Impact of NS5A Sequences of Hepatitis C Virus Genotype 1a on Early Viral Kinetics during Treatment with Peginterferon-α2a plus Ribavirin

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Background. Hepatitis C virus (HCV) genotype 1 is the most prevalent genotype in Western countries, and treatment with pegylated interferon (pegIFN) plus ribavirin fails in 50%–60% of patients. Genetic variability within the NS5A dsRNA-dependent protein kinase binding domain (PKRbd) of HCV-1b has been associated with responsiveness to IFN-α. Little is known about NS5A sequences of HCV-1a. We investigated whether genetic variability of HCV-1a NS5A correlates with the early HCV kinetics during treatment.

Methods. Twenty-four treatment-naive, HCV-1a–infected patients were treated with standard doses of pegIFN-α2a plus ribavirin. HCV viremia was quantitated at days 0, 1, 2, and 3 and weeks 1, 2, 4, 8, and 12 of treatment. According to HCV kinetics, patients were classified as early rapid responders, early moderate responders, or early slow responders. The full-length HCV NS5A was sequenced at baseline and at week 1.

Results. At baseline, variability of the NS5A C terminus (concentrated in the PKRbd) is associated with interferon efficacy but not with the second phase of the early viral decline that has been associated with a sustained virologic response. Comparisons between baseline and week-1 full-length sequences did not show significant increases in mutations.

Conclusions. Genetic variability of HCV-1a NS5A does not predict responsiveness to IFN-α. Differences in early kinetics during combination therapy are not due to selection of IFN-resistant HCV strains.
IFN-based therapies, and the importance of the NS5A variability in determining a durable therapeutic response has been intensely discussed [8, 9]. In particular, the ability of the HCV NS5A protein to functionally hamper the activation of dsRNA-dependent protein kinase (PKR), an important mediator of the IFN-induced cellular antiviral state, could allow HCV to escape the antiviral effects of IFN [10, 11]. Several studies have reported the observation that presence of circulating virus with a wild-type sequence in the putative IFN sensitivity–determining region (ISDR) associates with nonresponsiveness to IFN, whereas a mutated ISDR predicts response [12–14]. Furthermore, in vitro studies have shown that mutations within the PKR binding domain (PKRbd) of NS5A could inhibit the interaction between NS5A and PKR, leading to a block of viral replication [15]. Other studies have not confirmed these findings, and the role of NS5A in the response to IFN is still unclear.

NS5A could interfere with IFN-induced cellular signaling by interacting with other cellular proteins, as well as by modulating the transcription of many cellular genes [16–19]. In addition to the involvement of ISDR and PKRbd in IFN resistance, other functional domains of NS5A have been implicated. These include the variable region 3 (V3) in the C terminus, a cytoplasmic retention signal, and a nuclear localization signal (NLS). Finally, the NS5A transcription-activating domain contains 2 acidic regions (AR1 and AR2) and 1 proline-rich region (PRR) [20]. Furthermore, NS5A is the major phosphoprotein of HCV, and its phosphorylation grade may play an important role in regulating its functions, including HCV replication and/or cellular adaptation [21].

Most of these studies have been performed on NS5A of genotype 1b. Conversely, data regarding HCV-1a, the most prevalent genotype in Western countries and one of the most difficult to eradicate with therapy, are still very limited. To assess whether the selection of resistant variants of HCV might explain primary resistance to IFN therapy, we analyzed the full-length NS5A sequence of HCV genotype 1a before and after the first dose of pegIFN plus ribavirin in a group of patients showing different patterns of early HCV kinetics.

PATIENTS, MATERIALS, AND METHODS

Patients. Twenty-four consecutive and treatment-naive patients with chronic HCV-1a infection attending the Hepatitis Clinic, University College Hospital, London, were enrolled in the study if they satisfied the following criteria: HCV RNA positive by polymerase chain reaction (PCR) and liver biopsy obtained within 12 months and consistent with chronic hepatitis, with no established cirrhosis. All patients were hepatitis B surface antigen and anti-HIV negative. Additional exclusion criteria included average daily intake of alcohol >20 g of ethanol and injection drug use. Patients were treated weekly with 180 μg of pegIFN plus 1000–1200 mg of ribavirin daily. All patients were evaluated at baseline; at the end of treatment week (TW) 1, 2, and 4; and monthly thereafter. No patient required dose reductions in the first 12 weeks of treatment.

HCV RNA quantitation. HCV RNA was extracted from 1 mL of plasma batched at baseline, days 1, 2, and 3, and TW 1, 2, 4, and 8 using the QiAamp UltraSens Virus Kit (Qiagen). HCV RNA concentration was determined by real-time quantitative reverse-transcription (RT) PCR (limit of detection, 20 IU/mL). Briefly, purified HCV RNA was reverse transcribed, and PCR was amplified using the Quant iTect Probe RT-PCR Kit (Qiagen) and quantitated using TaqMan (Applied Biosystems). Sample quantitation values were determined from an internal quantitation standard curve, which had previously been validated against the World Health Organization International Standard for HCV [96/790] (National Institute for Biological Standards and Control). All samples were tested by RT-PCR in duplicate with negative controls. HCV genotyping was determined by restriction fragment-length polymorphism at the University of Edinburgh (Edinburgh, UK) [22].

Viral kinetic modeling. Standard compartment models for HCV kinetics during IFN-α–based therapy define compartments used for the free viral load (VL), productively infected hepatocytes, and uninfected hepatocytes. The influence of these compartments on each other can be approximated by constant rates that are assumed for free viral clearance (c), infected cell loss (δ), virus production (ρ), and de novo infection (β). When treatment is initiated using an antiviral agent, this model is disturbed, and the dynamic VL changes that ensue allow calculation of virus production and clearance rates.

The antiviral effectiveness of the IFN-α treatment is represented by efficiency rate c on virus production and η on de novo infection, where η was fixed to 0.5. A possible treatment-induced (delayed) immunologic effect on the infected cell loss was modeled by an inflation factor M = 1 (Mδ). Estimates were derived for c, δ, Mδ, εν, and the viremia levels v0 = v(t0) and vν = v(tν) from a nonlinear least-squares approach of the logarithmic VL [23].

Classification of response. Mathematical modeling of the early kinetics has shown that patients achieving a sustained viral response (SVR) have an estimated rate of infected cell loss (Mδ) >0.25 per day, and 80% patients without an end-of-treatment response had demonstrated Mδ <0.13 per day [23]. On the basis of these thresholds, we classified all patients into 3 groups: rapid responders (ERRs; Mδ ≥ 0.25/day), moderate responders (EMRs; 0.13 ≤ Mδ < 0.25/day), and slow/flat responders (ESRs; Mδ < 0.13/day). We also grouped patients according to whether they attained at least a 1-log10 reduction in VL from baseline at TW 1, a 2-log10 reductions from baseline at TW 2, a 3-log10 reductions from baseline at TW 4, and HCV RNA clearance at TW 8.

NS5A amplification. HCV RNA was extracted from 100 μL
of serum using RNAzol-B (Tel-Test), in accordance with the manufacturer’s instructions. RNA was reverse transcribed using 100 pmol of pd[N]6 and 160 U of M-MLV (Invitrogen). The full-length NS5A was amplified by nested PCR using a high-fidelity Taq polymerase (ABanalitica), 5 μmol/L of each primer (see table 1), and 10 μL of cDNA. For both rounds, the following conditions were used: 5 min at 95°C; 35 cycles of 30 s at 95°C, 20 s at 55°C, and 50 s at 72°C; followed by 3 min at 72°C.

**NS5A sequence analysis.** Amplicons were used for direct sequencing. Sequences were corrected using Chromas (version 2.23; http://www.telysium.com.au/chromas.html) and aligned with SeqManII (DNAStar). Deducible amino acid sequences (aa 1973–2420) were compared with the ESR-7 sequence (see Results); sequences from TW 1 were compared with the corresponding sequences from baseline.

Phylogenetic analysis was performed with Treecon software (version 1.3b; obtained from Y. van de Peer, VIB/Ghent University, Bioinformatics and Evolutionary Biology, Ghent, Belgium), using the Kimura correction and the neighbor-joining method. Phylogenetic analyses of each amino acid sequence were performed using the ProSite program (http://us.expasy.org/prosite/) and the NetPhos Server (version 2.0; http://www.cbs.dtu.dk/services/NetPhos/). NetPhos is a neural network–based method for predicting potential phosphorylation sites at serines, threonines, or tyrosines (sensitivity, 69%–96%). All analyzed NS5A sequences, baseline and TW 1, have been submitted to the EMBL database (accession numbers AM600912–AM600956).

**Data analysis.** Statistical analysis was performed using Statistica software (version 6.0). Groups were compared at baseline by the Mann-Whitney U test. A 2-sample paired Student’s t test was used to compare qualitative differences between time points. P ≤ .05 was considered to be significant.

**RESULTS**

**Classification of patients according to early HCV RNA kinetics during therapy.** Using M0 thresholds of 0.25 and 0.13/day, patients were grouped as ERR (14 patients), EMR (5 patients), or ESR (5 patients). There was no significant difference in the mean baseline viral load among ERRs (5.46 ± 0.64 log10 IU/mL), EMRs (5.69 ± 0.29 log10 IU/mL), and ESRs (5.16 ± 0.99 log10 IU/mL). The main baseline and on-treatment parameters in these 3 groups of patients are described in table 2.

**Analysis of baseline NS5A sequences in different viral kinetic groups.** Initially, the baseline full-length NS5A sequence was analyzed in all patients. Phylogenetic analysis at both nucleotide and amino acid levels showed no clustering among sequences derived from patients with different early virologic response (figure 1). Figure 2 shows the NS5A sequences alignment from all patients. Sequences were aligned and compared with a reference NS5A sequence isolated from patient 7 (ESR-7), the patient with the lowest viral decrease after 1 week of treatment, chosen as the early nonresponder prototype. This approach was chosen because there are no published NS5A prototype sequences derived from HCV-1a–infected patients characterized as nonresponders to IFN-based therapy. When the full-length NS5A sequences from the 3 groups were compared with the NS5A from ESR-7, the mean number of amino acid substitutions observed was similar in ERRs (31.3 ± 7) and ESRs (32.5 ± 2.6), with EMRs (22.4 ± 9.1) having slightly fewer mutations (no statistically significant difference). The same trend was found when the amino terminus (aa 1973–2208) and the carboxy terminus (aa 2209–2420) of NS5A were analyzed separately.

We then examined in detail some of the functional domains of NS5A. Analysis of the putative ISDR (aa 2209–2248) showed that the mean number of amino acid substitutions, compared with the ESR-7 ISDR sequence, was similar in ERRs (2.4 ± 1), EMRs (2.2 ± 1.5), and ESRs (2.5 ± 1). When the entire PKRbd (aa 2209–274) was considered, the mean number of mutations found in EMRs was slightly lower (mean ± SD, 2.8 ± 1.8) than that in both ERRs (4.1 ± 1.6) and ESRs (4 ± 1.6), but this difference was not statistically significant. Similarly, the extent of amino acid variability observed in the V3 (aa 2356–2379) region of HCV from EMRs was slightly smaller (mean ± SD, 3.8 ± 2.2) than that from both ERRs (5.7 ± 1.7) and ESRs (6.8 ± 1), again without reaching statistical significance.

We also analyzed AR1 (aa 2144–2185), AR2 (aa 2221–2272), and PRR (aa 2283–2372) sequences, which are known to be important for the transcription-activating function of the NS5A. The mean ± SD number of mutations compared with the ESR-7 prototype was extremely variable in different patients subgroups independently of their early virologic response (ERRs, 8.6 ± 2.1; EMRs, 6 ± 2; ESRs, 8.3 ± 1.3).

All of the analyses described above, including that of full-length NS5A and of each individual functional domain, were also performed using the HCV-1 (M62321) and the HCV-H (M67463) sequences as prototypes. The results obtained were similar to those obtained when the ESR-7 sequence was used as the prototype.

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**Table 1. Primers used for full-length NS5A amplification.**

<table>
<thead>
<tr>
<th>PCR round, primer name</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td><strong>First</strong></td>
<td></td>
</tr>
<tr>
<td>NS5A-6005s</td>
<td>5′-GCCCTGGAGCCCTTGTAGTC-3′</td>
</tr>
<tr>
<td>NS5A-7732as</td>
<td>5′-CTGGTGAAAGTGGTGGAATAC-3′</td>
</tr>
<tr>
<td><strong>Second</strong></td>
<td></td>
</tr>
<tr>
<td>NS5A-6125s</td>
<td>5′-TTTTTCCCCCAGCCGACTAC-3′</td>
</tr>
<tr>
<td>NS5A-7671as</td>
<td>5′-TGGATGGCGAGTTTGTCTTCC-3′</td>
</tr>
</tbody>
</table>

**NOTE.** The primer name indicates its 5′ nucleotide position with respect to the hepatitis C virus 1 (M62321) prototype. PCR, polymerase chain reaction.
Finally, given that NS5A is the major phosphoprotein of HCV, all of the sequences were analyzed using the ProSite program, which identifies the type and number of phosphorylation sites for protein kinase C (PKC), tyrosine kinase (TYR), casein kinase 2 (CK2), and cyclic AMP–dependent protein kinase kinases. The single TYR site found in the NS5A (aa 129) was highly conserved within isolates. With regard to the other kinases, only minor differences in the number of specific sites were observed among patients, which were not associated with the degree of HCV RNA decrease at TW 1. Furthermore, no significant differences were found in the total number of potential phosphorylation sites at serines, theonines, and tyrosines identified in these sequences using NetPhos. Moreover, sequences were analyzed for previously described basal phosphorylation regions (aa 2200–2250 and 2350–2419) and hyperphosphorylation sites (aa S197, S201, and S204) [24]. Hyperphosphorylation sites were fully conserved in all of our sequences. Phosphorylatable residues within the 2200–2250 basal phosphorylation region were highly conserved, whereas variations, both in the number and in the position, were observed in the 2350–2419 basal phosphorylation region, but those differences were not associated with the early viral kinetics.

Analysis of baseline NS5A sequences in different virologic response groups. Comparison of those patients who did and did not attain a 2-log decrease at TW 2, a 3-log decrease at TW 3, or HCV RNA negativity at TW 8 revealed no significant differences in the number of baseline amino acid substitutions (relative to the ESR-7 sequence). There was a trend toward a greater number of amino acid substitutions in the NS5A/V3 region in 11 of 24 patients who achieved at least a 1-log reduction in VL (mean ± SD, 6.1 ± 2.2), compared with those (13/24) who did not achieve this reduction (mean ± SD, 4.5 ± 2.1; P = .052), after receiving just 1 week of treatment. Baseline VL, age, and weight were similar in both groups.

A significant correlation was found between the initial log decrease in viral load or antiviral efficacy ε and the total NS5A mutation frequency for all analyzed functional domains in all patients (r = 0.59; P = .003), especially within the NS5A/V3 domain (r = 0.62; P = .001), as well as the AR1 and PRR regions. However, no correlation was identified in mutational frequency of other NS5A functional domains with the other measured viral kinetic parameters. NS5A variability was not analyzed in relation to SVR because of the small number of patients included and heterogeneity of treatment schedule used in relation to dose reduction and total duration.

Comparison of NS5A sequences at baseline and after 1 week of pegIFN plus ribavirin therapy. The NS5A was successfully amplified from 21 of 24 patients, using samples obtained after TW 1 in patients treated with pegIFN plus ribavirin. The full-length NS5A sequence was compared with that obtained at baseline. No amino acid mutations were detected within the functional domains previously described, and no pattern of sequence changes was found when patients were grouped according to the early virologic response (figure 3). In fact, only rare amino acid substitutions (mean ± SD, 2.3 ± 2.1 mutations over the entire gene) were found in TW 1 sequences from a large proportion of the patients, and only in 3 cases (2 ERRs and 1 EMR) did the baseline sequences remain unchanged. More important, changes were spread along the entire gene with no evidence of clustering in any of the analyzed functional domains.

When phosphorylation sites were analyzed using ProSite, few changes were observed after 1 week of treatment, and they were specific for 2 kinases. One patient, ERR-13, lost 1 PKC site (position 238), and patient ERR-36 lost 1 PKC (position 402) and 1 EMR (position 395) site. After 1 week of therapy, phosphorylation predicted values estimated by NetPhos were slightly lower, confirming the previous results obtained with ProSite. After 1 week of therapy, the NLS remained mostly unchanged, whereas the cytoplasmic retention signal was one of the NS5A domains in which most changes occurred, although they were limited in number.

**DISCUSSION**

Optimal treatment of chronic hepatitis C is currently based on the use of pegIFN-α in combination with ribavirin, but only ∼50% of treated patients with HCV genotype 1 achieve a sustained virologic response. Resistance to IFN remains a major problem for the treatment of chronic hepatitis C, and little is known about the exact pathogenetic mechanisms, although both virologic and host immunologic factors have been widely implicated. Previous studies have shown that sensitivity to IFN,
reflected by a rapid viral decay during the first phase of treatment, might be associated with sequence variability in the NS5A gene. These findings were derived from analysis of the NS5A sequences of genotype 1b, whereas, to date, very little has been reported concerning HCV-1a.

In the present study, entire NS5A sequences from a homogeneous group of patients with HCV-1a infection, all receiving the same pegIFN combination therapy, were analyzed in relation to early viral decay kinetics. Evolution of the NS5A sequence after a single dose of pegIFN was also investigated.

Mathematical modeling of the initial viral decrease indicates that the very rapid first phase of viral reduction during the first 24–48 h is a result of dose-dependent inhibition of viral production [5]. The antiviral efficacy (ε) during this phase is a direct measurement of the log decrease and, in the present study, was found to correlate with an increasing number of NS5A mutations relative to the prototype ESR-7 nonresponder sequence. This appeared to be most pronounced within the V3 domain in patients showing at least a 1-log reduction in VL after 1 week of treatment. Pretreatment NS5A/V3 sequence variability had previously been reported, with also more amino acid changes among IFN responders in HCV-1a–infected patients [25–27] but fewer mutations in IFN responders among HCV-1b–infected patients [28]. However, the V3 domain differences in the present study were not shown to be associated with the subsequent phases of viral decay, which are implicated in a more durable treatment response.

The second phase of the early viral decline is slower and reflects the treatment-induced infected cell death rate in the mathematical model. A significant correlation between this second-phase decay rate (M₀) and baseline aminotransferase levels was observed, which corroborates the model view that the second-phase decay represents the degradation rate of infected cells ($r = 0.42; P = .04$) [6]. Three patterns of early viral response to the antiviral treatment were differentiated, according to a previously validated calculation of the drug-induced loss
rate of virally infected cells (M6): a very rapid kinetic of HCV RNA decay occurred in 60% of patients, whereas the remaining patients showed a slow/minimum decline in HCV RNA.

Our results demonstrated that in patients infected with HCV-1a, the overall number of amino acid substitutions in the ISDR or/and PKRbd, relative to our nonresponder prototype NS5A sequence, did not correlate with the extent of HCV RNA decrease during the postprimary phase of viral kinetics, which

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**Figure 2.** Amino acid sequence alignment of pretreatment hepatitis C virus (HCV)–1a full-length NS5A. Early-slow responder (ESR)–7 is reported at the top as reference sequence. Sequences are arranged according to the virologic response by means of early HCV kinetics. Prototype sequences for HCV-1a, HCV-1 (M62321), HCV-H (M67463), and HCV-1b, HCV-J (D90208) are reported. Amino acid positions are numbered according to the published sequence of prototype HCV-1. Analyzed domains are indicated as well as previously published regions involved in basal phosphorylation and sites of hyperphosphorylation. ISDR, interferon sensitivity–determining region; PKR, dsRNA-dependent protein kinase; V3, variable region 3.
Figure 3. Sequence comparison of NS5A selected domains before and after 1 week of treatment in 21 patients. EMR, moderate responder; ERR, rapid responder; ESR, slow responder; ISDR, interferon sensitivity–determining region; PKR, dsRNA-dependent protein kinase; NLS, nuclear localization signal; V3, variable region 3.

has been reported to be associated with an SVR [6]. Thus, pretreatment NS5A sequences in these regions are not predictive of the likely pattern of early viral response. No specific pattern of amino acid substitutions associated with different sensitivity to IFN were identified when sequences of selected functional domains previously described or the complete NS5A gene were analyzed in detail. These findings with HCV-1a are in agreement with a recently published study of patients treated with IFN and infected with HCV-1b in which no relationship was found between antiviral efficacy at 24 h and the baseline sequence of any NS5A region [29].

For each of our patients, the NS5A sequence obtained 1 week after a single dose of pegIFN was compared with that obtained at baseline. The results showed that, despite only rare amino acid substitutions, the NS5A sequence was mostly conserved. No evidence of selection of specific resistant variants was observed after the first dose of IFN. Evolution toward a viral population carrying a resistant ISDR has been previously described for HCV-1b [12], but this might be the effect of a longer period of therapy with standard IFN rather than pegIFN. Furthermore, slight differences between our results and those of some of the previously published studies might be ascribed to a different methodologic approach, given that we performed direct sequencing of the NS5A gene, whereas others analyzed individual clones.

In conclusion, we have shown a relationship between the frequency of mutations in HCV-1a NS5A, early viral kinetics, and pegIFN efficacy that is represented mostly by aa substitutions in the V3 region at the C terminus of NS5A. These findings support the concept that NS5A mutations can impede interactions between NS5A and PKR and that NS5A can regulate the cellular PKR defense against viral protein translation. However, we did not find specific selection of particular NS5A sequences occurring in any patients after a single dose of pegIFN. No correlation was uncovered between the second viral kinetic phase, which is associated with more sustained responses, and altered amino acid configurations in putative NS5A functional domains. Therefore, the presence of higher NS5A mutational frequency might influence the ability of IFN to inhibit virus production very early, but other factors are necessary to facilitate a sustained IFN effect for antiviral success in patients with chronic HCV genotype 1a infection.
References