid decrease of viral load in responders, sequence data were available under therapy in only one patient, sampled at the first month of treatment.

Both sequence alignments and phylogenetic trees showed that the major sequences of the IRES, the capsid protein and the PePHD domain were genotype specific. The first 320 nucleotides of the pretherapeutic IRES sequences were highly conserved between responders, non responders and breakthrough patients infected by the same genotype and remained highly conserved during and after treatment in all patients, except for one breakthrough patient (data not shown).

Despite the fact that almost all patients showed substitutions in their pretreatment core sequences when compared to the HCV consensus sequence of their respective genotypes, no pretherapeutic sequence was associated with treatment response (data not shown). During or after treatment, changes in the major capsid sequence were observed in 4 breakthrough patients, 2 of whom showed disappearance of HCV RNA, and 1 non responder (patients #2, 4, 5, 6 and 15, Figure 2), but no specific substitution was seen (data not shown). Breakthrough strains of patients #2 and 5 showed sequence evolution in 2 of the highly conserved basic domains of HCV capsid protein (amino acids 38-43 and 58-71). In particular, in breakthrough patient #2, four amino acid changes, including a Thr to Arg substitution at position 43, appeared before breakthrough and were maintained thereafter. However, such modifications of a major variant during treatment may be explained by the presence of 2 distinct HCV strains whose proportion were modified by antiviral therapy (Figure 2). This may be also the case for non responder #15 whose capsid sequence showed sufficient alterations to cluster with another genotype after treatment (Figure 2).

The PePHD region was highly conserved between patients infected by the same genotype, whatever the treatment response, and showed no sequence change during treatment in any of

the patients. Interestingly, during treatment, modification of PePHD flanking regions (amino acids 622 to 696 according to HCV-J) was seen only in breakthrough patients: 2 with disappearance of HCV RNA during treatment (patients #6 and 7) and 2 with a reduction of HCV RNA titres (patients #1 and 9) (Figure 3). However, as for the capsid sequences, no specific mutation could be observed. Before breakthrough, the HCV sequence of patient #6 contained several substitutions and did not cluster in phylogenetic trees with sequences observed before treatment and after breakthrough. This also could reflect the presence of 2 co-infecting HCV strains, whose proportion varied during treatment, rather than drastic modification of a single strain.

For the NS5A and NS5B regions, pre- and post-treatment data were available for only the 13 genotype 1b-infected patients and, during treatment, for only 6 of them (1 responder, 3 non responders and 2 breakthrough patients including one with HCV RNA disappearance). Before treatment, the whole NS5A sequence (amino acids 1973-2419), including the PKR-binding domain (amino acids 2209-2274) and the ISDR (amino acids 2209-2248), showed no significant difference between the different treatment response patterns with respect to the number of mutated amino acids compared to the HCV-J prototype sequence (data not shown and Table II). The mean number of mutations in the PKR-binding domain and the ISDR was similar between breakthroughs and responders, but was greater for both of these groups than in non responders. It is worth noting that the overall increase of the mean values for the breakthrough group was mainly due to patient #4 whose HCV strain contained more mutations than other breakthrough strains. However, the sequence of this HCV strain was identical after breakthrough, suggesting that the accumulation of mutations in the PKR-binding domain was not associated with sensitivity to IFN. Interestingly, the HCV strains of the responder group contained more mutations than the strains found in the other groups with respect to the pre-treatment sequence of the V3 region (amino acid 2356-2379) (Table II).

This is mainly due to the N-terminal portion of the V3 domain (amino acids 2356-2366) where HCV strains from responders had from 4 to 6 mutations compared to HCV-J while breakthrough strains showed only 1 to 3 mutations and non responder strains none (Figure 4). No significant difference was seen in the number of mutations in V3 compared to HCV-J between breakthrough patients whose HCV RNA become undetectable and the other breakthrough patients. After treatment, the V3 sequence showed mutations compared to pretherapeutic samples in 2 breakthrough patients but no specific mutation could be identified (patients #4 and 6, Figure 4).

The analysis of NS5B sequences (amino acids 2421-3011) in these genotype 1b-infected patients did not show differences in the mutation rate between the groups and no specific mutation was associated with a treatment response pattern (data not shown).

Analysis of the viral quasispecies. In order to analyse the relationship between the modification of viral quasispecies and response to treatment, the pattern of HCV quasispecies was studied by SSCP in samples obtained before, during and after treatment in all patients (data not shown). For the IRES sequences, SSCP patterns indicated a stability of quasispecies during treatment, with the exception of 2 breakthrough patients including patient #2. The capsid, ISDR and PKR-binding domains showed less stability and changes of SSCP patterns during treatment were more frequent in breakthrough patients than in non responders. In V3 and E2, the majority of non responders and breakthrough patients showed replacement of bands under treatment. Interestingly, the analysis of the SSCP patterns of 2 successive samples for patient #4 taken 3 months apart before breakthrough, revealed that modification of the E2 quasispecies occurred in the second sample, just before the viral breakthrough.

To analyse the viral quasispecies of the capsid, E2 and V3 regions from 3 breakthroughs, 2 non responders and 2 responders (all of genotype 1b), PCR products were cloned. Clonal

frequency was analysed by SSCP and 1 to 2 clones representative of each pattern were sequenced. In all patients, the dominant sequence was the PePHD sequence associated with *in vitro* resistance (⁶⁵⁹RSELSPLLLSTT⁶⁷⁰), with the exception of Ser660Thr and Ser660Ala substitutions in a breakthrough (patient #4) and a responder (patient #12), respectively. During treatment, all other minor sequences were cleared in non responders and breakthrough patients, although 2 minor variants appeared after breakthrough in patient #4 (data not shown). The analysis of V3 and the flanking regions of PePHD (E2 region) did not reveal any selection of variants except for patient #6. For the E2 region, before breakthrough, patient #6 showed sequences not present before treatment or after breakthrough, while the major sequence observed after breakthrough was present as a minor species in the pretherapeutic viral population (data not shown). This was consistent with the hypothesis of 2 co-infecting HCV strains, as mentioned above for the analysis of the major variant (Figure 3). For the V3 region, more than 80% of the quasispecies of patient #6 evolved towards the prototype HCV-J sequence. No mutation was selected in viral capsid quasispecies during treatment except that mentioned in direct sequencing for breakthrough patient #2 and non responder #15.

The quasispecies variability of capsid, V3 and region 622-696 of E2 was then analysed in more detail (Tables III, IV and data not shown). The mean complexity and the mean within- and between-sample genetic distances of the 3 regions were similar for all groups, except that there was slightly greater E2 distances in non responders and breakthrough patients and slightly greater capsid amino acid complexity before treatment in responders. For E2, the differences were restricted to the nucleotide sequences as assessed by the proportion of synonymous substitutions per synonymous site (dS) and non synonymous substitutions per non synonymous site (dN). While dN was comparable between groups, dS was greater for non responders and breakthrough patients. Capsid and V3 regions showed no difference in dN and dS with respect to treatment response. Non responder and breakthrough strains showed in

E2 more synonymous mutations and less non synonymous mutations than in capsid and V3. Altogether, these results suggest that, in the E2 region, non responder and breakthrough strains present more variability than responder strains. However this variability remained limited to the nucleotide level, suggesting structural and/or functional constraints at the peptide level. Compared to E2, capsid and V3 showed lower variability. In patient #6, E2 quasispecies showed dS, dN and between-sample distances 3 to 4 times greater than in other breakthroughs, suggesting an important divergence of the viral population before breakthrough (Table III). These results are in agreement with the likely existence of a second co-infecting HCV strain whose sequences were only detected when replication of the first strain was decreased by the treatment and before less sensitive minor variants were selected. With the exception of this patient, breakthrough strains were no more variable than strains of primary non responders (Table III). Moreover, analysis of the evolution of complexity and distances during treatment confirmed that no specific profile was observed for breakthrough strains, some of them even showing patterns close to non responder strains (data not shown).

Humoral immune response against HCV proteins. Response to treatment may be influenced by the host immune response. We therefore analysed the humoral immune response in all samples using the INNO-LIA HCV Ab IV assay. For all groups of patients, the strongest response was directed against the NS3 protein and the lowest against the envelope glycoprotein E1. Reactivities were similar between groups except for the anti-E2 response: antibody levels were lower for breakthroughs, but this difference was not statistically significant (data not shown). For all 3 groups of patients, anti-HCV antibody levels were relatively stable during and after treatment.

DISCUSSION

The causes and mechanisms of viral breakthrough during treatment of chronic hepatitis C are still poorly understood. Only few longitudinal studies during treatment have been conducted, although it is a unique *in vivo* model for the study of drug resistance. Furthermore, resistance factors were mainly analysed during IFN monotherapy, but current therapy combines IFN and ribavirin. For these reasons, we analysed the HCV genome sequences and host humoral immune responses in 9 patients who experienced a viral breakthrough during IFN-ribavirin combination therapy.

All patients classified as breakthrough patients in this study showed a complete biochemical response during treatment, except patient #5 who showed a significant ALT value decrease without normalisation. Three of these patients (#5, 6, 7) could be defined as “true” breakthrough patients with a drop of HCV RNA levels to under the detection limit of the PCR assay. The others showed HCV RNA titre reduction from 1.3 log₁₀ to levels close to the detection threshold. However, no significant difference with respect to pretherapeutic clinical and virological features, genetic variability before, during and after treatment, and anti-HCV humoral immune responses could be seen between these two profiles of breakthrough patients.

Several viral genome regions were chosen for their role in viral replication and their potential implication in response to treatment, i. e. the IRES and regions coding for the capsid protein, the PePHD domain of E2, and the NS5A and NS5B proteins. The analysis of major variants of viral quasispecies demonstrated the absence of selection of mutants in the IRES and NS5B regions before or after viral breakthrough. In particular, the IRES sequence was conserved and did not influence the treatment efficacy, as already shown for non responders (Soler et al., 2002).

Since the identification of the ISDR in the PKR-binding domain (Enomoto et al., 1995), the role of mutations within this region in the sensitivity to treatment has not been confirmed during IFN monotherapy and IFN-ribavirin bitherapy (Enomoto et al., 1996; Pawlotsky et al., 1998; Nousbaum et al., 2000; Murphy et al., 2002). Previous studies including 1 or 2 cases of viral breakthrough during IFN monotherapy also showed conflicting results (Zeuzem et al., 1997; Polyak et al., 1998). A recent report from Franco and colleagues (Franco et al., 2003) confirmed that the NS5A viral populations vary widely between patients, but no differences were observed in the complexity, diversity, types of nucleotide changes, or evolutionary pattern of the quasispecies according to the stage of liver disease. This suggests that many of the “mutations” observed in our study may correspond to spontaneous variations. Our results indicate that the PKR-binding domain and particularly the ISDR do not seem to play a role in viral breakthrough (Table II). In contrast, our results suggest that the variable region V3 might influence response to IFN, as already published by other groups (Figure 4)(Nousbaum et al., 2000; Murphy et al., 2002).

By comparison with the consensus sequence, pretherapeutic amino acid changes at position 660 in PePHD, observed here in 1 breakthrough and 1 responder, were also observed in non responders in the study of Chayama and colleagues (Chayama et al., 2000) and therefore did not seem to play a role in treatment response. Interestingly, during treatment, the evolution of the major variant for capsid protein and E2, except PePHD which was highly conserved, was more frequent in breakthrough patients than in others (Figures 2 and 3). It has been suggested that the capacity of the PePHD domain to inhibit IFN-induced PKR, at least *in vitro*, may explain the higher general resistance of genotype 1 strains to IFN therapies (Taylor et al., 1999). However, the role of this domain in resistance to IFN with or without ribavirin is still controversial (Berg et al., 2000; Cochrane et al., 2000; Polyak et al., 2000; Sarrazin et al., 2000, Sarrazin et al., 2001; Lo and Lin, 2001). In our study, the PePHD region clearly does

not play a role in the development of viral breakthrough, but mutations in the vicinity of PePHD may affect the function of this domain by changing the secondary and tertiary structures of this region of E2. However, no specific mutation associated with resistance could be identified in the regions flanking PePHD. The capsid protein has a conserved nucleotide and amino acid sequence (Ina et al., 1994; Smith and Simmonds, 1997), especially through 4 basic clusters which may be important for its' interactions with HCV RNA, or may contain potential nuclear localisation sites (Shimoike et al., 1999; Santolini et al., 1994; Bukh et al., 1994). Our data revealed that these clusters remained stable during treatment in the three groups, with the exception of some HCV strains from breakthrough patients which showed 1 to 2 mutations. However, as for E2, no specific mutation was identified.

In selected patients, a detailed analysis of viral quasispecies was performed (Tables III and IV). Quasispecies heterogeneity in capsid protein and V3 was comparable, regardless of the treatment response. The amino acid sequence 622-696 of E2 showed a greater heterogeneity in breakthroughs and non responders as compared to responders. However, this increased variability was only tolerated at the nucleotide level, suggesting structural and/or functional constraints on amino acid changes, which may account for an essential role of this E2 region in the viral life cycle. For patient #6, important changes of the major variant during treatment, confirmed by an increased quasispecies heterogeneity, reflected most likely a co-infection with two HCV strains, as already observed in other studies (Soler et al., 2002). No characteristic variation of quasispecies heterogeneity was seen in breakthrough patients during treatment, some of them having a profile similar to non responders. Similarly, no particular variant was selected in the quasispecies of capsid protein, V3 and the amino acids 622-696 of E2, except for patient #2 in the capsid protein and patient #6 in V3 and E2. However, the PePHD sequence associated with *in vitro* resistance was selected in genotype 1b-infected non responders and breakthroughs, as already described for non responders in

another study (Chayama et al., 2000). Therefore, the PePHD sequence may be one of the determinants involved in resistance, but is insufficient to induce resistance on its own.

Response to treatment may also be influenced by hosts factors, and in particular by the vigour of humoral response (Fujita et al., 2003). The analysis of anti-HCV humoral responses in breakthroughs did not reveal any defect of the host immune response, apart from a slightly weaker response against HCV E2 protein. However, no particular evolution of antibody levels could be observed during treatment. Sustained viral clearance induced by antiviral therapy has been shown to be associated with a subsequent decrease of antibodies against Core, NS3 and E1 (Yoshioka et al., 1991; Yuki et al., 1993; Urushihara et al., 1994; Depraetere et al., 2000). No significant decrease of these antibodies in responders as well as no difference in the evolution of the general antibody levels in the 3 groups of patients were observed in this study. However, our study did not involve a detailed analysis of multiple epitopes within each of the HCV proteins. Decrease of antibody levels in sustained responders, as measured by the INNO-LIA assay, is also seen when patients are monitored for longer periods after treatment, although antibodies to certain HCV proteins may persist for extended periods of time (Depraetere et al., 2000).

In conclusion, the analysis of HCV genetic variability in patients with a viral breakthrough during IFN-ribavirin bitherapy showed a complex pattern of evolution of HCV quasispecies. There was no common HCV genomic profile in breakthrough patients. Concerning the capsid and E2 proteins and the V3 region, the selection of strains with a higher fitness may play a role in the acquisition of resistance in some patients, but no selection of specific mutations was observed. Studies involving larger cohorts of patients are needed in order to elucidate the mechanism of this type of resistance to IFN-ribavirin bitherapy.

Acknowledgements

I. Vuillermoz was the recipient of a fellowship from the Ligue Contre le Cancer and from the ARC. E. Khattab was the recipient of a fellowship from the Egyptian government. The authors thank Dr Eberle for providing the Amplicor HCV Monitor 2.0 kit and Dr A. Kay for reviewing the manuscript.

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Figure Legends

Figure 1: Evolution of viral load (histogram) and ALT (black line) levels in the 3 groups of patients during the study. Viral load and ALT scales are indicated on the left and on the right, respectively. The ALT upper limit of the normal (ULN) value is indicated by a dotted line. BT, breakthrough.

Figure 2: Phylogenetic tree of the predominant amino acid sequences of the core region in all patients analysed. Patients whose predominant sequence evolved during treatment are in bold type. Each patient is designated by a number. BT: breakthrough patient; NR: non responder; R: responder; preT: before treatment; bt: breakthrough; M: month of treatment; postT: after treatment. For patient #4, two successive samples were available before breakthrough (at 5 and 8 months of treatment respectively).

Figure 3: Phylogenetic tree of the predominant amino acid sequences of the PePHD domain and its flanking regions in all patients analysed. Patients whose predominant sequence evolved during treatment are in bold type. Each patient is designated by a number. BT: breakthrough patient; NR: non responder; R: responder; preT: before treatment; bt: breakthrough; W and M: week or month of treatment; postT:., after treatment. For patient #12, two predominant sequences were present before treatment (named 1 and 2). For patient #4, two successive samples were available before breakthrough (at 5 and 8 months of treatment respectively). For patient #6, two different sequences (before bt (1) and (2)) were obtained in 2 separate amplifications of the sample. Pre- and post-treatment samples of patient #2 were not in the same cluster because three residues could not be determined by direct sequencing

in the latter sequence. It is therefore not clear that the strain has evolved between both analyses.

Figure 4: Major V3 sequences in genotype 1b-infected patients. Each sequence was compared to the HCV-J 1b prototype sequence. The positions of the first and last amino acids of the V3 domain in the HCV polyprotein are indicated under HCV-J sequence. Each patient is designated by a number. BT: breakthrough patient; R: responder; NR: non responder; preT: before treatment; bt: breakthrough; W and M; week and month of treatment; post: after treatment.

Table I: Clinical and virological characteristics of the patients prior to therapy.

	Breakthrough patients			P^{\dagger}	Non responders	Responders	P^{\ddagger}
	total	ARN VHC (-)	↘ ARN VHC > 1 log ₁₀				
Number of patients	9	3	6		5	5	
Age (year) *	50.22 ± 10.74	59.00 ± 3.61	45.83 ± 10.50	NS [¶]	53.20 ± 8.98	39.60 ± 12.74	NS [§]
Sex (M/F)	6/3	2/1	4/2	NS [#]	5/0	3/2	NS [#]
Routes of infection							
Transfusion	2	1	1		2	1	
IVDU	1	0	1		1	3	
Professional	2	1	1		0	0	
Medical exposure	0	0	0		1	0	
Unknown	4	1	3		1	1	
Duration of disease (years) *	20.67 ± 6.71	19.00 ± 6.25	22.33 ± 8.08	NS [¶]	19.67 ± 6.42	14.5 ± 6.86	NS [§]
Pretreatment ALT (xULN) *	3.04 ± 1.36	3.00 ± 1.23	3.07 ± 1.53	NS [¶]	3.00 ± 1.30	1.58 ± 1.09	NS [§]
Pretreatment histology *							
Activity (METAVIR score)	1.78 ± 0.97	2.33 ± 0.58	1.50 ± 1.05	NS [¶]	1.20 ± 0.45	1.20 ± 0.45	NS [§]
Fibrosis (METAVIR score)	2.83 ± 1.12	3.00 ± 1.00	2.75 ± 1.26	NS [¶]	3.00 ± 1.00	1.20 ± 0.45	0.0176 [§]
Genotype 1a / 1b / 3a	2 / 6 / 1	0 / 3 / 0	2 / 3 / 1		0 / 5 / 0	1 / 2 / 2	
Pretreatment viral load (x 10⁴ IU HCV/ml) *	123.22 ± 177.77	73.47 ± 89.42	148.09 ± 212.46	NS [¶]	90.60 ± 83.07	80.86 ± 121.18	NS [§]
Treatment protocol							
Standard IFN + ribavirin	7	3	4		5	2	
PegIFN + ribavirin	2	0	2		0	3	
Duration of treatment (months) *	14.44 ± 4.82	13.00 ± 4.36	15.17 ± 5.27	NS [¶]	8.80 ± 2.77	11.40 ± 0.55	NS [§]

* Data expressed as mean ± SD; [†] comparison between the 2 profiles of breakthrough patients; [‡] comparison between the 3 groups of patients;

[§] Kruskal-Wallis test, NS, non significant ($P>0.05$); [¶] Mann-Whitney test, NS, non significant ($P>0.05$); [#] χ^2 test, NS, non significant ($P>0.05$).

Table II: Number of mutations in the NS5A pretreatment predominant sequences of genotype 1b-infected patients compared to the HCV-J sequence.

	PKR-binding domain (2209-73) *	ISDR (2209-48) *	V3 (2356-79) *
Breakthrough patients			
Total (N=6)	6.83 ± 4.07	2.67 ± 3.14	5.00 ± 1.10
HCV RNA (-) (N=3)	6.33 ± 4.04	2.33 ± 2.31	4.67 ± 0.58
⊘ ARN VHC > 1 log ₁₀ (N=3)	7.33 ± 4.93	3.00 ± 4.36	5.33 ± 1.53
<i>P-value</i> ‡	0.4867	0.8166	0.6374
Non responders (N=5)	4.00 ± 1.87	0.40 ± 0.55	3.50 ± 1.73
Responders (N=2)	6.50 ± 2.12	2.50 ± 2.12	10.5 ± 0.71
<i>P-value</i> §	0.4863	0.1412	0.0425 ¶

* Data expressed as the mean number of mutations ± standard deviation,

‡ The mean number of mutations were compared between the 2 profiles of breakthrough patients using the non parametric Mann-Whitney test,

§ The mean number of mutations were compared between the 3 groups using the non parametric Kruskal-Wallis test,

¶ Statistically significant ($P \leq 0.05$).

Table III: Analysis of PePHD and flanking regions quasispecies in genotype 1b-infected patients.

Group	Patient #	Mean complexity (SD) *		Mean genetic distance (SD)		Type of mutational changes		
		nucleotide	amino acid	Within-sample †	Between-sample ‡	Mean (SD) dS §	Mean (SD) dN ¶	dN/dS ratio
R	11	0.4355 (0.0445)	0.3025 (0.0601)	0.0075 (0.0021)	0.0082 (0.0024)	0.0115 (0.0059)	0.0061 (0.0023)	0.5304
	12 £	0.6410	0.4690	0.0101 (0.0042)	-	0.0119 (0.0096)	0.0093 (0.0046)	0.7815
NR	17	0.7273 (0.1906)	0.4818 (0.0631)	0.0248 (0.0041)	0.0319 (0.0076)	0.0883 (0.0184)	0.0086 (0.0020) ^a	0.0974
	16	0.71 (0.0981)	0.285 (0.2975)	0.0211 (0.0139)	0.0243 (0.0183)	0.0637 (0.0131)	0.0077 (0.0020) ^b	0.1099
BT	8	0.7905 (0.1174)	0.4058 (0.1987)	0.0233 (0.0125)	0.0276 (0.0086)	0.0759 (0.0162)	0.0069 (0.0020) ^a	0.0909
	6	0.4412 (0.2643)	0.2478 (0.2114)	0.0213 (0.0188)	0.0950 (0.0430)	0.2658 (0.0395)	0.0215 (0.0074) ^b	0.0809
	4	0.711 (0.1322)	0.2626 (0.1048)	0.0175 (0.0080)	0.0302 (0.0070)	0.0855 (0.0174)	0.0046 (0.0011) ^b	0.0538

* Mean Shannon entropy calculated for all samples obtained for each patient.

† Mean within-sample genetic distance calculated for all samples obtained for each patient.

‡ Mean genetic distance between all samples obtained for each patient.

§ Mean proportion of synonymous substitutions per synonymous site among all quasispecies of each patient.

¶ Mean proportion of non synonymous substitutions per non synonymous site among all quasispecies of each patient. It was compared to the proportion of synonymous substitutions per synonymous site by using a Z-test (^a $P < 0.001$, ^b $P < 0.0001$).

£ Because of the rapid decrease of viraemia during treatment, only the pretreatment sample was analysed for patient #12.

Table IV: Analysis of core region quasispecies in genotype 1b-infected patients.

Group	Patient #	Mean complexity (SD) *		Mean genetic distance (SD)		Type of mutational changes		
		Nucleotide	amino acid	Within samples †	Between samples ‡	Mean (SD) dS §	Mean (SD) dN ¶	dN/dS ratio
R	11 £	0.42	0.42	0.04 (0)	-	0.05 (0.01)	0.03 (0.01)	0.6
	12 £	0.43	0.36	0.01 (0)	-	0.01 (0)	0.01 (0)	1.00
NR	17	0.35 (0.01)	0.22 (0.01)	0.01 (0.012)	0.016 (0.008)	0.03 (0.01)	0.01 (0)	0.33
	15	0.34 (0)	0.19 (0)	0.026 (0.005)	0.02 (0)	0.02 (0.01)	0.02 (0.01)	1.00
BT	8	0.29 (0.02)	0.20 (0.01)	0.008 (0.005)	0.01 (0)	0.01 (0)	0.01 (0)	1.00
	4	0.27 (0.005)	0.17 (0.005)	0.017 (0.016)	0.031 (0.015)	0.025	0.03 (0.006)	1.33

* Mean Shannon entropy calculated for all samples obtained for each patient.

† Mean within-sample genetic distance calculated for all samples obtained for each patient.

‡ Mean genetic distance between all samples obtained for each patient.

§ Mean proportion of synonymous substitution per synonymous site among all quasispecies of each patient.

¶ Mean proportion of non synonymous substitution per non synonymous site among all quasispecies of each patient.

£ Because of rapid decrease of viraemia during treatment, only pretreatment samples were analysed for patients FeM and LaM.