An Analysis of Acute Changes in Interleukin-6 Levels After Treatment of Hepatitis C with Consensus Interferon

SCOTT J. COTLER,1 K. RAJENDER REDDY,2 JON MCCONE,3 DARIN L. WOLFE,4 ANGUO LIU,4 TERESA R. CRAFT,4 MARY W. FERRIS,4 ANDREW J. CONRAD,5 JEFF ALBRECHT,5 MARY MORRISSEY,1 DANIEL R. GANGER,1 HOWARD ROSENBLATE,1 LAWRENCE M. BLATT,6 DONALD M. JENSEN,1 and MILTON W. TAYLOR4

ABSTRACT

Cytokine production has been implicated in the antiviral response to interferon-α (IFN-α) in hepatitis C and in the development of IFN-α-related side effects. We characterized acute changes in serum cytokine levels following administration of a single dose of consensus IFN (IFN-con1) and during continuous treatment of chronic hepatitis C patients. Serum samples were collected at baseline, at multiple times early after IFN administration, and weekly thereafter. Viral RNA titers were assessed by RT-PCR, and viral kinetics were followed. ELISA assays were used to measure IFN-γ, tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-6, and IL-16. Serum cytokine levels were low at baseline. IL-6 was detected in patients with hepatitis C but not in healthy control subjects by either ELISA or RT-PCR, indicating that low levels of circulating IL-6 were associated with hepatitis C infection. None of the cytokines measured increased significantly after IFN administration except for IL-6. IL-6 levels rose rapidly, peaked at 6–15 h in a dose-dependent manner, and returned to baseline by 48 h in both patients receiving a single dose of IFN and those receiving continuous treatment. This was confirmed by RT-PCR. Pretreatment IL-6 levels were directly correlated with area under the curve (AUC) for IL-6 during the 24 h after IFN dosing ($r = 0.611, p = 0.007$). Viral titers decreased within 24–48 h after a single dose of IFN-con1. Changes in hepatitis C RNA titers were not significantly associated with pretreatment IL-6 levels or with changes in IL-6 levels. In conclusion, (1) baseline serum cytokine levels, except for IL-6, were low or within the normal range in patients with hepatitis C, (2) IL-6 levels were detected in some patients with hepatitis C before treatment but not in healthy controls, (3) IL-6 levels increased acutely after a single dose of IFN-α, and IL-6 induction was related to baseline IL-6 level, and (4) changes in IL-6 levels did not correlate with the early virologic response to IFN.

INTRODUCTION

Hepatitis C virus (HCV) is the major cause of chronic hepatitis and hepatocellular carcinoma in the Western world. Chronic viremia develops in approximately 75% of persons who contract the infection,1 a minority of whom progress to cirrhosis and hepatocellular carcinoma.2–5 Approved treatments for HCV include various type I interferons (IFN) and, more recently, the combination of IFN-α and ribavirin. Type I IFN has direct antiviral activity as well as immunomodulating, anti-inflammatory, and antifibrotic effects. Forty-eight weeks of IFN-α monotherapy for HCV is associated with a sustained response rate of 13%–19%.6,7 Combination therapy with IFN-α and ribavirin increases sustained response rates to approximately 35%–40%.6,7 IFN-α tends to reduce serum aminotransferase levels and to decrease inflammation, as assessed by liver biopsy, even in patients who remain viremic on therapy.8,9

1Section of Hepatology and Department of Preventive Medicine, Rush-Presbyterian-St. Luke’s Medical Center, Chicago, IL 60612.
2University of Miami Medical School, Miami, FL 23124.
3Mc Cone Endoscopy Centers, Alexandria, VA 22306.
4Department of Biology, Indiana University, Bloomington, IN 47405.
5National Genetics Institute, Los Angeles, CA 90064.
6Ribozyme Pharmaceuticals, Boulder, CO 80301.
Cytokine profiles have not been well characterized in patients with hepatitis C. Studies of cytokine production in patients receiving antiviral therapy have yielded variable results. IFN-α-induced cytokine production was proposed as a mechanism for the reduction in viral load that occurs with antiviral therapy through the activation of cytotoxic T cells. Cytokine responses may also contribute to the development of side effects to IFN-α.

We have initiated studies to analyze the cytokine response in patients with hepatitis C undergoing IFN therapy. In the present study, we examined circulating cytokine levels previously identified as possibly modulating the immune response in HCV infection. This was accomplished in patients with hepatitis C at baseline, after a single dose of consensus interferon (IFN-con1) and during continuous daily treatment with IFN-con1. Whereas the levels of most of the cytokines measured did not change, IL-6 levels increased within 6 h of IFN administration and returned to baseline within 25–48 h.

### Table 1. Cytokine Levels at Baseline in Control and Hepatitis C Patients

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Hepatitis C patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE (pg/ml)</td>
<td>Range (pg/ml)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.31 ± 0.05</td>
<td>0.009-0.56</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.37 ± 0.7</td>
<td>0.64-7.71</td>
</tr>
<tr>
<td>IL-6</td>
<td>Group 1: 4.88 ± 2.6</td>
<td>0-19</td>
</tr>
<tr>
<td></td>
<td>Group 2: 6.25 ± 1.6</td>
<td>0-23</td>
</tr>
<tr>
<td>IL-16</td>
<td>412 ± 169</td>
<td>151-2768</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

The Institutional Review Board at RUSH-Presbyterian-St. Luke’s Medical School, University of Miami Medical School, The Copernicus Group Independent Review Board, and Indiana University approved the research protocol. All subjects provided written informed consent before study entry.

**Subjects**

*Group 1: Previous nonresponders.* Eighteen patients with chronic hepatitis C who were previous virologic nonresponders to at least a 3-month course of IFN therapy were included in this study. Their mean age was 54 ± 11 years. All subjects had detectable hepatitis C viremia, genotype 1 infection, and features of chronic hepatitis C on liver biopsy, without evidence of other causes of liver disease. They had at least a 3-month washout period between completing their previous course of

**FIG. 1.** IL-6 induction in group 1 patients after a single 15-μg (A) or 30-μg (B) dose of IFN-con1.
IFN-α and enrollment in this study. Nine subjects received a single 15-μg dose of IFN-con1, and 9 received a single 30-μg dose. Serum samples were collected at 0 time (baseline), and at 1, 2, 4, 6, 8, 24, 48, 72, and 96 h postinjection.

**Group 2: Treatment-naive patients.** Twenty-seven previously untreated patients were evaluated. All had detectable hepatitis C viremia, genotype 1 infection, and histologic features of chronic hepatitis C. Their mean age was 48 ± 6 years. Seven patients received 15 μg IFN-con1 daily and had serum samples collected at baseline, 6, 15, 21, 24, 48, and 96 h, and at week 1 and week 2. Twenty subjects received 15 μg IFN-con1 t.i.w. and had serum samples taken at baseline and at weeks 1, 2, 12, and 24.

![Graph showing changes in IL-6 levels with IFN therapy](image)

**FIG. 2.** (A) Relationship between serum IL-6 levels and onset of fever in group 1. Median values are presented for both parameters. Nine of eighteen patients developed a fever over 100°F. (B) Frequency of side effects in group 1 patients 8 h after IFN administration, corresponding to the time of peak IL-6 levels.
Control subjects. Fifty healthy students and faculty members from Indiana University served as controls. None had a history of hepatitis or other causes of liver disease. Their mean age was 32 ± 12 years. Serum samples were treated identically to those of subjects in the experimental groups.

Cytokine assays

Serum samples were assayed for cytokines using regular and high-sensitivity ELISA. The following ELISA were used in this study: IFN-γ, tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-6, and IL-16. All ELISA kits were purchased from R&D Systems (Minneapolis, MN). The assays were performed with standard cytokines supplied by the manufacturer. The lower limits of detection using standards provided by R&D Systems for the ELISA were as follows: IFN-γ, 15 pg ml⁻¹; TNF-α, 0.25 pg ml⁻¹; IL-2, 15.6 pg ml⁻¹; IL-4, 0.25 pg ml⁻¹; IL-6, 0.7 pg ml⁻¹; IL-16, 31.2 pg ml⁻¹. IL-6 was measured in all subjects at each time that serum was collected. IFN-γ, TNF-α, IL-2, IL-4, and IL-16 measurements were limited to 9–15 subjects per cytokine at 0, 6, 8, 24, and 48 h for group 1 and at early time points for group 2 and 3 because sera were limited and levels were consistently within the normal range (Table 1). Serum from control subjects was chosen at random from the 50 participants to assay for each cytokine.

RNA preparation and RT-PCR for IL-6

Total RNA was isolated from peripheral blood lymph (PBL) cell pellets from patients of groups 2 and 3 using Trizol reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. RNA was quantitated by measuring absorbance at 260 nm and 280 nm in a Shimadzu 260U spectrophotometer. The integrity of the RNA and the possibility of DNA contamination were assessed by agarose gel electrophoresis prior to the synthesis of cDNA. If necessary, the RNA sample was treated with RNase-free DNase 1. The quality of the RNA was also judged by the presence of intact 28S and 18S ribosomal RNA bands on a formaldehyde/agarose/ethidium bromide gel. RNA was stored in small aliquots at −80°C. Aliquots of RNA (2 μg) were denatured at 80°C for 5 min, then reverse-transcribed in 40 μl reactions (50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.01 μg/μl random hexamer primers, 2 U/μl RNase inhibitor, 1 mM dNTP, and 40 U/μl Moloney murine leukemia virus [MMuLV] for 1 h at 42°C, followed by 15 min at 65°C.

For each PCR reaction, 5 μl of the resulting cDNA was used as template. All amplifications were performed in 50-μl reaction volumes (20 mM Tris, pH 8.8, 1.5 mM MgCl₂, 25 mM KCl, 0.05% NP-40, 0.01 μg/μl bovine serum albumin [BSA], 0.25 mM dNTP, 0.004 μg/μl each primer, 0.025 U/μl Taq). Manual hot start was used for IL-6 primer pairs. Cycling parameters were as follows: initial denaturation at 94°C for 4 min, then 40 cycles of 30 sec at 94°C, 30 sec at specific annealing temperature, 1 min at 72°C; final extension of 6 min at 72°C. Optimal annealing temperatures were determined for each primer pair and were as follows: 55°C for GAPDH and 45°C for IL-6.

Reactions were sampled after 20, 25, 30, 35, and 40 cycles to ensure that amplification had not reached saturation. Amplification products were electrophoresed on 1.8% agarose gels, stained with ethidium bromide, and visualized under UV light. Bands were quantitated using the Bio-Rad Quantity One imager and software (Bio-Rad, Hercules, CA). Primers used were: IL-6, upper 5’-CAG CTA TGA ACT CCT TCT CCA CAA G-3’, and lower 5’-TGC CCA TGC TAC ATT TGC CGA AGA G-3’. Primers for GAPDH were upper GGT CGG AGT CAA CGG ATT TG, and lower ATG AGC CCC AGC TTT CTC CAT.

IL-8 primers were run on the same gels because we had previously noted a change in IL-8 mRNA levels in 1 patient after treatment with IFN (data not shown). However, this was not seen in other patients.

Virologic testing

Viral titers were assayed by quantitative RT-PCR by National Genetics Institute (Los Angeles, CA). Hepatitis C genotyping was performed by line probe assay (LiPA) (Innogenetics, Zwijnaarde, Belgium) as described previously(18) Genotypes were grouped according to the Simmonds classification(19).

Statistical analysis

The median IL-6 levels at all time points were compared independently for each dose using a Friedman’s test. We estimated and tested the difference between baseline and each consecutive measure to determine where the maximum change in IL-6 levels occurred and when they returned to baseline, using pairwise Mann-Whitney tests adjusted for multiple comparisons. The concentrations of IL-6 were measured over time, and area under the curve (AUC) analysis was performed to test if the 24-h induction of IL-6 was correlated with baseline mea-
sures. Because the distributions of baseline IL-6 and 24-h induction of IL-6 were nonnormally distributed, Spearman’s rank correlations were used.

RESULTS

Serum cytokine levels

At baseline, serum levels of IL-4, IL-16, and TNF-α were not significantly different between patients with hepatitis C and healthy controls (Table 1). IL-6 levels were detected in some patients with hepatitis C but not in uninfected control patients. IL-2 levels were below the limits of detection in both hepatitis C patients and controls, and IFN-γ was barely detectable.

IL-2 levels remained undetectable, and IL-4, IL-16, TNF-α, and IFN-γ levels remained in the normal range at all time points after IFN administration.

IL-6

In group 1, baseline IL-6 levels increased rapidly after a single IFN-con1 dose and peaked at 6–8 h after IFN-con1 administration (Fig. 1). Subjects who received 15 µg IFN-con1 had a median IL-6 level of 34.7 pg ml\(^{-1}\) (interquartile range 27.6–50.1 pg ml\(^{-1}\)) at 6 h compared with a median level of 50.6 pg ml\(^{-1}\) (interquartile range 42.3–67.5 pg ml\(^{-1}\)) for those given 30 µg (\(p = 0.058\)). IL-6 levels returned to baseline between 24 and 48 h.

The induction of IL-6 in group 1 patients was further evaluated using AUC analysis. Pretreatment IL-6 levels were directly correlated with AUC for IL-6 during the 24 h after IFN dosing (\(r = 0.611, p = 0.007\)). Peak IL-6 levels were temporally related to the onset of fever and other IFN-associated side effects (Fig. 2). Likewise, IL-6 levels increased rapidly and peaked between 6 and 24 h in the group 2 patients who had measurements taken at early time points. Although such patients received continuous IFN-con1 dosing, there was no elevation of IL-6 levels at later time points (Fig. 3), nor were there daily variations. Samples from group 2 patients taken at 1, 2, 12, and 24 weeks of IFN-con1 therapy did not show enhancement of IL-6 levels, which remained within the normal range. Thus, IL-6 levels increased transiently with initiation of IFN therapy and quickly returned to baseline.

We examined whether increased serum cytokine levels were correlated with de novo transcription of this cytokine in peripheral blood mononuclear cells (PBMC) by measuring changes in cytokine mRNA levels in group 2 patients using RT-PCR. As can be seen from Figure 4 and Table 2, there was a

![RT-PCR of IL-6 mRNA levels in patients receiving 30 µg IFN-con1 daily. The apparent increase in IL-8 mRNA is found only in 1 patient. IL-6 mRNA is barely detectable in less than 40 rounds of PCR amplification. There is a correlation between amounts of IL-6 detectable by ELISA and the presence of mRNA. IL-6 mRNA was not detected in a group of control patients.](image)
correlation between the detection of bands of IL-6 by RT-PCR and the ELISA measurements. Patient 101019 had no detectable IL-6 after 40 rounds of amplification at 0 time but had detectable IL-6 at 6 h of treatment. This band was not detectable at 15 h. No IL-6 was detected by ELISA at 0 time, but 30.97 pg was detected in the sera at 6 h, and only 3 pg was detected at 15 h. The presence of the IL-8 band indicates that the RNA was not degraded. In patient 101114, IL-6 mRNA was detected at 0 time and at other time points. In a series of RT-PCR reactions from healthy controls, IL-6 was not detectable even at 40 rounds of amplification under conditions in which GAPDH was present (at 20 rounds). Most hepatitis C patients had detectable IL-6, as measured by ELISA before the initiation of treatment (Table 1).

**HCV RNA kinetics**

Figure 5 illustrates the kinetics of the HCV RNA decrease and increase during the first 96 h after a single injection of IFN-con1 in group 1 patients who were previously nonresponders to IFN-α. Viral titer decreased by a mean of 1.1 logs (SD = 0.62 logs) during the first 24 h after IFN-con1 administration and then recovered steadily to near baseline by 96 h. The change in viral titer was not significantly associated with pretreatment IL-6 level or the change in IL-6 levels. HCV titers in patients of group 2 showed typical biphasic patterns of inhibition, as previously reported.  

**DISCUSSION**

We found that IL-6 levels increased acutely following IFN-con1 administration. Although previous studies of IFN-α-induced IL-6 production have yielded conflicting results, our data confirm a report that showed an increase in IL-6 levels after 6 h of IFN treatment. IL-6 is a pleiotropic cytokine. It influences B cell maturation, antigen-specific immune responses, and hepatic synthesis of acute-phase proteins. Inhibition of IFN-α-related IL-6 production reduced the severity of side effects, including fever, in one study. Our data provide further evidence that IL-6 contributes to the adverse effects that develop with initiation of IFN therapy. Flu-like symptoms, including fever, developed in concert with rising IL-6 levels while other serum IL levels measured remained at low or undetectable levels.

The relationship observed between pretreatment IL-6 levels and the AUC for IL-6 is particularly interesting; that is, the IL-6 response to IFN was directly related to basal IL-6 level. IL-6 genetic polymorphisms appear to be one determinant of IL-6 expression. Further study is needed to evaluate whether genetic polymorphisms or other mechanisms are associated with individual differences in the IL-6 response to IFN. Such research could improve our understanding of why some patients develop more marked side effects to IFN therapy.

IL-6 production also appears to be associated with hepatitis C infection, as IL-6 was detected in patients with HCV by ELISA and by RT-PCR (Fig. 4) but was not detected in controls. The levels of IL-6 mRNA are very low in patients with HCV. It was not detectable in most patients before 35–40 rounds of amplification, whereas another cytokine, IL-8, was detectable as a strong band after 20 rounds. There is heterogeneity in the IL-6 response among patients, as shown both by direct ELISA measurements and by RT-PCR (Table 2 and Fig. 4). These data also suggest that IL-6 mRNA has a very short half-life.

IL-6 has been reported to be produced by a large number of cell types, including macrophages, T cells, B cells, keratinocytes, and endothelial cells, among others. IL-6 was reported to be induced by IL-1 and TNF-α, although we could not detect either of these cytokines by ELISA in serum samples. In cell culture, IL-6 is induced in fibroblasts by IFN-β, TNF-α, platelet-derived growth factor (PDGF), and certain viral infections. Production of IL-6 can be superinduced by cycloheximide treatment, suggesting that regulation of this gene is under the control of a labile repressor. The IL-6 promoter region contains a multisresponse element (MRE), a nuclear factor-κB (NF-κB) binding site, an NF-IL-6 site, and an activator protein-1 (AP-1) binding site. Determination of whether these sites are activated by IFN-α awaits further research.

The current study is also novel in that it evaluates cytokine responses in patients receiving IFN-con1. This synthetic, recombinant type-1 IFN was shown to produce greater gene induction, natural killer (NK) cell activation, and antiviral activity on an equal mass basis relative to IFN-α2a and IFN-α2b in vitro. IFN-con1 dosing is expressed in micrograms and not in international units, which are used to measure IFN-α2a and IFN-α2b activity. The specific activity of IFN-con1 is approximately 1–2×10⁹ U/mg protein. In a large clinical trial, IFN-con1 dosed at 9 μg t.i.w. for 24 weeks was associated with a sustained response rate and a side effect profile similar to that of 15 μg (3 MU) IFN-α2b. Retreatment of relapsers and nonresponders with 15 μg IFN-con1 led to increased response rates without additional side effects. No clinical data are available about the 30-μg dose of IFN-con1 administered as a single injection in group 1 patients in this study.

We found that baseline serum IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-16 levels were within normal range in all patients with hepatitis C. With the exception of IL-6, these low levels were maintained after IFN-con1 administration. Previous studies documented that some HCV-infected patients had elevated serum cytokine levels, including IFN-γ, IL-4, and IL-6. Potential explanations for the variations in our findings from those reported elsewhere include differences in such factors as the cytokine assays used, HCV genotype, stage of liver disease, heterogeneity in cytokine production, and differences in IFN.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time (h)</th>
<th>pg/ml IL-6</th>
<th>pg/ml IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>101017</td>
<td>0</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>177</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>101019</td>
<td>0</td>
<td>0 (not detectable)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>101114</td>
<td>0</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>69</td>
<td></td>
</tr>
</tbody>
</table>
FIG. 5. Hepatitis C RNA levels in group 1 patients as measured by RT-PCR at various times after injection of a single dose of IFN-con1. (A) 15 μg IFN-con1. (B) 30 μg IFN-con1.
In one study, IL-4 levels decreased from baseline at the completion of a course of IFN-α therapy (dose not specified), although IL-4 levels were not significantly reduced at early time points during treatment.\(^{(12)}\) In the current study, IL-4 levels did not change appreciably during treatment. Our data do agree with those of Corssmit et al.,\(^{(23)}\) in which healthy human volunteers were administered a single 5-MU/m\(^2\) injection of IFN-α2B, and the inflammatory response and cytokine response were measured.

Some reports have identified an acute reduction in serum HCV RNA levels with initiation of IFN-α therapy.\(^{(20,21,31)}\) Similarly, we observed a mean 1.1 log decrease in HCV RNA level at 24 h in previous nonresponder patients after a single dose of IFN-con-1. The lack of a significant relationship between acute changes in serum cytokine and HCV RNA levels suggests that cytokine responses do not play a major role in the development of the first-phase viral decline. Mathematical modeling suggests that the acute reduction in viremia is related to direct inhibition of viral production or release by IFN.\(^{(20,21)}\) In contrast, a slower second-phase viral decline observed with continued therapy has been postulated to reflect immune-mediated killing of infected hepatocytes.\(^{(32)}\)

In conclusion, we find that IL-6 appears to be produced in some patients in response to hepatitis C infection and may reflect ongoing inflammation. IL-6 increases rapidly in serum after a single dose of IFN-con-1, and peak IL-6 levels are associated with the onset of IFN-related flu-like symptoms. IL-6 levels are not correlated with early virologic response and decrease to baseline with continued treatment. IL-6 production after IFN-con-1 administration correlated with baseline IL-6 level, implying that some persons are predisposed to a more vigorous IL-6 response.

**ACKNOWLEDGMENTS**

This work was supported in part by a grant from Amgen Inc., Thousand Oaks, CA, and National Genetics Institute, Los Angeles, CA.

**REFERENCES**


Address reprint requests to:
Dr. Milton W. Taylor
Department of Biology
Indiana University
Bloomington, IN 47405
Tel: (812) 855-3340
Fax: (812) 855-6750
E-mail: taylor@indiana.edu

Received 3 May 2001/Accepted 25 July 2001