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Antiviral Effect of Human Recombinant Interleukin-12 in Patients Infected With Hepatitis C Virus

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The heterogeneity of hepatitis C virus (HCV) is due to the continuous and high replication rate, the low fidelity of the RNA-dependent RNA polymerase, and the immune surveillance of the host. Interleukin-12 (IL-12) plays a central role in mounting an effective cellular immune response directed towards elimination of intracellular pathogens. The effect of IL-12 on hepatitis C viremia and the HCV quasispecies population is unknown. In this study, 12 patients (9 males, 3 females; mean age: 44 ± 11 years), all virological non-responders to previous IFN-α treatment, received recombinant human IL-12 s.c. once weekly for 10 weeks stratified to three dose schedules (0.03 μg/kg, 0.1 μg/kg, and 0.5 μg/kg body weight, respectively). Fourteen IFN-α non-responders and 14 untreated patients served as age- and sex-matched controls. Serum HCV RNA concentrations and HCV quasispecies distribution were measured serially by quantitative reverse transcription - polymerase chain reaction and single strand conformation polymorphism analysis of the hypervariable region of the second envelope gene, respectively. Serum ALT and median HCV RNA levels before treatment (52.7 ± 21.7 U/L; 2.6×10^6 copies/mL) showed no significant changes during IL-12 treatment (57.3 ± 58.8 U/L and 3.2×10^6 copies/mL, 50.3 ± 46.2 U/L and 3.1×10^6 copies/mL, and 46.8 ± 35.3 U/L and 3.9×10^6 copies/mL at weeks 1, 4, and 10, respectively). Similar results were observed in 14 IFN-α non-responders and 14 untreated patients. However, changes in HCV quasispecies occurred in 10/12 (83%) and 9/14 (64%) patients treated with interleukin-12 and interferon-α, respectively, but only in 3/14 (21%) untreated subjects ($P < 0.003$ and $P < 0.03$). These results imply that interleukin-12 exerts only limited antiviral activity against certain HCV quasispecies in vivo. ***J. Med. Virol. 60:264–268, 2000.***

KEY WORDS: hepatitis C virus; interleukin-12; hypervariable region; quasispecies; single strand conformation polymorphism

INTRODUCTION

A heterogeneous population of viruses, so-called quasispecies exists in vivo in patients infected chronically with hepatitis C virus (HCV) [Martell et al., 1992]. The heterogeneity of HCV is due to the continuous and high replication rate, the low fidelity of the RNA-dependent RNA polymerase, and the immune surveillance of the host. The hypervariable regions (HVR) within the second envelope gene (HCV-E2) show particularly high intratypic variability and are considered to be target of neutralizing antibodies. Sequence variations of HVR-1 during the natural course of chronic HCV infection were described previously [Kumar et al., 1993; Kurosaki et al., 1994]. Limited data suggest that changes of the quasispecies population are accelerated during recombinant interferon-α (rIFN-α) therapy which may influence the responsiveness to treatment [Sakuma et al., 1996].

Interleukin-12 (IL-12) is a heterodimeric cytokine which is produced by antigen-presenting cells in response to diverse stimuli, and promotes cell-mediated immunity by facilitating type 1 helper T (T_H1) lymphocyte responses, including the secretion of interferon-γ from both T and natural killer cells, enhancing the lytic activity of natural killer cells, and augmenting specific cytolytic T lymphocyte responses [Trinchieri, 1995]. In

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TABLE I. Clinical, Biochemical, Serological, Molecular, and Histological Characteristics of Chronically HCV Infected Patients

Characteristics	IL-12-treated patients (n = 12)	IFN-α-treated patients (n = 14)	Untreated controls (n = 14)
Age (yr)[a]	44 ± 11	50 ± 10	44 ± 9
Sex (M/F)	9/3	9/5	9/5
Pretreatment aminotransferases:[a,b]			
ALT (U/L)	52.7 ± 21.7	72.3 ± 69.4	72.9 ± 69.4
AST (U/L)	29.3 ± 16.0	50.8 ± 64.1	51.4 ± 64.0
Serology:			
anti-HCV	12	14	14
HBsAg	0	0	0
anti-HBc	7	5	4
anti-HIV	0	0	0
HCV genotype:			
1	8	12	14
2	2	1	0
3	1	1	0
4	1	0	0
Pretreatment HCV RNA[c] (copies/mL)	2.6×10^6 [$3.2 \times 10^5 - 5.4 \times 10^7$]	3.3×10^6 [$5.0 \times 10^5 - 1.0 \times 10^8$]	5.0×10^6 [$1.5 \times 10^5 - 5.0 \times 10^7$]
Liver histology:			
minimal activity	0	1	2
moderate activity	3	4	6
severe activity	5	5	4
active cirrhosis	4	4	2

[a]Mean ± SD.
[b]Normal reference ranges: 4–22 U/L for ALT and 6–18 U/L for AST.
[c]Median [range].

addition, IL-12 can increase the production of some subclasses of IgG antibodies [Germann et al., 1995]. IL-12 has been shown to have potent therapeutic effects in a number of infectious diseases, including several viral infections [Gately et al., 1998].

In the present study, the total viral load and changes of the HCV quasispecies population were investigated in untreated patients with chronic hepatitis C, patients treated with interferon-α and, for the first time, in patients treated with recombinant human IL-12 (rHuIL-12). The data provide convincing evidence that sequence variations of the HVR-1 are more pronounced in patients treated with rHuIL-12 or rIFN-α than during the natural course in untreated patients.

PATIENTS AND METHODS

Patients

Forty patients with chronic hepatitis C were enrolled in the present study. The diagnosis of chronic hepatitis C was based on biochemical, serological, virological, and histological findings. All patients had elevated aminotransferases, were positive for anti-HCV antibody (3rd generation enzyme-linked immunosorbent assay), HCV RNA by reverse transcription-polymerase chain reaction (RT-PCR), and negative for hepatitis B surface antigen and anti-HIV type 1 and 2 antibodies. Liver histology showed minimal to moderate inflammatory activity, severe inflammatory activity, or active cirrhosis in 16, 14, and 10 patients, respectively (Table I). Twelve patients (9 males, 3 females; mean age 44 ± 11 years), all non-responders to previous interferon-α therapy, were treated with rHuIL-12 once weekly. Patients were assigned to different cohorts, receiving 0.03 μg/kg (n = 4), 0.1 μg/kg (n = 6), or 0.5 μg/kg rHuIL-12 (n = 2) for 10 consecutive weeks [Zeuzem et al., 1999]. Fourteen virological non-responders (9 males, 5 females; mean age 50 ± 10 years) treated with 3 MU rIFN-α thrice weekly for 12 months and 14 untreated patients with chronic hepatitis C (9 males, 5 females; mean age 44 ± 9 years) served as controls. Blood samples in patients treated with rHuIL-12 were obtained twice within four weeks before initiation of treatment and during therapy at week 1, 2, 3, 4, and 10. In patients treated with rIFN-α and in untreated controls serum samples were drawn less frequently at weeks –4, 0, 4, and 10. This study was approved by the local Ethics Committee and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient.

Measurement of HCV RNA and HCV Genotyping

Blood samples for HCV RNA quantitation were centrifuged within 2 hours and immediately frozen at –80°C to achieve optimal conditions. HCV RNA was quantified by bDNA assay (Quantiplex HCV RNA 2.0, Chiron Diagnostics, Emeryville, CA) according to the manufacturer's instructions. All serum samples of a respective patient were thawed and assayed in parallel to control for interassay variability. Genotyping of HCV was undertaken by reverse hybridization assay (INNO LiPA HCV II, Innogenetics, Gent, Belgium) [Lee et al., 1997a].

RT-PCR of the Hypervariable Region (HVR)-1

The amplification of HVR-1 within the HCV envelope gene was carried out as described previously [Lee et al., 1997b]. Briefly, after extraction of HCV RNA from serum, cDNA synthesis was carried out using random hexamer oligonucleotides (Boehringer Mannheim, Mannheim, Germany). HCV-cDNA was amplified using an optimized genotype-independent "nested" primer set specific for the HCV-E1/E2 region, which generated a PCR product of 307 base pairs (bp) (position +956 to +1262). The PCR product was analyzed on a 3% agarose gel stained with ethidium bromide. Throughout PCR application, previously described measures to avoid contamination were strictly followed [Kwok and Higuchi, 1989].

Single Strand Conformation Polymorphism (SSCP) Analysis

SSCP analysis was carried out by the Phast™ system (Pharmacia, Freiburg, Germany) as recently described [Lee et al., 1997b]. Briefly, two microliters of the HVR-1 PCR product were mixed with two microliters of 94% formamide, denatured at 90°C for 2 min, and chilled immediately on ice. The denatured PCR product was applied on a 12.5% non-denaturing polyacrylamide minigel (Pharmacia) and the electrophoresis was performed at 15°C for 2 hours (150 V for 300 Vh). The DNA bands were subsequently visualized by silver staining and photographed for documentation. Reproducibility of the band pattern was confirmed for each time point by repeating PCR-SSCP. Gels were interpreted independently by two experienced investigators in a "blinded" manner and assigned either to changed or constant band pattern.

Statistical Analysis

Data are presented as mean ± standard deviation (SD). Dichotomous variables were compared by the χ^2 test. Differences in the serum aminotransferase and HCV RNA levels over time were analyzed by the Wilcoxon signed-rank test. P values of $< .05$ were considered significant.

RESULTS

Viral load and changes of the HCV quasispecies population were investigated in patients treated with rHuIL-12 (n = 12) or rIFN-α (n = 14) as well as in untreated patients with chronic hepatitis C (n = 14). The groups were matched for sex, age, serum virus titer and liver histology. Pretreatment viremia was 2.6 × 10^6 copies/mL in patients treated with rHuIL-12 (range 0.3 – 54 × 10^6 copies/mL), 3.3 × 10^6 copies/mL in patients treated with rIFN-α (range 0.5 – 100 × 10^6 copies/mL), and 5.0 × 10^6 copies/mL in untreated patients before initiation of the study (range 0.2 – 50 × 10^6 copies/mL). HCV genotype 1 was identified in 8 of 12 patients treated with rHuIL-12, 12 of 14 patients treated with rIFN-α and in all 14 untreated patients with chronic hepatitis C. Further clinical, biochemical, serological and histological characteristics of the patients are summarized in Table I.

In patients with chronic hepatitis C, who were all virological non-responders to previous interferon-α therapy, median HCV RNA levels remained unchanged during treatment with 0.03 to 0.5 μg/kg rHuIL-12 once weekly. In neither patient a decline of more than one $\log_{10}$ was observed. Serum ALT levels slightly declined from 68 ± 26 U/L to 64 ± 9 U/L (0.03 μg/kg rHuIL-12), from 51 ± 13 U/L to 45 ± 35 U/L (0.1 μg/kg rHuIL-12), and from 37 ± 3 U/L before treatment to 18 ± 13 U/L at week 10 (0.5 μg/kg rHuIL-12), respectively. In virological non-responders treated with interferon-α as well as in untreated controls, hepatitis C viremia revealed only minor fluctuations of less than one $\log_{10}$. In patients treated with rIFN-α ALT levels slightly declined from 72 ± 69 U/L before to 63 ± 40 U/L 10 weeks after initiation of treatment. Similarly, serum ALT levels of untreated patients were lower at the end compared with the beginning of the study period (55 ± 33 vs. 73 ± 33 U/L).

Alterations of quasispecies populations were assessed by an optimized PCR-SSCP analysis of the hypervariable region (HVR)-1 within the second envelope gene (E2). In untreated patients, who never had received any antiviral therapy, SSCP analysis revealed a change of the band pattern in 3 of 14 patients (21%) within 10 weeks (Fig. 1A). The change of the SSCP band pattern in these three patients occurred already within the initial 4 weeks of the study period. In accordance with these data in untreated patients, we observed also alterations of the quasispecies population in two patients within 4 weeks before initiation of treatment with rIFN-α or rHuIL-12. SSCP analysis of HVR-1 revealed an evolved band pattern 10 weeks after initiation of rIFN-α treatment in 9 of 14 patients with chronic hepatitis C (64%; $P < 0.03$ compared with untreated controls). Representative SSCP analyses are shown in Figure 1B.

During 10 weeks of rHuIL-12 treatment, changes of the SSCP band pattern occurred in 10 of 12 patients (83%). This rate was significantly higher compared with untreated controls ($P < 0.003$), but did not achieve statistical significance compared with patients treated with rIFN-α. Representative SSCP analyses of patients before initiation and after 10 weeks of rHuIL-12 therapy are shown in Figure 1C. An evolved SSCP band pattern was already observed within 4 weeks after initiation of therapy in 7 of 12 patients. In two patients the SSCP band pattern changed already before initiation of treatment and remained constant thereafter in one patient and revealed additional changes within the initial 4 weeks of treatment in the other patient. In two patients a different SSCP band pattern evolved between week 4 and 10 of therapy. In patients with change of SSCP band pattern, the median viral load at baseline (5.0 × 10^6 copies/mL in the

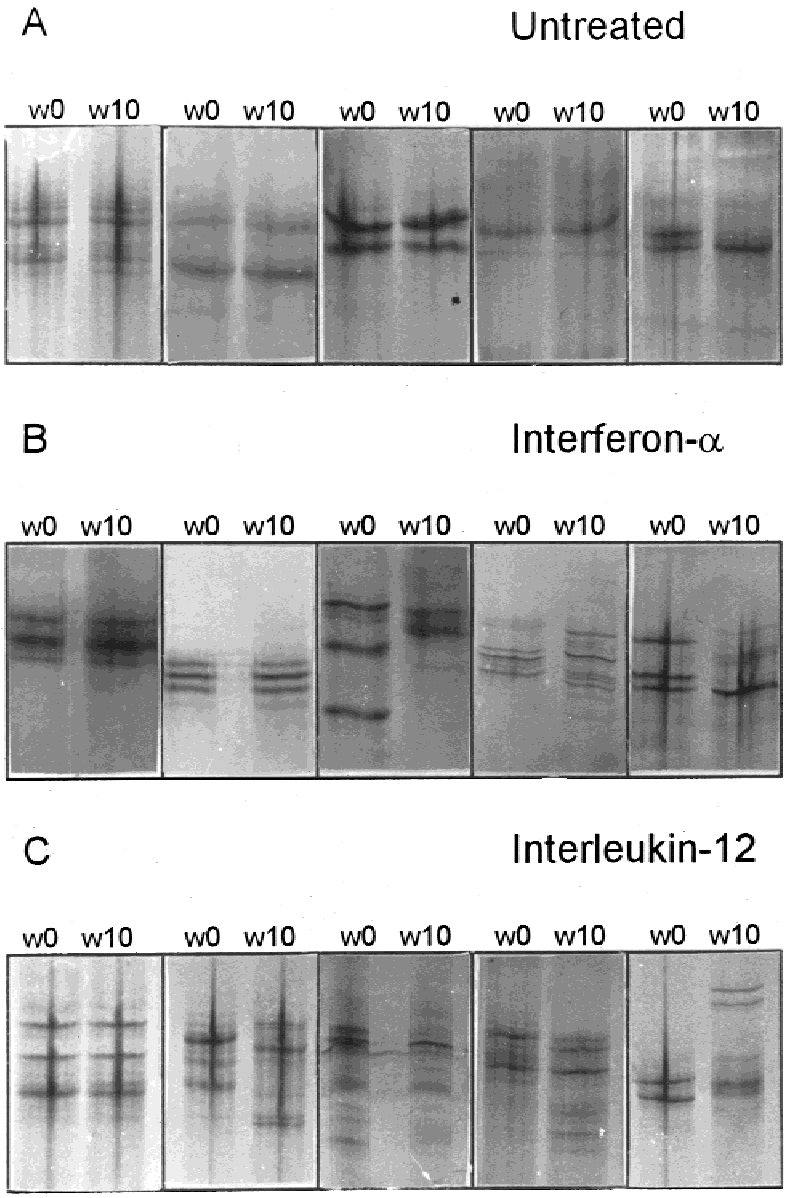

Fig. 1. Single strand conformation polymorphism analysis of the hypervariable region-1 was applied to observe changes in the HCV quasispecies population in untreated patients with chronic hepatitis C **(A)**, or patients treated with recombinant interferon-α **(B)**, or interleukin-12 **(C)**. Five representative analyses at baseline (w0) and week 10 (w10) are shown from each cohort.

untreated group (3/14), 5.0×10^6 copies/mL in the interferon-α-treated group (9/14), and 3.9×10^6 copies/mL in the IL-12-treated group (10/12)) was not significantly different to the median viral load after 10 weeks (2.5×10^6 copies/mL, 5.0×10^6 copies/mL and 3.4×10^6 copies/mL, respectively).

DISCUSSION

Interleukin-12 (IL-12) is synthesized by antigen-presenting cells including monocytes, macrophages, and dendritic cells. Studies in animal systems indicate that this cytokine exerts a number of regulatory effects on T lymphocytes and natural killer (NK)-cells [Gately et al., 1998]. The effects on viral load and changes of the HCV quasispecies population were investigated in 12 patients during rHuIL-12 therapy using quantitative RT-PCR and an optimized PCR-SSCP analysis of the hypervariable region (HVR)-1 within the second envelope gene (E2), respectively. As controls, viremia, and evolution of quasispecies population were analyzed in 14 patients during rIFN-α therapy and in 14 untreated patients with chronic hepatitis C. Because of the high genetic heterogeneity within HVR-1, analysis of this region should sufficiently reflect the genetic complexity and diversity of HCV in an individual patient [Kurosaki et al., 1994].

Serial measurements of serum transaminases and HCV RNA concentration during rHuIL-12 treatment revealed no significant effects. A possible reason for this poor antiviral response rate to rHuIL-12 therapy in patients with chronic hepatitis C might be that these patients failed to respond to previous rIFN-α treatment [Zeuzem et al., 1999]. In contrast to the total viral load, profound effects of rHuIL-12 on the HCV quasispecies

population were observed in 10 of 12 patients (83%). Changes in quasispecies were also observed in 9 of 14 rIFN-α nonresponders (64%) indicating selection of HCV quasispecies during rIFN-α and probably more pronounced during rHuIL-12 treatment. Because clones may have overlapping migration pattern despite different nucleotide sequences, the number of SSCP bands does not necessarily reflect the number of major variants [Lee et al., 1997b]. We therefore did not quantify the number of SSCP bands but directly compared SSCP migration patterns. The reliability of the SSCP analysis was confirmed by multiple runs of the amplification reaction and SSCP gel electrophoresis of the same serum specimen.

IL-12 leads to a T_H-shift of CD4+ cells toward T_H1. T_H1 cells release interferon-γ, which is an essential component for antiviral and anti-proliferative effects of IL-12 [Gately et al., 1998]. Thus, as proposed for interferon-α [Sakuma et al., 1996; Yun et al., 1996], elimination of interferon-sensitive and selection of interferon-resistant quasispecies may occur during rHuIL-12 treatment. However, pharmacodynamic assessments revealed detectable IFN-γ serum levels only in patients treated with 0.5 μg/kg rHuIL-12 [Zeuzem et al., 1999], while in the present study quasispecies alterations were also observed in 8 of 10 patients treated with lower rHuIL-12 doses.

Additional biological IL-12 activities include stimulation of non-specific lytic activity of natural killer cells and activation of specific cytolytic T-cell responses [Gately et al., 1998]. The quasispecies distribution within the liver is heterogeneous, suggesting possible differences in the HCV replication rate and antigen presentation in various hepatocytes [Cabot et al., 1997]. In addition, CTL escape mechanisms due to viral sequence variations were proposed in previous studies [Koziel et al., 1993; Rehermann et al., 1996]. By promoting the cellular immune response, treatment with rHuIL-12 could lead to a selective elimination of infected hepatocytes and a subsequent shift of the serum quasispecies population.

IL-12 is also able to stimulate the production of some subclasses of IgG antibodies [Germann et al., 1995]. The mutation rate of the hepatitis C virus genome is significantly reduced in chronically infected patients with hypogammaglobulinemia compared to patients with an intact humoral immune system [Booth et al., 1998]. The hypervariable regions of HCV are known targets for neutralizing antibodies [Weiner et al., 1992; Farci et al., 1994]. Thus, stimulated antibody production during treatment with rHuIL-12 could lead to an accelerated evolution of the HCV HVR-1 quasispecies population.

Taken together, despite a lack of antiviral efficacy on the entire HCV population, the effect of rHuIL-12 on quasispecies fluctuation was significant in the present study. The underlying mechanism for this phenomenon, however, remains unknown. It is of considerable interest whether IL-12 has different and/or additional antiviral effects compared with interferon-α. Assuming that resistant variants to interferon-α might be sensitive to IL-12 and vice versa, a combination of rIFN-α and rHuIL-12 could possibly act synergistically against a broader spectrum of HCV variants.

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