Evolution of hepatitis C virus quasispecies during therapy with IL2 combined to alpha interferon and ribavirin

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This study analyses the impact of interleukin 2 (IL2) combined with alpha interferon (IFN-α) and ribavirin on the heterogeneity of hepatitis C virus (HCV). We studied 10 patients who took part in a clinical trial that assessed the effects of retreatment with IL2, IFN-α and ribavirin in patients who failed to clear the virus after a previous bitherapy. The heterogeneity of HCV quasispecies was assessed by cloning and sequencing the hypervariable region 1 (HVR1) in samples obtained at baseline (W0), after 12 weeks of treatment with IFN-α and ribavirin (W12), after a cycle of administration of IL2 in combination with the classical bitherapy (W21 and W24) in the eight patients who failed to clear the virus under treatment. The mean viral load at W21 and at W24 was not different from that at W12. The heterogeneity of HVR1 quasispecies after the administration of IL2 was not different from that at baseline or after 12 weeks of bitherapy. Furthermore, the proportion of nonsynonymous substitutions was unchanged after the IL2 cycles. Thus, the efficacy of the tritherapy with IL2, IFN-α and ribavirin is similar to that of the classical bitherapy. Treatment with IL2 in combination with IFN-α and ribavirin had no effect on the selective pressure on HCV quasispecies. IL2 is not the best option to treat hepatitis C.

Introduction

Infection by the hepatitis C virus (HCV) is the major cause of non-A, non-B hepatitis and is highly prevalent worldwide [1]. The disease often progresses to chronic hepatitis, which may lead to cirrhosis and hepatocarcinoma, and is a major cause of liver transplantation [1].

Treatment is based on alpha interferon (IFN-α), which succeeds in eliminating the virus in only 20% of cases when it is used alone [2]. The combination of IFN-α plus ribavirin (RBV) improves hepatitis C treatment, with eradication rates up to 40% [3,4]. Current treatment uses a combination of pegylated IFN-α plus RBV [5,6], but some patients still fail to eradicate the virus. Most of them are infected by HCV genotype 1 and/or have a severe liver disease. Better, more potent, antiviral agents are needed to increase the eradication rates.

Studies on immune responses in anti HCV positive/HCV RNA negative patients have shown that a strong virus-specific CD4+ Th cell response with the production of Th1 cytokines is associated with the spontaneous clearance of HCV RNA [7]. The immune response also plays an important part in HCV eradication after treatment. IFN-α is a cytokine with both antiviral and immunomodulatory properties [8], and RBV may act as an immunomodulator to activate cellular immunity [9]. Patients treated with IFN-α plus RBV exhibited a predominant Th1 response in peripheral blood mononuclear cells (PBMC) and a decreased Th2 response [10]. Viral eradication was associated with a great proliferation of HCV-specific T cells with increased IFN-α production [10]. High levels of Th1-type cytokines such as IFN-α, interleukin 2 (IL2) and
tumour necrosis factor (TNF-α) were found in virological responders [10,11], whereas the production of the Th2-type cytokine IL10 was suppressed [10]. Th1 predominance was also reported in patients given pegylated IFN alone or in combination with RBV [12].

IL2 has been used to improve the function of the immune system in HIV-infected subjects [13], and in combination with IFN-α in patients with chronic hepatitis B [14]: immunomodulatory and antiviral effects were observed, and the treatment was reasonably well tolerated. The effects of a combined therapy of IL2 with IFN-α and RBV for chronic HCV carriers has been recently investigated in nonresponders [15].

The quasispecies nature of HCV has been documented for the whole genome, but the variability is most marked at the 5’ end of the E2 gene, in the so-called hypervariable region 1 (HVR1) [16,17]. The biological function of HVR1 quasispecies remains controversial. HVR1 is reported to be a target for the immune system, as it contains epitopes for both B and T cell responses [18,19]. Other studies suggest that HVR1 functions as an immune decoy [20]. Thus, HVR1 is currently used to assess HCV quasispecies heterogeneity. Several studies have linked HVR1 heterogeneity to the response to IFN-α treatment [21,22]. The selective pressure on HVR1 was shown to be greater in patients who eliminate the virus after treatment than in nonresponders [23,24].

This study analyses the impact of IL2 on HCV quasispecies in patients treated with IL2 in combination with IFN-α and RBV. The genetic heterogeneity of the hypervariable 1 (HVR1) region of HCV E2 gene was studied in sequential samples taken from these patients during the various phases of treatment.

Materials and methods

Patients and samples

Serum samples were collected from 10 patients chronically infected by HCV, who were part of a clinical trial to assess the beneficial effects of IL2 combined with IFN-α and RBV [15]. All the patients were infected with HCV genotype 1, had severe liver disease and failed to clear the virus when previously treated with IFN-α and RBV. All 10 patients were given a bitherapy with IFN-α (3 million units (MU) every 2 days) plus ribavirin (1000–1200 mg per day) for 12 weeks, followed by a tritherapy with IL2, IFN-α and RBV, with four cycles of IL2 every 3 weeks (W12, W15, W18, W21). IL2 was given at the dose of 3 MU subcutaneously for 5 consecutive days. The genetic heterogeneity of HCV was studied in samples collected just before starting therapy (W0), after 12 weeks of the classical bitherapy (W12), after the third cycle of IL2 (W21) and at the end of treatment, after the last cycle of IL2 (W24). HCV viral load was quantified at W0, W12, W15, W18, W21, W24 and 8 weeks after the withdrawal of therapy (W36).

HCV RNA quantification

Serum HCV RNA concentrations were measured by quantitative reverse-transcription polymerase chain reaction (RT-PCR) with an internal standard (Amplicor HCV Monitor, Roche Diagnostics, Meylan, France), according to the manufacturer’s instructions.

E2-HVR1 quasispecies analysis

Amplification of the E2-HVR1 region

Total nucleic acids were extracted from 100µl of each serum sample using the Nuclisens kit (NASBA diagnostics, Organon Teknika, Boxtel, NL). RT-PCR was carried out with the Qiagen One Step RT-PCR kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions, under the following conditions: 30 min RT at 50°C, 15 min denaturation at 95°C, followed by 35 cycles (95°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec) and a final extension at 72°C for 10 min, using the external primers KS1 (5’-CAGGACTGCAATTGGCTCAATCTA; nt 1245–1266) and KS2 (5’-TTGCAAGTATTAAAGCAGTCC; nt 1612–1630). The nested PCR was performed under the same conditions with the internal primers KS3 (5’-CAGTGAGGTCTTACCAGGG; nt 1395–1414) and KS4 (5’-ATGTGCGAGCTGCCATTGGT; nt 1587–1606).

Cloning and sequencing

PCR products were purified with QIAmp Columns (Qiagen) as specified by the manufacturer. Purified products were quantified by spectrophotometry: 10 ng of products were ligated directly into 50 ng of PCR II vector (original TA cloning kit; Invitrogen, Leek, The Netherlands) at 14°C overnight. The recombinant plasmids were used to transform competent Escherichia coli cells according to the manufacturer’s protocol, and the transformants were grown on ampicillin plates. Twenty clones containing HVR1 inserts were prepared and sequenced on both strands by the dyeoxy chain termination method (ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction, Applied Biosystems, Paris, France) on a model 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). A total of 680 clones were generated and sequenced. Electropherogram data were analysed using the Sequence Navigator Program.

Sequence analysis

Nucleotide sequences were aligned with the CLUSTAL W [25] program (version 1.8). The complexity of the
HCV strain in the region of interest was quantified by calculating the Shannon entropy as follows: \( S = -\sum_i (p_i \ln p_i) \), where \( p_i \) is the frequency of each sequence in the virus quasispecies. The normalized entropy \( S_n \) was calculated as \( S_n = S/\ln N \), where \( N \) is the total number of sequences analysed per sample. Diversity was calculated for all pairs of nucleotide sequences, using the DNADIST module in the PHYLIP package, version 3.575. The calculation was based on a Kimura two-parameter distance matrix with a transition-to-transversion ratio of 2.0. The numbers of synonymous substitutions per synonymous site and nonsynonymous substitutions per nonsynonymous site were calculated with the Jukes-Cantor correction for multiple substitutions with the MEGA program [26].

Statistical analysis
Differences between the initial and follow-up samples were evaluated using the Wilcoxon rank test. \( P \) values less than 0.05 were considered significant.

Results

Patient characteristics
The clinical and demographic characteristics of the 10 patients (seven men and three women) are summarized in Table 1. All were infected with HCV genotype 1 and their mean fibrosis score was (3.3 ±0.9).

Changes in serum viral load during the different phases of the treatment
Of the 10, eight patients failed to clear the virus during the various phases of treatment (nonresponders); the remaining two had no detectable HCV RNA after the first 12 weeks of treatment with IFN-\( \alpha \) and ribavirin (W12: 4.7 ±0.6 log copies/mL, \( P <0.01 \)) (Figure 1). The viral load during the IL2 treatment at W15 (4.6 ± 0.6), W18 (4.4 ±0.7) and W21 (4.3 ±0.7) was not different from that at W12. The viral load at the end of the treatment was smaller than that at baseline (W24: 4.4 ±0.7 vs W0: 5.8 ±0.1, \( P <0.05 \)), but was not different from that at W12. At W36, 2 months after the withdrawal of therapy, the viral load increased back to baseline values in all patients (mean HCV viral load: 5.8 ±0.06 log copies/mL). Nevertheless, the alanine aminotransferase level at week 24 (76.4 ±55) was significantly lower than at baseline (133.7 ±79, \( P <0.05 \)).

HVR1 quasispecies heterogeneity during IFN-\( \alpha \) plus RBV treatment
The change in HCV quasispecies during the different phases of treatment was studied in the eight patients who failed to clear the virus. The complexity at W12 was similar to that at W0, in terms of both amino acids and the nucleotides (data not shown). The diversity was calculated as the mean genetic distance (gd) between variants. The diversity at W12 (0.073 ±0.020) was not different from that at W0 (0.071 ±0.030) (Figure 2). The number of nonsynonymous substitutions per nonsynonymous site (Ka) was not different from the number of synonymous substitutions per synonymous site (Ks) at both W0 (Ka: 0.0705 ±0.031 vs Ks: 0.0853 ±0.034) and W12 (Ka: 0.0775 ±0.023 vs Ks: 0.0779 ±0.023) (Figure 2).

Analysis of the mutational changes at baseline showed that Ka was significantly greater than Ks in five patients, the two responder-relapsers (patients nine and

<table>
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<tr>
<th>Patient, n</th>
<th>Sex</th>
<th>Age</th>
<th>Fibrosis score (Metavir)</th>
<th>Baseline HCV RNA (log copies/mL)</th>
<th>End-of-treatment response</th>
<th>Sustained virological response</th>
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<tr>
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</tr>
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<td>Mean (SEM)</td>
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<td>3.3 (0.9)</td>
<td>5.8 (0.1)</td>
<td></td>
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</tr>
</tbody>
</table>

F, female; M, male.
10) and three nonresponders (patients one, six and seven). In the remaining five patients, Ka was either similar to Ks (patient two) or significantly smaller than Ks (patients three, four, five and eight) (data not shown).

HVR1 quasispecies heterogeneity after the third cycle of IL2

The complexity at W21 was similar to those at W12 and W0 for both the amino acid and the nucleotide sequences (data not shown). The diversity at W21 (0.0697 ±0.030) was also similar to those at W12 and W0 (Figure 2). The number of nonsynonymous mutations (Ka: 0.0765 ±0.033) at W21 was similar to the number of synonymous mutations (Ks: 0.0651 ±0.027) (Figure 2).

The between-sample diversity, calculated between W12 and W21 (0.0934 ±0.028), was not different from the W0-W12 between-sample diversity (0.0952 ±0.023). Regarding the between-sample mutational changes, the number of nonsynonymous mutations (rKa) was not different from the number of synonymous mutations (rKs), when calculated between W12 and W21 (rKa: 0.097 ±0.031 vs rKs: 0.082 ±0.024) and between W0 and W12 (rKa: 0.097 ±0.036 vs rKs: 0.095 ±0.028).

HCV quasispecies heterogeneity at the end of treatment, after the last cycle of IL2

The complexity at W24 was not different from those at W21, W12 or W0, for either the amino acid or the nucleotide sequences. The W24 diversity (0.0623 ±0.028) was also not different from the diversities at W21, W12 and W0 (Figure 2). The Ka (0.0649 ±0.027) and the Ks (0.0651 ±0.035) at W24 were also similar (Figure 2).

The W12-W24 between-sample diversity (0.1142 ±0.031) was not different from the W12-W21 and the W0-W12 between-sample diversities. The W12-W24 rKa (0.1210 ±0.036) was not different from the rKs (0.095 ±0.033).

Discussion

About 55% of chronic HCV carriers achieve virus eradication with the bitherapy based on pegylated IFN and RBV. New strategies are needed for the remaining 45% of patients. As some patients with acute hepatitis spontaneously clear HCV, an immunotherapy, producing an efficient antiviral immune response could be a new treatment option for chronic hepatitis. Thus, IL2 is reported to block HCV replication in patients infected with HIV and HCV [27], but this is controversial [28]. The effects of IL2 combined with classical bitherapy on chronic HCV carriers has been recently assessed [15]. The impact of IL2 on HCV may be reflected in changes in the quasispecies.

The heterogeneity of HCV quasispecies was characterized in the HVR1 region. This region was used because of its great variability, enabling the optimal detection of the different variants [16] and because it is a target for the immune response. Although the influence of the host immune response on HCV genetic drift is controversial [29], a comparison of the heterogeneity of HVR1 and of the flanking regions was consistent with immune pressure on this hypervariable region[30].
In this study, the viral load decreased slightly after the first 12 weeks of therapy, at this time only two patients cleared the virus; thereafter, it remained almost unchanged until the withdrawal of treatment, at which time it increased back to the baseline value in all patients. These results suggest that the efficacy of the tritherapy with IL2, IFN-α and RBV is similar to that of the classical bitherapy with IFN-α and RBV in term of virological response. A biochemical response was observed, which is in agreement with other studies [31]. Thus, the patients recruited to the initial clinical trial failed to clear the virus during a previous treatment with IFN-α and RBV [15]. They were also infected with genotype 1, had severe liver disease and most of them were men. Greater decreases in viral load may have been obtained in patients having characteristics associated with a sustained viral response and/or in naive patients.

The diversity did not change after the first 12 weeks of treatment with IFN-α and RBV, in agreement with other studies [32]. The introduction of IL2 did not lead to a significant change in the genetic diversity. Viral eradication after treatment with IFN-α alone has been associated with early RNA clearance [33] and with a great decrease in diversity [34]. The lack of viral clearance or any change in genetic diversity could be associated with the lack of response to multi drug therapies.

The study of mutational changes (synonymous and nonsynonymous substitutions) provides an indirect estimation of the selective pressure. Synonymous mutations do not alter the amino acid encoded, whereas nonsynonymous mutations result in a different amino acid. More nonsynonymous mutations than synonymous substitutions can be interpreted as evidence of positive selection [35], which is mainly produced by the host immune system [36]. A stronger immune pressure, reflected in a greater number of nonsynonymous mutations before treatment with IFN-α alone has been associated with a sustained virological response [23,24]. The two patients who cleared the virus under treatment and three of the eight nonresponders had the same profiles at baseline as those who responded to treatment with IFN-α alone in term of mutational changes: a greater number of nonsynonymous mutations appears to be a factor involved in viral clearance but it alone is not sufficient to predict sustained virus eradication. The first 12 weeks of treatment with IFN-α and RBV did not change the number of nonsynonymous substitutions and so did not modify the selective pressure on HCV. This was not surprising as these patients failed to clear HCV RNA during a previous treatment with IFN-α and RBV.

The proportions of nonsynonymous and synonymous substitutions were not modified after a cycle of IL2, as illustrated by points W21 and W24. Thus the selection pressure remained unchanged during this phase of treatment. The change in selective pressure produced by IL2 was similar to that caused by IFN-α and RBV, as shown by the study of between-sample mutational changes. These results suggest that IL2 in combination with IFN-α and RBV does not increase the positive selection pressure on HCV.

These data indicate that the efficacy of the tritherapy with IL2, IFN-α and RBV is similar to that of standard bitherapy. Further studies are needed to better characterize any changes in the intensity and/or quality of the immune response induced by the administration of IL2 in combination with IFN-α and RBV. Therefore, IL2 does not enhance the efficacy of the conventional bitherapy so does not appear to be the best treatment option against hepatitis C.

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