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Direct evidence for immunomodulatory properties of ribavirin on T-cell reactivity to hepatitis C virus

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Abstract

Background/Aim: An understanding of ribavirin's beneficial effects on treatment outcome in chronic hepatitis C (CH-C) may help to develop new treatment approaches. Here we investigated whether ribavirin directly affects HCV-specific reactivity of CD4 + T-lymphocytes from patients with CH-C.

Methods: Peripheral blood mononuclear cells from forty HCV RNA positive patients were cultured ex vivo with HCV core, NS3, NS4 alone, and with different concentrations of ribavirin. Virus-specific CD4+ T-cell reactivity was analysed by a proliferation assay; quantitation of cytokine (interferon-gamma, IL-10, IL-5, IL-12p35, IL-12p40) mRNA levels; measurement of interferon-gamma and IL-10 production (by ELISA) and enumeration of interferon-gamma and IL-10 producing T-cells by Elispot assays.

Results: At 2–5 μM ribavirin induced de novo or enhanced T-cell proliferation to HCV antigens in a proportion of patients. Increased T-cell proliferation was associated with decreased IL-10 production in response to HCV core and reduced frequency of IL-10 producing CD4+ T-cells, while interferon-gamma levels remained unchanged. At 20 μM ribavirin markedly suppressed T-cell proliferation, and interferon-gamma mRNA expression to HCV antigens.

Conclusions: Ribavirin, at clinically achievable plasma levels, modulates directly the T-cell responses to HCV antigens in some CH-C patients. Suppression of IL-10 production may represent a useful strategy to induce/augment T-cell reactivity to HCV.

Keywords: Ribavirin; Chronic hepatitis C; IL-10; IFN-γ

1. Introduction

The use of ribavirin in combination with interferon-alpha has markedly improved the rate of sustained virological response in patients with chronic hepatitis C (CH-C) (Poynard et al., 1998; McHutchison et al., 1998). The success of this combination regimen, in comparison to interferon-alpha monotherapy, has stimulated the research efforts to better understand the mechanisms of action of ribavirin, which will help to develop new therapeutic strategies (J.Y. Lau et al., 2002).

Placebo-controlled trials have demonstrated that, when given alone, ribavirin does not affect significantly serum HCV RNA levels (Di Bisceglie et al., 1995; Dusheiko et al., 1996). The lack of a direct antiviral effect of ribavirin monotherapy is also supported by recent studies, using improved molecular assays, which showed no effect on HCV viraemia, nor on HCV quasispecies (Lee et al., 1998; Querenghi et al., 2001).

In vitro studies, using mitogen-stimulated peripheral blood mononuclear cells (PBMC) or in animal models, have suggested that ribavirin has immunomodulatory properties and promotes T helper 1 (Th1) reactivity (Tam et al., 1999; Ning et al., 1998; Hultgren et al., 1998; Fang et al., 2000). However, the direct effect of ribavirin on HCV-specific T-cell reactivity from patients with CH-C has not been analyzed. Importantly, the immunomodulatory properties of ribavirin appear to vary at different concentrations. At low concentrations (0.1–1.0 μg/ml; 1 μg/ml equivalent to 4 μM), ribavirin was shown to enhance B-lymphocyte reactivity (Powers et al., 1982), potentiate Th1 and suppress Th2-pattern of immune responses (Tam et al., 1999). In contrast, at higher concentrations (above 5 μg/ml or 20 μM) ribavirin inhibits lymphocyte proliferation and has an immunosuppressive effect (Tam et al., 1999; Powers et al., 1982; Peavy et al., 1980). The aim of this study was to determine directly the effects that ribavirin exerts on HCV-specific reactivity of T-cells, obtained from patients with CH-C with various stages

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of liver disease. For this purpose, PBMC were cultured ex vivo with HCV antigens alone and in the presence of ribavirin at concentrations encompassing the clinically achievable plasma levels 1.25–2.5 μg/ml (5–10 μM)(Lertora et al., 1991). T-cell reactivity was then analyzed by a proliferation assay, cytokine gene expression, cytokine production and enumeration of HCV-specific T-cells.

2. Material and methods

2.1. Material

PBMC were isolated from 40 patients with chronic hepatitis C, monitored at University College London Hospitals. The study was approved by the UCLH Ethics Committee. All patients were seropositive for anti-HCV (Abbott Diagnostics, Maidenhead, UK) and HCV-RNA (Amplicor; Roche, Basel, Switzerland) and have not received antiviral treatment. All patients were seronegative for HBsAg and anti-HIV (Abbott). Liver biopsy was performed in 33 of 40 patients and the liver fibrosis score (Ishak et al., 1995), identified 10 patients with mild hepatitis (stages 0–1), 13 with moderate hepatitis (stages 2–4) and 10 with advanced fibrosis or cirrhosis (stages 5–6).

2.2. Methods

2.2.1. T-cell proliferation assay

T-cell proliferative response to HCV antigens was analysed as previously described (Cramp et al., 2000). Briefly, three recombinant HCV proteins (Mikrogen, Munich, Germany) were used: HCV core (amino acids 1–115); NS3 (aa 1007–1534); NS4 (aa 1616–1862). Tetanus toxoid (Connaught Laboratories, Ontario, Canada) and phytohemagglutinin (PHA) (Sigma, Dorset, UK) were included as positive controls. PBMC were incubated with HCV antigens alone and with HCV antigens in the presence of different concentrations of ribavirin (0.5, 1.25, 2.5 and 5 μg/ml) using three replicates for each condition with 2×10^5 cells/well in 96-well, flat bottom-plates for 6 days. A stock solution of ribavirin, kindly provided by Schering–Plough Research Institute (Kenilworth, NJ), was prepared at 0.5 mg/ml and stored immediately at −80 °C until use. After thawing, the final preparations of ribavirin were tested for endotoxin levels by the Limulus Amebocyte Lysate (LAL) assay and the levels were <10 eu/ml. The thawed stock preparation was not re-used. The proliferative response was evaluated by [^{3}H]-thymidine uptake and measured in counts per minute (cpm) by a β-counter (Wallac, Turku, Finland). The number of patients tested for proliferative responses to individual HCV antigens with different concentrations of ribavirin was based on the availability of PBMC. The HCV antigens were also tested in 20 controls without HCV infection. The cut-off for a significant proliferative response was defined as a stimulation index (SI) >2.5, which was greater then the mean cpm plus 3 S.D. in the negative controls. By depletion experiments, we have established that the proliferative response is due to CD4+ T-cells (Cramp et al., 2000).

2.2.2. Ex vivo cytokine production

In parallel with the T-cell proliferation assay, PBMC (3×10^5 cells/well) were cultured in three replicates per condition with HCV antigens alone and with HCV antigens plus ribavirin for 6 days. After this period, the plates were centrifuged; supernatants and cells were collected separately, and stored at −20 or −80 °C, respectively. Interleukin-10 (IL-10) and interferon-γ (IFN-γ) in the supernatants were measured by enzyme-linked immunoassays (ELISA) (Quantikine R and D Systems, Abingdon, UK). PBMC from the same plates were used for RNA extraction and analysis of cytokine gene expression.

2.2.3. Enumeration of HCV-specific, IFN-γ and IL-10 producing cells

To determine the effect of ribavirin on the number of IFN-γ producing CD4+ T cells specific for HCV, we employed an Elispot assay as previously described (G.K. Lau et al., 2002). Briefly, nitrocellulose-bottom 96-well millititer plates (Millipore, Bedford, MA) were coated with 100 μl/well of the IFN-γ monoclonal antibody (anti-IFN-γ, Mabtech AB, 1-DIK, Stockholm, Sweden) at a concentration of 10 μg/ml in PBS overnight at 4 °C. In parallel, PBMC (2×10^5/well) were incubated with HCV antigens (2 μg/ml) alone and in the presence of different ribavirin concentrations in 96-well round-bottom plates (Becton Dickinson, Oxford, UK) at 37 °C for 28 h. PHA was used as a positive control. Subsequently, PBMC were transferred to the antibody-coated plate for further 20 h. Nitro-blue tetrazolium chloride/bromo-chloro-indolyl-phosphatetoluidune (NBT/BCIP, Roche Diagnostics, Lewes, UK) was used as a substrate and the spots were counted with an Elispot reader (AID, Strassberg, Germany). The number of specific spot-forming cells (SFCs) was determined as the mean number of spots in the presence of an antigen minus the mean number of spots in the wells with medium only and expressed per 1×10^6 PBMCs (G.K. Lau et al., 2002). The Elispot assay for IL-10 producing cells was similar with minor modifications. The plates were coated with a monoclonal antibody to IL-10 (Mabtech) at 10 μg/ml in PBS at 4 °C overnight. In parallel, PBMC (5×10^4/well) were incubated with HCV antigens alone and with different concentrations of ribavirin in round bottom plates for 14–16 h. PBMC were transferred to coated membrane and incubated for further 12 h at 37 °C. After washings, biotin-conjugated antibody to IL-10 (Mabtech) was added and incubated at room temperature for 2 h. The subsequent steps with streptavidin–alkaline phosphatase and NBT/BCIP were the same as described above. By depletion of CD4+ T cell from PBMC, using immunomagnetic separation (Dynabeads; Dynal, Oslo, Norway), the number of SFC decreased significantly, confirming that IFN-γ and IL-10 is due to the CD4+ T cells.

2.2.4. Quantitation of cytokine gene expression

Total RNA was extracted from PBMC using the RNAeasy kit (Qiagen, Crawley,UK). The cDNA was synthesized using random hexamers (Promega, Southampton, UK) and reverse transcriptase (Promega). The mRNA of IFN-γ, IL-10, IL-5, IL12p40 and IL12p35 genes was quantitated by

a real time polymerase chain reaction (PCR) using TaqMan reagents (Applied Biosystems, Foster City, CA) and the ABI PRISM 7700 sequence detector (Applied Biosystems). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to control for variations in the amount and quality of total RNA. The mRNA levels of each cytokine in the presence of an HCV antigen alone and with different concentrations of ribavirin were determined by the difference between the Ct value of GAPDH mRNA and the Ct value of cytokine mRNA (ΔC_t). The ΔC_t was then expressed as cytokine mRNA molecules per 10,000 molecules of GAPDH mRNA. The coefficient of variation of the C_t values calculated from standard curves of four experiments was 2.4%.

2.2.5. Statistical analysis

The results in the presence or absence of ribavirin were compared using parametric (*t*-test) and non-parametric tests (Wilcoxon, Friedman or McNemar), as appropriate. Correlations between variables were assessed using the Spearman rank-order correlation coefficient. Statistical analyses were performed using the SAS (SAS Institute, Cary, NC) statistical package.

3. Results

3.1. Effect of ribavirin on HCV-specific CD4+ T-cell proliferation

In the absence of ribavirin, 4 of the 40 (10%) patients responded to HCV core, 7 of 39 (18%) to NS3 and 7 of 40 (18%) to NS4. Overall, a positive T-cell proliferative response to at least one HCV antigen was present in 10 of 40 (25%) patients with CH-C.

In the presence of ribavirin (0.5 or 1.25 μg/ml) the number of patients showing significant T-cell proliferation to HCV core increased from 4 of 40 (10%) to 12 of 40 (30%) patients ($p = 0.03$, McNemar test, Fig. 1a). In the remaining 28 cases there was no proliferative response to HCV core, either with or without ribavirin (Fig. 1b). In the subgroup of cases with increased T-cell reactivity in the presence of ribavirin ($n = 12$), the SI with 0.5 μg/ml ribavirin was significantly higher than the SI with no ribavirin (mean ± S.E.M.: 6.2 ± 1.9 versus 2.7 ± 0.6, $p = 0.02$, Table 1) and was also higher than the SI in the presence of 5.0 μg/ml ribavirin (6.2 ± 1.9 versus 1.7 ± 0.3, $p = 0.0009$, Table 1).

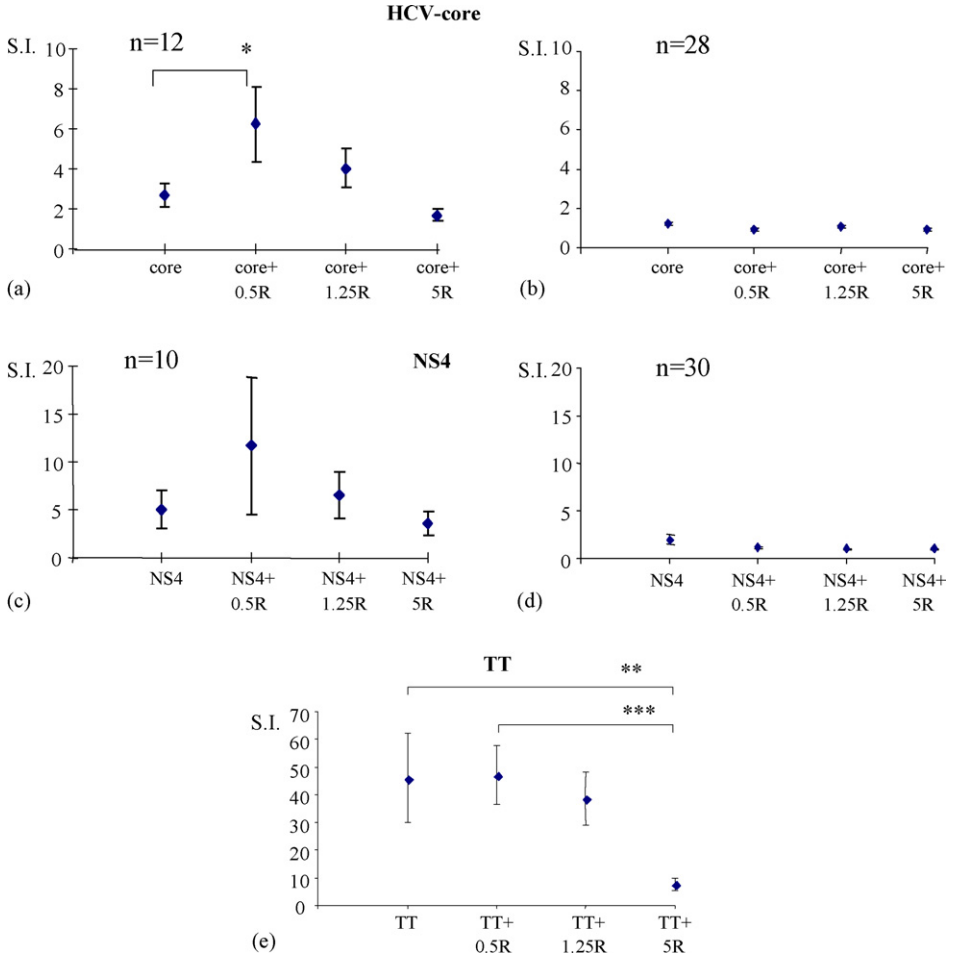

Fig. 1. Ex vivo effect of ribavirin on HCV core-, NS4- and tetanus toxoid (TT)-specific T cell responses in patients with chronic hepatitis C (CH-C). (a) Stimulation index (SI) in 12 CH-C patients with a significant response to HCV core ($^{*}p = 0.02$), (b) SI in 28 CH-C patients with no significant response to HCV core, (c) SI in 10 CH-C patients with a significant response to NS4, (d) SI in 30 CH-C patients with no significant response to NS4, (e) SI in 20 CH-C patients with a significant response to tetanus toxoid (TT) ($^{**}p = 0.02$, $^{***}p = 0.05$). 0.5R = 0.5 μg/ml ribavirin, 1.25R = 1.25 μg/ml ribavirin, 5R = 5 μg/ml ribavirin.

Table 1
Effect of ribavirin on stimulation index to HCV antigens in patients with significant proliferative responses

HCV antigens	HCV antigen alone	HCV antigens plus ribavirin			
		0.5 μg/ml	1.25 μg/ml	2.5 μg/ml[a]	5 μg/ml
HCV core ($n=12$)	2.7 ± 0.6[*]	6.2 ± 1.9[*,§]	4.0 ± 1.0	1.7 ± 0.5	1.7 ± 0.3[§]
NS3 ($n=4$)	6.8 ± 3.8	18.0 ± 15.5	5.4 ± 3.1	1.9 ± 0.7	1.3 ± 0.3
NS4 ($n=10$)	5.1 ± 1.1	11.6 ± 7.1	6.6 ± 2.5	5.0 ± 2.0	3.6 ± 1.2

The numbers in parenthesis represent the number of patients with significant T-cell reactivity to individual HCV antigens. Results are expressed as mean SI ± S.E.M. Patients studied at all concentrations were 12 for HCV core, 4 for NS3 and 10 for NS4.

[a] Due to a limited number of PBMC from some patients ribavirin at 2.5 μg/ml was tested in a proportion of cases 10 for HCV core, 2 for NS3, 7 for NS4.

[*] $p=0.02$.

[§] $p=0.0009$.

In the presence of ribavirin the number of patients showing significant T-cell reactivity to NS4 increased from 7 of 40 (17.5%) to 10 of 40 (25%) patients ($p>0.05$). The strength of T-cell reactivity to NS4 in these 10 patients was highest with 0.5 μg/ml ribavirin, but the difference did not reach statistical significance ($p>0.05$) (Fig. 1c). Importantly, 7 of these 10 cases also belonged to the subset of 12 patients, who showed significant T-cell reactivity to HCV core in the presence of ribavirin. The remaining 30 cases showed no significant T-cell proliferation to NS4 alone or with any ribavirin concentration used (Fig. 1d). The ex vivo effect of ribavirin on T-cell proliferative responses did not differ between patients with different stage of CH-C.

At 5 μg/ml, ribavirin markedly inhibited also T-cell proliferative response to tetanus toxoid, used as a recall antigen (Fig. 1e). The possibility that the anti-proliferative effect of high ribavirin concentrations was due to a toxic effect, was assessed by measuring PBMC viability with the use of trypan blue exclusion at the end of the culture period. After six days in culture, the PBMC viability was the same in the control wells and in the presence of all ribavirin concentrations used (between 96 and 98%), demonstrating that ribavirin had no toxic effect.

3.2. Ex vivo analysis of HCV-specific cytokine production

We further investigated the effect of ribavirin on IFN-γ and IL-10 production in response to HCV core antigen in PBMC from patients with CH-C. The cytokine levels were measured in the supernatants of the same PBMC which were used for gene expression analysis, cultured with HCV core antigen alone and in the presence of 0.5, 1.25 and 5 μg/ml ribavirin. This analysis included 15 patients, with supernatants available from all conditions studied. Four of these 15 patients had significant T-cell proliferation to core antigen. At the concentrations tested, ribavirin did not change significantly the IFN-γ protein levels ($p=0.48$, Fig. 2a). The production of IL-10 from PBMC incubated with HCV core in the presence of ribavirin at 0.5 μg/ml (219.43 ± 43.25 pg/ml) was significantly lower compared to IL-10 levels produced by PBMC cultured with HCV core alone (288.34 ± 34.59 pg/ml) ($p=0.05$) (Fig. 2b). In the presence of 0.5 μg/ml ribavirin, lower IL-10 levels were found mainly in patients with significant T-cell proliferation. In particular, IL-10 levels in the supernatants of patients with increased

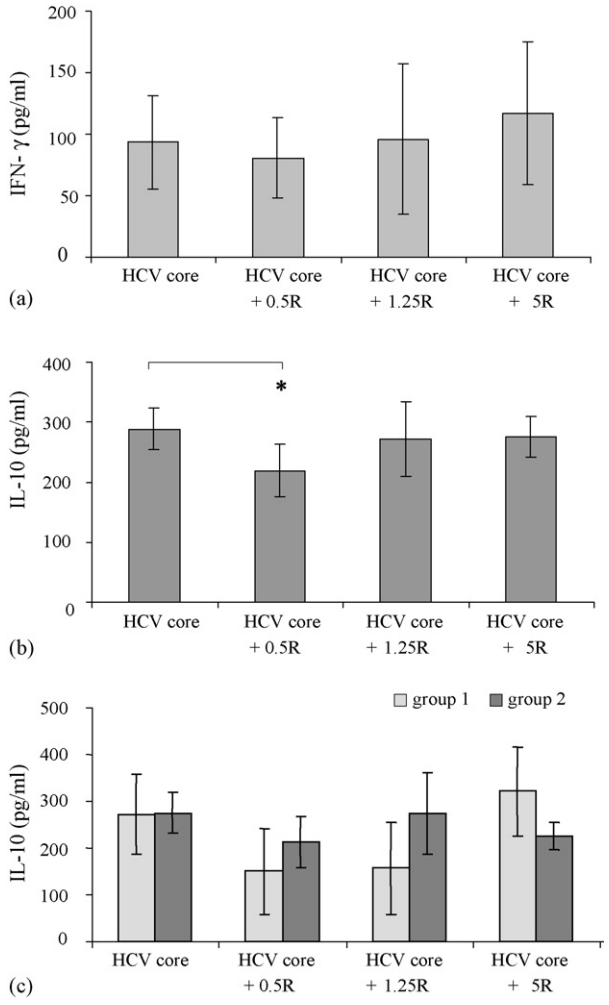

Fig. 2. Ex vivo effect of ribavirin on cytokine production in response to HCV core from PBMC in 15 patients with CHC tested in all conditions. (a) IFN-γ production in response to HCV core; (b) IL-10 production in response to HCV core in all patients ([*] $p=0.05$); (c) IL-10 production in response to HCV core in patients with enhanced HCV-specific T cell reactivity (group 1, $n=4$) and patients with no enhanced HCV-specific T cell reactivity (group 2, $n=11$). The bars represent the mean (±S.E.M.) IL-10 production. 0.5R = 0.5 μg/ml ribavirin, 1.25R = 1.25 μg/ml ribavirin, 5R = 5 μg/ml ribavirin. Bars represent the mean values ± S.E.M. for each condition.

Table 2
Effect of ribavirin on the frequency of HCV-core specific IL-10 producing T-cells in five patients with significant proliferative responses to HCV core

	HCV core alone	HCV antigens plus ribavirin		
		0.5 μg/ml	1.25 μg/ml	5 μg/ml
Patient 1	40	50	70	109
Patient 2	226	25	142	195
Patient 3	148	126	150	233
Patient 4	110	91	95	83
Patient 5	82	46	74	74
Mean ± S.E.M.	121.2 ± 31.6	67.5 ± 18.1	106.2 ± 16.8	138.7 ± 31.8

S.E.M.: standard error of the mean.

SI to HCV core ($n = 4$) were 272.33 ± 85.98, 150.96 ± 91.57 and 157.89 ± 98.57 pg/ml in response to HCV core alone, HCV core plus 0.5 μg/ml ribavirin and HCV core plus 1.25 μg/ml ribavirin, respectively. In contrast, the IL-10 levels in supernatants of patients with no T-cell proliferation to HCV core ($n = 11$) were unchanged −294.17 ± 38.28, 244.33 ± 49.23 and 295.01 ± 70.72 pg/ml in response to HCV core alone, HCV core plus 0.5 μg/ml ribavirin and HCV core plus 1.25 μg/ml ribavirin respectively (Fig. 2c). The difference between the two groups was not significant due to the small number of cases with significant T-cell proliferation to HCV core.

3.3. Quantitation of cytokine-producing cells

Next we investigated whether the increase in HCV-specific T cell proliferation in the presence of 0.5 μg/ml of ribavirin is associated with changes in the frequency of T-lymphocytes, which produce IFN-γ or IL-10 in response to HCV core antigen. For this analysis we selected five patients whose PBMC had shown an increase in the HCV-specific T-cell proliferation in the presence of 0.5 μg/ml ribavirin. Using an IFN-γ Elispot assay we found no significant changes in the number of HCV-specific, IFN-γ producing, CD4+ T cells without and with ribavirin: the mean number of specific spot forming cells (SFC) per 1×10^6 PBMC in response to HCV core alone was 96.6 ± 54.6; core plus 0.5 μg/ml ribavirin: 103.3 ± 46.7; core plus 1.25 μg/ml ribavirin: 81.6 ± 51.7. In the same patients the presence of ribavirin altered the frequency of IL-10 producing CD4+ T cells in response to HCV-core antigen (Table 2). The lowest number of IL-10 producing cells was detected in the presence of 0.5 μg/ml of ribavirin (mean ± S.E.M.: 67.5 ± 18.1), while the number of IL-10 producing cells without ribavirin or with 5 μg/ml ribavirin was 121.2 ± 31.6 and 138.7 ± 31.8, respectively. This finding is in line with the effect of the low ribavirin concentration on IL-10 production, which we found by measuring IL-10 levels in culture supernatants.

3.4. Quantitation of cytokine gene expression

To investigate whether the presence of ribavirin affects the gene expression of immunoregulatory cytokines in response to HCV antigens, the mRNA of IFN-γ, IL-5, IL-10, IL-12 p40, IL-12 p35 was quantitated in PBMC cultured with HCV-core or NS4 alone and also in the presence of these antigens plus ribavirin at 0.5, 1.25 and 5 μg/ml. The comparisons between cytokine mRNA levels in the presence of HCV antigens alone

Table 3
In vitro effect of Ribavirin on cytokine mRNA expession in PBMC in response to HCV antigens[a]

HCV antigens	Cytokine mRNA	HCV antigen alone[b]	HCV antigen plus ribavirin[c]		
			0.5 μg/ml	1.25 μg/ml	5 μg/ml
HCV core	IFN-γ	25.8 ± 9.1	1.4 ± 0.4	1.2 ± 0.6	0.7 ± 0.2*
	IL-10	253.8 ± 96.2	2.3 ± 0.5	1.3 ± 0.2	2.4 ± 0.6
	IL-5	2.0 ± 1.0	2.4 ± 0.9	3.4 ± 1.4	1.9 ± 0.6
	IL-12 p40	19.5 ± 15.9	1.2 ± 0.3	1.1 ± 0.3	0.9 ± 0.2
	IL-12 p35	0.9 ± 0.6	3.0 ± 1.0	1.4 ± 0.5	2.5 ± 1.1
HCV NS4	IFN-γ	31.7 ± 15.9	1.6 ± 0.9	0.5 ± 0.3	0.5 ± 0.2*
	IL-10	292.6 ± 110.5	3.2 ± 1.5	1.8 ± 0.8	3.0 ± 1.0
	IL-5	6.0 ± 2.0	3.5 ± 2.7	0.5 ± 0.1	0.8 ± 0.5
	IL-12 p40	4.0 ± 0.9	1.1 ± 0.2	9.4 ± 8.5	0.8 ± 0.2
	IL-12 p35	0.3 ± 0.2	6.6 ± 5.0	2.1 ± 1.1	3.2 ± 1.3

[a] The level of each cytokine transcript was determined by the difference between the C_t value of GAPDH mRNA and the C_t value of cytokine mRNA (ΔC_t). The ΔC_t was then expressed as cytokine mRNA molecules per 10,000 molecules of GAPDH mRNA.

[b] The level of each cytokine transcript in PBMC incubated with HCV antigen alone are shown in the table as mRNA molecules per 10,000 molecules of GAPDH mRNA.

[c] The numbers in the columns of HCV antigen plus different concentrations of ribavirin represent the ratio between cytokine mRNA with HCV antigen plus ribavirin and cytokine mRNA with the same HCV antigen without ribavirin.

* $p < 0.05$ in comparison to IFN-γ mRNA in PBMC with HCV antigen alone.

and in the presence of viral antigens plus ribavirin included cases with complete data for all concentrations tested for HCV core ($n = 15$); for NS4 ($n = 14$) (Table 3).

There was no significant difference in IFN-γ mRNA levels at any of the concentrations tested in response to HCV core or NS4 (Table 3). With the high ribavirin concentration (5.0 μg/ml), the IFN-γ mRNA levels were significantly reduced in comparison to transcript levels in PBMC incubated with HCV antigens alone ($p < 0.05$). In the presence of HCV core or NS4, the IL-10 mRNA molecules were on average 10-fold higher than the IFN-γ mRNA molecules (IL-10 mRNA/IFN-γ mRNA ratio-mean ± S.E.M. for HCV core 9.8 ± 10.6; for HCV NS4 9.2 ± 6.9). There was no significant change in the mean IL-10 mRNA level in PBMC with any of the ribavirin concentrations used, in comparison to the mRNA levels in PBMC cultured without ribavirin (Table 3). With increasing concentrations of ribavirin, the ratio of IL-10 mRNA/ IFN-γ mRNA increased, which was due to a decrease in IFN-γ mRNA levels, especially in the presence of 5 μg/ml of ribavirin. Amongst patients who showed significant T-cell proliferation in the presence of ribavirin, there was a significant negative correlation between changes in the SI (ΔSI) and changes in IL-10 mRNA/IFN-γ mRNA ratio ($r = -0.473$, $p = 0.001$). The mean levels of IL-5, IL-12 p40 and IL-12 p 35 mRNAs did not differ significantly in PBMC cultured with HCV antigens alone and in the presence of any ribavirin concentration.

4. Discussion

The present study provides direct evidence that ribavirin, at concentrations between 2 and 5 μM (0.5 and 1.25 μg/ml, respectively), maintains T-cell proliferative response to HCV antigens and in a proportion of CH-C patients significantly enhances HCV-specific T-cell reactivity. The increased T-cell proliferation to HCV antigens is associated with a significant decrease in IL-10 production in response to HCV antigens, in parallel with a decreased number of HCV-specific IL-10 producing CD4+ T cells, while the levels of IFN-γ production remain unchanged. These data support our previous in vivo data that patients treated with the combination of interferon-alpha plus ribavirin show lower IL-10 production in comparison to those treated with interferon alone (Cramp et al., 2000). At higher concentrations (5 μg/ml or 20 μM) we found that ribavirin significantly inhibits T-cell proliferation and IFN-γ gene expression in response to HCV antigens. A dose-dependent, anti-proliferative effect of ribavirin, at concentrations between 5 and 60 μg/mL (or 20–240 μM), has previously been demonstrated with mitogen-stimulated PBMC or in cultures of rat and human hepatocytes (Tam et al., 1999; Powers et al., 1982; Peavy et al., 1980; Ilyin et al., 1998). The suppression of cell proliferation is due to inhibition of DNA synthesis and is entirely reversible by addition of guanosine (Peavy et al., 1980; Ilyin et al., 1998).

Ribavirin is a pleiotropic agent, with a range of biological activities, which have been considered as a possible explanation for the synergistic effect of ribavirin and interferon-alpha combination treatment of patients with CH-C (J.Y. Lau et al., 2002; Crotty et al., 2002). One of the proposed mechanisms has been inhibition of host's inosine 5′-monophosphate dehydrogenase (IMPDH), which decreases the intracellular concentration of guanosine triphosphate (GTP). This ribavirin activity is unlikely to have a significant impact against HCV because there is no correlation between the GTP pool reduction and the antiviral effect (Lee et al., 1998). Furthermore, recent attempts to use mycophenolate mofetil, another potent IMPDH inhibitor, in the treatment of CH-C, have shown no antiviral activity (Firpi et al., 2003; Cornberg et al., 2002; Jain et al., 2002).

Another hypothesis, based on a poliovirus model, is that ribavirin could induce lethal mutagenesis of RNA viruses as a result of its misincorporation into the viral genome (Crotty et al., 2000). Two recent studies, using the HCV replicon system, have demonstrated that ribavirin indeed has a capacity to affect HCV-RNA replication through a mechanism of error-prone replication (Contreras et al., 2002; Lanford et al., 2003). However, this was achieved only at concentrations greater than 200 μM, which are much higher than the plasma level of the drug during treatment (Lertora et al., 1991). In both studies, the reduction of replicon RNA was minimal at ribavirin concentrations below 100 μM.

As ribavirin is a purine analogue, it may exert immunomodulatory activities via toll-like receptor (TLR). However, ribavirin did not stimulate TLR7 or TLR8-mediated signaling in cells transfected with these TLRs at the biologically active concentrations (1–5 μM) (Vollmer et al., 2004). Previous studies using mitogen-stimulated PBMC or animal models have shown that ribavirin promotes Th1 type response either by suppressing Th2 cytokine profile (Ning et al., 1998) and/or by increasing Th1 cytokines (Tam et al., 1999; Hultgren et al., 1998; Fang et al., 2000). The present study extends these observations by demonstrating ex vivo that ribavirin does modulate the reactivity to HCV of T-cells from patients with CH-C. The immunomodulatory effect of ribavirin involved enhanced T-cell proliferation and decreased IL-10 production in a proportion of patients. Our finding of selective inhibition of IL-10 producing CD4+ T-cells may indicate that ribavirin inhibits T-regulatory cells, which deserves future investigations. Interestingly, the enhanced lymphocyte reactivity by low ribavirin concentrations was previously suggested to result from selective interference with suppressor T-cells (Powers et al., 1982). Ribavirin-induced inhibition of IL-10 production may be particularly beneficial in the combination regimen with interferon-alpha. We have shown that interferon-alpha treatment increases IL-10 production, while this is significantly reduced in patients received interferon/ribavirin combination therapy (Cramp et al., 2000). A suppressed IL-10 production to HCV will alter the balance in favour of Th1 type of T-cell reactivity, which is essential for resolution of HCV infection (Lechner et al., 2000; Gerlach et al., 1999; Tsai et al., 1997).

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