total RNA using random hexamers and M-MRV (Invitrogen, Carlsbad, CA).

2.3. Realtime RT-PCR

Quantifications of mRNA for IFN-γ, IL-4, and IL-12R β1 and β2 chains were performed on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA) using predeveloped Taqman reagents including primers and probes for IFN-γ, IL-4 and GAPDH according to the manufacturer's instructions [11]. Primers and probes for the amplification and quantification of the IL-12R β1 chain and the β2 chain mRNA were deduced from the reported nucleotide sequence [12]: β1 chain: forward (nt.1049–1069) AACCAGACGTGGCACATTCCT, reverse (nt.1138–1159) CCAATACATGGTGGTCCCGTT, probe (nt.1087–1116) ACCAGTGGCTCTGAATATCAGCGTCGGAAC; β2 chain: forward (nt.3113–3136) CAGCACATCTCCCTTTC-TGTTTTC, reverse (nt.3254–3276) ACTTTAAGGCTTG-AAGCCTCACC, probe (nt.3140–3169) TCAAGTTCTC-TTCACCCACTCACCTTCTCC. Primers and probes for IFN-γ and IL-4 mRNA were as follows: IFN-γ: forward (nt.463–486) CGAGATGACTTCGAAAAGCTGACT, reverse (nt.570–592) TTTTGTCCCTTCGCTTTTTTC, probe (nt.541–566) CAAGTGATGGCTGAACTGTCGCCAGC; IL-4: forward (nt.66–85) GGGTCTCACCTCCCAACTGC, reverse (nt.285–305) ACACGAGGCCGTCAAGATGTC, probe (nt.114–135) TGCCGGCAACTTTGTCCACGGA. Amplification of GAPDH was performed for each experimental sample as an endogenous control. Threshold cycle C1, which is inversely correlated with the target mRNA levels, was determined as the cycle number at which the reporter fluorescent emission increased above the threshold level. The amount of target mRNA was compared with that of standard DNA, which was obtained by the cloning of PCR products into plasmid vectors, and expressed as log copies/μg total RNA. Variations between experiments were verified to be less than 10% by four repeated measurements on the same cDNA preparation from a healthy subject. Lower detection level of each cytokine mRNA by this assay was 100 copies/μg total RNA.

Changes of mRNAs between weeks 0, 4 and 24 were calculated as follows: changes between weeks 0 and 4 (Δ0–4) = ((mRNA level at week 4 − mRNA level at week 0)/mRNA level at week 0) × 100, and changes between weeks 0 and 24 (Δ0–24) = ((mRNA level at week 24 − mRNA level at week 0)/mRNA level at week 0) × 100. Changes of levels of these mRNA were compared between the two groups of patients to evaluate the possibility of clinical usability in estimating the viral relapse after the therapy.

2.4. HCV RNA

Quantification of HCV RNA in serum samples was performed using a commercially available kit: Amplicor HCV monitorTM test (Roche Diagnostic Systems, Branchburg, NJ). The lowest level of detection of this test is 200 HCV RNA copies/ml of sample.

2.5. Statistical analysis

Non-parametrical analysis of sequential changes in cytokine mRNA levels was performed using Friedman's test. For the analysis of percent increase in cytokine mRNA level as a factor of viral responses, the Mann–Whitney *U*-test was employed. For comparison of mRNA level at each time point among patients with different HCV genotypes, oneway ANOVA or Dunnett's *t*-test was used. *P*-values of <0.05 were considered to be statistically significant.

3. Results

3.1. Virological responses

All 13 patients completed the 24-week ribavirin/IFN-α therapy. At the end of the treatment, serum HCV RNA was detected in none of the 13 patients. At the end of the 24-week follow-up after the treatment, serum HCV RNA became positive in six patients (relapse, REL) and remained negative in seven patients (sustained viral response, SVR). Two baseline factors were found to be significantly different between patients with SVR and those with REL, namely HCV genotype (1b versus others, $p = 0.029$) and age (>40 years old versus ≤40 years old, $p = 0.046$, Table 1).

3.2. Cytokine mRNA level in CD4+ cells

Expression levels of IL-12R β1 and β2 chains, IFN-γ and IL-4 at pretreatment in 13 patients with chronic hepatitis C were compared with those of 15 normal subjects. The comparison showed no statistical difference in mRNAs for IL-12R β1 and β2 chains between patients and healthy subjects (Table 2). Expression of IFN-γ mRNA was significantly higher in patients with chronic hepatitis C ($p < 0.001$). Baseline cytokine mRNA level in CD4+ cells in patients with chronic hepatitis C was not correlated with clinical findings such as HCV virus load, HCV genotypes, serum levels of ALT, liver fibrosis grade or liver histological activity (Table 2).

Sequential measurement of changes in mRNA level in CD4+ cells during the treatment showed no substantial changes in the level of mRNA for IL-12R β1 chain, IL-12R β2 chain, IFN-γ and IL-4 when the average of 13 patients was compared between three sampling points (Table 3). Expression level of each mRNA was not significantly different between SVR and REL (Table 3 and Fig. 1). IL-4 mRNA was detectable in seven patients at week 0, 10 patients at week 4 and 11 patients at week 24, although expression of IL-4 mRNA was at low levels near detection limits in most cases. Due to the possibility of unstable results near detection

Table 2
Baseline level of cytokine mRNA and clinical factors

Characteristics	N	IL-12R β1 chain[a]	IL-12R β2 chain[a]	IFN-γ	IL-4[a,b]
Patient group					
Hepatitis C	13	5.23 (0.12)	5.01 (0.11)	5.31 (0.11)	2.08 (0.06)
Normal subjects	15	3.01 (1.26)	3.02 (1.05)	2.02 (0.76)	ND
Age in years					
>40	4	4.95 (0.32)	5.00 (0.25)	5.03 (0.25)	<2.00
≤40	9	5.36 (0.10)	5.02 (0.12)	5.44 (0.10)	2.12 (0.09)
ALT (IU/l)					
>100	7	5.19 (0.13)	5.01 (0.18)	5.36 (0.13)	<2.00
≤100	6	5.71 (0.09)	4.98 (0.25)	5.42 (0.11)	2.10 (0.10)
HCV genotype					
Genotype 1	6	5.27 (0.14)	4.97(0.15)	5.40 (0.15)	2.18 (0.13)
Genotype 2 + 3	7	5.20 (0.21)	5.06 (0.16)	5.24 (0.19)	<2.00
HCV RNA (kcopies/ml)					
>400	6	5.04 (0.12)	4.85 (0.28)	5.30 (0.13)	<2.00
≤400	7	5.34 (0021)	2.10 (0.12)	5.30 (0.19)	2.04 (0.04)
Fibrosis score					
0, 1, 2	7	5.55 (0.17)	4.81 (0.01)	5.31 (0.06)	2.15 (0.15)
3, 4	6	5.34 (0.16)	5.04 (0.20)	5.45 (0.12)	<2.00
Activity score					
1	5	5.24 (0.20)	4.97 (0.26)	5.47 (0.17)	2.06 (0.06)
2	8	5.58 (0.10)	50.0 (0.18)	5.37 (0.09)	<2.00

Baseline level of each cytokine was compared in association with various clinical factors by Student's *t* test; ND: not detected.
[a] log copies/μg RNA. Data indicate mean and standard error in parentheses.
[b] Comparison was made using seven cases with detectable IL-4 mRNA.

Table 3
Level of mRNA for cytokines at three sampling points during ribavirin and IFN-α therapy

mRNA[a]	Weeks in therapy		
	0	4	24
IL-12R β1 chain			
Total	5.23 (0.12)	5.30 (0.16)	5.02 (0.10)
SVR	5.17 (0.20)	5.19 (0.12)	4.96 (0.16)
REL	5.29 (0.15)	5.43 (0.32)	5.08 (0.11)
IL-12R β2 chain			
Total	5.01 (0.11)	5.38 (0.18)	5.36 (0.19)
SVR	4.93 (0.15)	5.27 (0.27)	5.66 (0.29)
REL	5.11 (0.16)	5.51 (0.22)	5.00 (0.215)
IFN-γ			
Total	5.31 (0.11)	5.21 (0.12)	5.26 (0.10)
SVR	5.14 (0.15)	5.03 (0.13)	5.28 (0.16)
REL	5.52 (0.14)	5.42 (0.20)	5.21 (0.10)
IL-4[b]			
Total	2.08 (0.06)	2.06 (0.05)	2.15 (0.05)
SVR	2.00 (0.00)	2.09 (0.09)	2.16 (0.08)
REL	2.18 (0.13)	2.03 (0.03)	2.13 (0.08)

[a] log copies/μg RNA. Data indicate mean, and standard error in parentheses.
[b] Data for IL-4 mRNA level was positive in 7 (2 SVR and 5 REL) of 13 cases at week 0, 10 (5 SVR and 5 REL) at week 4 and 11 (5 SVR and 6 REL) at week 24.

limits of realtime PCR, statistical analysis was not performed on the expression of IL-4 mRNA.

Comparison was made to find the difference of changes in mRNA using the percent change of mRNA level. Percent increase in level of mRNA for IL-12R β2 chain from baseline to the end of the treatment was significantly higher in patients with SVR (15.3 ± 6.1%) than in those with REL (1.6 ± 4.7%, $p < 0.05$, Table 4). Percent changes in the level of mRNA for IFN-γ from the baseline to the end of the treatment was 3.1 ± 3.3% in patients with SVR and −5.3 ± 2.7% in REL, but the difference was not statistically significant. There was no significant difference in percent changes in the level of mRNA for the IL-12R β1 chain between the two groups (−3.8 ± 2.1% for SVR versus −3.5 ± 4.0% for REL, data not shown). Since mRNA for IL-4 was detected in only one subject at week 4, in four of the seven subjects with SVR at the end of the treatment, in one subject at week 4, and four of the six subjects with REL at the end of the treatment (data not shown), percent increase was not calculated for IL-4 mRNA.

3.3. Relevance of changes in cytokine mRNA level and relapse of HCV

The sensitivity and specificity to estimate HCV relapse after the therapy were calculated by using kinetics of mRNA levels of IL-12R β2 chain mRNA and IFN-γ: 71% and 67% for Δ0–24 of IL-12R β2 chain mRNA, respectively, and 57% and 67% for Δ0–24 of IFN-γ mRNA, respectively. When fig-

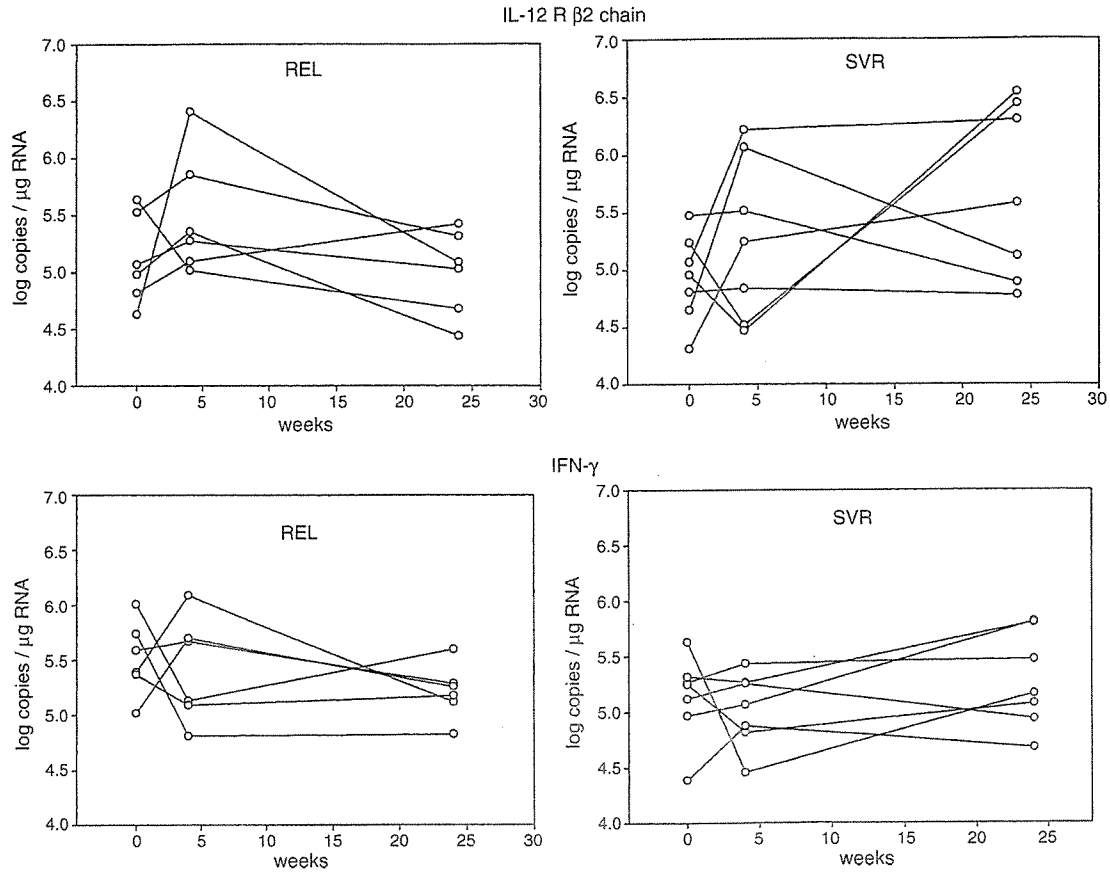

Fig. 1. Changes in mRNA expression of IL-12R β2 chain and IFN-γ during ribavirin and IFN-α combination therapy. Serial changes in mRNA levels of IL-12R β2 chain (upper panel) and IFN-γ (lower panel) were compared between patients with SVR and those with REL. Each point indicates an individual case. Blood samples were obtained before (week 0), at week 4 and at the end (week 24) of the ribavirin and IFN-α combination therapy.

Table 4
Level of mRNA for cytokines during ribavirin and IFN-α therapy: comparison by three time points between SVR and REL

	Weeks in therapy					p
	0	4	Δ0–4[a]	24	Δ0–24[a]	
IL-12R β2 chain[b]						
SVR	4.93 (0.15)	5.27 (0.27)	7.54 (6.57)	5.66 (0.29)	15.31 (6.12)	< 0.05
REL	5.11 (0.16)	5.51 (0.22)	8.50 (6.61)	5.00 (0.15)	−1.64 (4.71)	
IFN-γ[b]						
SVR	5.14 (0.15)	5.03 (0.13)	−1.54 (3.87)	5.28 (0.16)	3.14 (3.30)	
REL	5.52 (0.14)	5.42 (0.20)	−1.35 (5.31)	5.21 (0.10)	−5.30 (2.70)	

Abbreviations used: SVR, sustained viral response; REL: relapse of HCV.
[a] Percent changes of mRNA level at weeks 4 and 24 from week 0.
[b] log copies/ma RNA. Data indicate mean and standard error in parentheses.

ure for both mRNA were combined, sensitivity and specificity became 57% and 100%, respectively.

Changes in clinical parameters were compared between patients with SVR and those with REL at three sampling points (Table 5). Levels of leukocytes and lymphocytes were not different between the two groups.

4. Discussion

In this study, we evaluated the Th1 response ex vivo during treatment with ribavirin and IFN-α and found an increase of Th1 response at the end of the treatment in patients with sustained loss of HCV.

Table 5
Changes in the level of biochemical and hematological parameters during ribavirin and IFN-α therapy: Comparison by 3 time points between SVR and REL

	Weeks in therapy		
	0	4	24
Leukocytes[a] (μl^{-1})			
SVR	3800.0 ± 333.1	3257.1 ± 416.8	3185.7 ± 173.8
REL	4700.0 ± 349.3	4180.0 ± 661.4	3080.0 ± 483.1
Lymphocytes[a] (%)			
SVR	14.7 ± 6.3	16.7 ± 7.5	15.5 ± 6.8
REL	22.4 ± 6.0	19.7 ± 7.2	21.0 ± 6.2
ALT[a] (IU/l)			
SVR	164.7 ± 75.3	31.3 ± 6.1	17.6 ± 5.1
REL	146.6 ± 34.5	64.8 ± 29.8	48.6 ± 15.9
HCV RNA positive[b] (%)			
SVR	100	0	0
REL	100	33.3	0

[a] Data, except for serum HCV RNA, indicate mean and standard error of 7 SVR patients and 6 REL patients.
[b] Percentage of patients with serum HCV RNA positive at indicated time points.

Pretreatment levels of IL-12R β2 chain and IFN-γ were higher in patients with chronic hepatitis C than those in healthy subjects, indicating the possibility that a Th1 response was evoked in the liver with chronic HCV infection. Differentiation of uncommitted CD4+ T cells is affected by various factors including signals from costimulatory molecules on antigen presenting cells, as well as the nature and the concentration of antigen and the cytokines present under local circumstances [13,14]. Although there were no significant changes in the IL-12R β1 chain mRNA in our study, nitric oxide and IFN-α upregulates the IL-12R β2 chain independently of the IL-12R β1 chain [15,16]. Our previous studies in vitro in human T cells have shown that both ribavirin and IFN-α have the potential to induce upregulation of IL-12R β2 chain mRNA [10,11], which plays a key role in the differentiation of CD4+ T cells towards Th1 [17,18]. During the therapy, although a decrease in inflammation activity may attenuate stimulation of CD4+ T cells, administered ribavirin and IFN-α induces upregulation of IL-12R β2 chain mRNA.

The results of this study also suggest that combination therapy of ribavirin and IFN-α may activate CD4+ cells and lead to IFN-γ activity in some patients. Since IFN-γ has been reported to suppress HBV and HCV replication [19–21], production of IFN-γ by CD4+ T cells during ribavirin and IFN-α therapy may contribute to sustained virus loss and the induction of cytotoxic T lymphocytes [22].

In a long-term study, Shinohara et al. have demonstrated that baseline Th1 and Th2 cells may be related to the HCV response to IFN therapy [23]. Our results suggest for the possibility for using the kinetics of IL-12R β2 and IFN-γ mRNA levels to predict patients with relapse of HCV after the therapy in addition to viral analysis [24].

We were unable to analyze the kinetics of cytokine mRNA in patients with null viral response. Detailed study on such patients would be beneficial for the future development of an improved regimen of anti-viral therapy in patients with chronic hepatitis C, since the non-virological response has been reported to be as high as 40% in patients with genotype 1b and high viral load [25].

In conclusion, the results of this study suggest that the increase of Th1 response may be induced by combination therapy with ribavirin and IFN-α, and such a therapy may contribute to the eradication of HCV in these patients. The mechanisms which cause differences in the response of these mRNAs in CD4+ T cells between patients with SVR and those with REL are currently unclear. However, these results may suggest the possibility of using the Th1 response to distinguish patients with relapse of HCV after the therapy from those who achieve sustained virus response.

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