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Expression of interferon-α subtypes in peripheral mononuclear cells from patients with chronic hepatitis C: a role for interferon-α5

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SUMMARY. Interferon (IFN)-α is a family of antiviral proteins encoded by different genes. The biological significance of the existence of various IFN-α subtypes is not clear. We have investigated the interferon system in chronic hepatitis C virus (HCV) infection, a disease that responds to interferon-α2 therapy in only a limited proportion of cases. We analysed the expression of interferon regulatory factor (IRF)-1, IRF-2, and IFN-α subtypes in nonstimulated and Sendai virus-stimulated peripheral blood mononuclear cells (PBMC) from HCV infected patients and healthy controls. We observed that the IRF-1 mRNA and IRF-1/IRF-2 ratios were increased in PBMC from hepatitis C patients with respect to normal subjects. Sendai virus stimulation of PBMC led to a significant increase in the levels of IRF-1, IRF-2 and IFN-α mRNAs and in the production of IFN-α protein with respect to basal values in healthy controls as well as in patients with HCV infection. In addition, we found that while natural HCV infection induced increased IFN-α5 expression in PBMC, *in vitro* infection of these cells with Sendai virus caused a raise in the expression of IFN-α8 in both patients and normal controls. In summary, our results indicate that virus-induced activation of the IFN system in human PBMC is associated with selective expression of individual IFN-α subtypes, IFN-α5 being the specific subtype induced in PBMC from patients with chronic HCV infection.

Keywords: hepatitis C virus, interferon regulatory factor, interferon-α subtypes, peripheral blood mononuclear cells, Sendai virus.

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus that infects both the liver and lymphoid cells [1,2]. HCV infection has a strong tendency to evolve to chronicity and often progresses to cirrhosis and eventually to hepatocellular carcinoma [3,4]. The mechanisms by which this virus establishes such a high rate of persistent infection are still poorly understood. Some evidence suggests that the alteration in the interferon (IFN) system may be involved in the chronicity of this infection [5]. In addition, the function of peripheral blood lymphoid cells has been closely related to viral load [6,7].Treatment with recombinant IFN-α2, which is the current therapy, leads to sustained viral clearance only in a small proportion of treated patients [8].

Abbreviations: HCV, hepatitis C virus; IFN-α, interferon-α; IRF, interferon regulatory factor; PBMC, peripheral blood mononuclear cells.

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Human IFN-α comprises a family of extracellular signalling cytokines which confer resistance to viral infection [9–11] and have antiproliferative and immunomodulatory activities [12,13]. In a previous publication we described that in patients with chronic hepatitis C, IFN-α gene expression is downregulated in the liver but upregulated in peripheral blood mononuclear cells (PBMC) [14]. Since HCV can infect lymphoid cells [2] and these cells play an important role in the clearance of viral infection, we decided to analyse the interferon responsive factors (IRF) and the expression of specific IFN-α subtypes in PBMC from HCV infected patients.

IRF-1 and IRF-2 belong to a family of DNA-binding proteins which regulate interferon genes and interferon-inducible genes [15,16]. They bind to the same DNA element where IRF-1 acts as a positive regulator and IRF-2 is as a negative transcription factor that lacks the transactivation domain [17]. The increase in the IRF-1/IRF-2 ratio seems to be a critical event in the transcriptional activation of IFN and subsequent development of the cellular antiviral response [18,19].

IFN-α proteins are encoded by a multigene family comprising 13 intronless genes which show 78–94% homology at the nucleotide level [20] and are clustered in human chromosome 9 [21]. Individual IFN-α genes seem to be differentially expressed depending on the stimulus and on the producer cell type [15,22]. The biological significance of the large abundance of IFN-α genes is not clear. Although all IFN-α subtypes bind to a common receptor, several reports suggest that they show quantitatively distinct patterns of antiviral, growth inhibitory and immunomodulatory activities [23–25]. A combination of different IFN-α subtypes has been found in normal human unstimulated PBMC [14,26–28] and in Sendai virus-induced human PBMC [26,27,29]. However the expression of the different IFN-α subtypes in human viral infections has been poorly studied.

In the present report we demonstrate that the interferon system is activated in PBMC from HCV infected patients. In addition we show that while IFN-α5 seems to be induced in PBMC in HCV infection, IFN-α8 is the subtype produced by human PBMC in response to Sendai virus.

MATERIALS AND METHODS

Patients

We analysed PBMC samples from 41 patients with chronic hepatitis C. When diagnosed, chronic hepatitis C patients had raised serum transaminases for more than 6 months, positivity for anti-HCV antibodies (ELISA second-generation, Ortho Diagnostic System, Raritan, NJ, USA), presence of HCV-RNA by RT-PCR in serum and histological evidence of chronic hepatitis. Other causes of chronic hepatitis were excluded. Twelve patients were sustained responders to interferon treatment (persistent normal serum transaminases and negative HCV-RNA in serum for at least 10 months after completion of 1 years IFN treatment) when studied. None of the patients had received treatment with IFN-α in the 10-month period prior to the study. Table 1 shows the biochemical, histological and virological characteristics of the patients.

As controls, a group of 41 healthy volunteers (20 male, 21 female, age range from 24 to 55 years) were studied.

Detection, quantification and genotyping of HCV-RNA was performed as previously described [30].

Cell culture and stimulation

PBMC (1×10^7) resuspended in 5 mL of RPMI 1640 medium (Bio-Whittaker, Verviers, Belgium), supplemented with 1% of fetal calf serum and penicillin-streptomycin (100 U and 100 μg/mL, respectively), were incubated for 6 h at 37 °C and 5% CO_2 with or without 16 haemagglutination units per mL of Sendai virus. Following induction, PBMC were pelleted, washed once in 0.9% NaCl and lysed in Ultraspec (Biotecx, Houston, TX, USA). The samples were stored at −80 °C until extraction of total RNA, which was performed according to the methods of Chomczynski and Sacchi [31].

Simultaneously, other aliquots of 5×10^6 PBMC were cultured for 24 h under the same conditions with and without Sendai virus. Supernatants of PBMC were collected and stored at −80 °C until IFN-α protein was determined.

Under these conditions cell viability, tested with Trypan blue, was 100%.

Analysis of IFN-α mRNA levels and identification of IFN-α subtypes

Total RNA was extracted from PBMC samples and IFN-α mRNA levels were determined by semiquantitative RT-PCR

Table 1 Biochemical, histological and virological characteristics of patients with chronic hepatitis C

	HCV-RNA (+) ($n = 29$)	HCV-RNA (−) ($n = 12$)
Age range (years)	26–74	24–62
Sex	13 male, 16 female	10 male, 2 female
Serum ALT (IU/L)	62.5 ± 7.4*	13.4 ± 1.2
Serum AST (IU/L)	42.2 ± 5.9	12.3 ± 0.8
GGTP (IU/L)	31.2 ± 4.4	12.4 ± 1.1
Liver histological activity		
Total Knodell's score	9.7 ± 0.6	6.3 ± 0.6
Cirrhosis (*n*)	5	0
Serum HCV-RNA (copies/mL)	$1.6 \times 10^8 \pm 0.6 \times 10^8$	Non-detected
Viral genotype (*n*)		
1b	25	5**
1a	2	0**
3	2	6**
2	0	1**

*Means ± SD. **Pretreatment viral genotype.

in a Perkin Elmer Gene Amp PCR System 2400 (Perkin Elmer, Foster City, CA, USA). Before the reverse transcription, 1 μg of total RNA was treated with 1 U of deoxyribonuclease (DNase I amplification grade, Gibco-BRL, Gaithersburg, MD, USA) to remove all the contaminating DNA. In all cases the presence of traces of DNA was excluded by performing control reactions without reverse transcriptase. RNA was reverse transcribed (60 min at 37 °C) with 200 U of M-MuLV reverse transcriptase (Gibco-BRL) in 20 μL volume of 5 × RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) supplemented with 5 mM dithiothreitol (DDT), 0.5 mM deoxynucleoside triphosphate (Boehringer Mannheim, Mannheim, Germany), 25 U ribonuclease inhibitor (Promega Corporation, Madison, WI, USA) and 200 ng random hexamers (Boehringer Mannheim). After heating (95 °C, 1 min) and quick-chilling on ice, an aliquot of 6 μL (0.3 μg) of the cDNA pool was used for PCR amplification in 50 μL of 10 × buffer solution (100 mM Tris-HCl pH 9.3, 500 mM KCl, 1% Triton X-100) containing 0.08 mM dNTP, 3.5 μCi (α-^{32}P)-dCTP (Amersham, Buckinghamshire, UK), upstream and downstream primers (40 ng each), 1.5 mM $MgCl_2$ and 2 U of *Taq* DNA polymerase (Promega Corporation). Blank reactions with no RNA were performed in all experiments. As an internal control for each sample, PCR amplification of a fragment of β-actin cDNA (using 6 μL aliquot of the cDNA pool) was performed. IFN-α cDNA fragments were amplified by 20 cycles (94 °C, 60 °C and 72 °C, 1 min per step) and β-actin fragments were amplified by 20 cycles (94 °C, 55 °C and 72 °C, 1 min per step) a protocol that avoided interference of the plateau effect. We used universal primers designed to amplify all IFN-α subtypes. Oligonucleotides (5′–3′) d(TCCATGAGATGATCCAGCAG) and d(ATTTCTGCTCTGACAACCTCCC) were the upstream and downstream primers, respectively, for amplification of 274 base pair (bp) fragment from human IFN-α cDNA [20] that is located between nucleotides 240–514 [32]. Primers were designed to obtain the same amplification efficiency for all IFN-α subtypes. The two primers correspond to the two most conserved regions among all subtypes. The sequence for annealing of the downstream primer is identical for all of them. The annealing sequence of the upstream primer is the same for subtypes 2, 4, 5, 7, 8, 10, 16, 17 and 21 and it differs in only one nucleotide in subtypes 1, 14 and 22 (this change does not occur in the 3′ end of the primer). d(TCTACAATGAGCTGCGTGTG) and d(GGTGAGGATCTTCATGAGGT) were the primers used for amplification of a 314-bp fragment (nucleotides 1319–2079) from the reported human β-actin gene sequence [33].

After PCR amplification, 20 μL aliquots of the PCR reactions were electrophoresed in 2% agarose gel stained with ethidium bromide, and bands were visualized with an UV lamp. Equal size bands were excised and radioactivities were determined. Obtained values were corrected with background radioactivity from blank reactions with no RNA. Finally, values corresponding to IFN-α mRNA were normalized to those of β-actin mRNA and results were expressed as cpm ratio of IFN-α to β-actin. β-Actin is used as internal control because we have previously shown that β-actin mRNA is constantly expressed in PBMC of healthy controls and patients with chronic hepatitis C [30].

Validation experiments on PCR assays using known quantities of total RNA showed linearity of the counts of amplification bands from 0.125 to 1 μg for IFN-α and β-actin. Thus 0.3 μg for IFN-α and β-actin, respectively, were chosen as a suitable amount of total RNA to avoid the plateau effect. The coefficient of interassay variation for IFNα/β-actin was 15%. The identity of the PCR product from IFN-α cDNA amplification was verified by automatic sequencing (ABI PRISM™ 310 Genetic Analyser, Perkin Elmer).

The identification of IFN-α subtype transcripts was performed by cloning the RT-PCR products using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Clones from each insert were sequenced in ABI PRISM™ 310 Genetic Analyser (Perkin Elmer) using the dye Rhodamine terminator cycle sequencing kit (Perkin Elmer).

Analysis of IFN-α protein

To quantify IFN-α protein levels in PBMC supernatants of unstimulated and Sendai virus-stimulated PBMC, we used an ELISA (Cytosceen™, Biosource International, Camarillo, CA, USA) using specific antihuman IFN-α antibodies. The assay was performed following the instructions of the manufacturers. The sensitivity limit of the assay was 25 pg/mL.

Analysis of IRF-1 and IRF-2 mRNA levels

Estimation of IRF-1 and IRF-2 mRNA levels was carried out using previously described RT-PCR methodology. Total RNA from PBMC was reverse-transcribed (0.2 and 0.3 μg for IRF-1 and IRF-2, respectively) and the cDNA pool was used for PCR amplification, as above, but using 200 ng for each IRF-1 and IRF-2 upstream and downstream primers. β-Actin was also used as an internal control for each sample. IRF-1 and IRF-2 cDNA fragments were amplified by 19 and 28 cycles, respectively (94 °C, 55 °C and 72 °C, 1 min per step), to avoid the plateau effect. Oligonucleotides d(CTGATACCTTCTCTGATGG) and d(TCCAGGTTCATTGAGTAGG) were the upstream and downstream primers used to amplify a 353-bp fragment from human IRF-1 cDNA, that is located between nucleotides 631–983 [34]. d(TTTTCAGATCCCCTGGATGC) and d(TCAGTGGTGACCTCTACAAC) were the primers used for amplification of 525 bp fragment from human IRF-2 cDNA that is located between nucleotides 197–721 [35].

Validation experiments of PCR assays using known quantities of total RNA (from 0 to 1 μg) were carried out, and 0.3 μg for IRF-1 and 0.2 μg for IRF-2 were chosen as the suitable amount of total RNA to stay in the linear part of

the curve. The coefficient of interassay variation for IRF-1/β-actin and IRF-2/β-actin was 7.5% and 14%, respectively. The identity of the PCR product from IRF-1 and IRF-2 cDNA amplification was further verified by enzymatic digestion. We used *Eco*O109 I (for IRF-1) and *Eco*R I (for IRF-2) that yielded the predicted restriction fragments, while *Sac* I did not digest the amplified PCR products (no restriction sites for this endonuclease are present in the amplified regions).

Statistical analysis

Results of IFN-α, IRF-1 and IRF-2 are given as mean ± standard errors of the mean. Normality was assessed with the Shapiro–Wilks' test. Statistical analysis of IFN-α, IRF-1 and IRF-2 levels in PBMC were performed using nonparametric test (Kruskal–Wallis and Mann–Whitney *U*-tests). Associations between quantitative variables were studied with Spearman's correlation coefficient. IFN-α subtypes expressed in PBMC, before and after Sendai virus stimulation, were analysed with Wilcoxon test. All *P*-values were two-tailed. SPSS 6.0 for Windows was used for the statistical analysis.

RESULTS

Expression of interferon regulatory factors in PBMC from normal subjects and patients with present and past HCV infection

We have previously demonstrated that the values of IFN-α mRNA in unstimulated PBMC from HCV-RNA positive patients was higher than healthy controls [14]. To further analyse the activation of the interferon system in PBMC we have now determined the level of IRF-1 and IRF-2 gene expression in healthy subjects, patients with chronic hepatitis C and in patients who cleared HCV infection after IFN therapy. We found that in unstimulated PBMC, IRF-1 mRNA levels (Fig. 1a) and the IRF-1/IRF-2 ratio (Fig. 1b) were higher in HCV-RNA positive patients than in healthy controls. Patients with sustained complete virological and biochemical response after IFN therapy showed IRF-1 mRNA levels (Fig. 1a) and IRF-1/IRF-2 ratio similar to healthy controls (Fig. 1b). The levels of IRF-2 mRNA were comparable in the three groups studied (0.67 ± 0.06; 0.87 ± 0.07 and 0.85 ± 0.1; healthy controls, HCV-RNA (+) patients and HCV-RNA (–) patients, respectively).

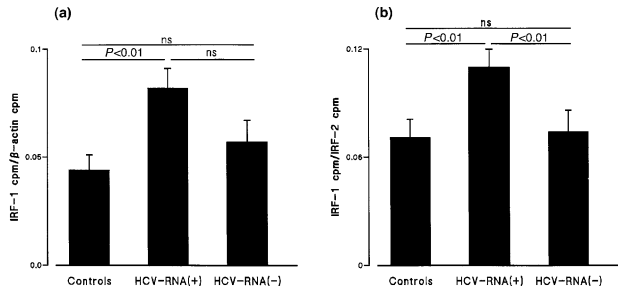

Fig. 1 (a) mRNA levels of IRF-1 and (b) IRF-1/IRF-2 ratio (as a ratio to β-actin) in unstimulated PBMC from healthy controls, patients with chronic HCV infection (HCV-RNA (+)) and patients who cleared HCV after interferon therapy (HCV-RNA (–)).

No correlation was observed between IRF-1 or IRF-2 levels and serum transaminases nor between IRF-1 or IRF-2-values and liver damage as estimated in liver biopsies by the Knodell's score of histological activity. Also no correlation was found between IRF-1 or IRF-2 mRNA levels and viral load as determined by competitive PCR in viraemic patients. Moreover, no differences in IRF-1 or IRF-2 mRNA values were found in patients infected with different HCV genotypes.

The effect of Sendai virus stimulation on IFN-α, IRF-1 and IRF-2 gene expression in PBMC from healthy controls, patients with chronic hepatitis C and patients who cleared HCV after interferon therapy

Sendai virus stimulation led to a significant increase in the levels of IRF-1, IRF-2 and IFN-α mRNA in PBMC with respect to basal values in healthy controls as well as in patients with present and past HCV infection (Table 2). Stimulated IRF-1, IRF-2 and IFN-α mRNA levels were similar in the three groups studied (Table 2).

We also measured IFN-α protein in supernatants from PBMC with and without Sendai virus stimulation. IFN-α protein could not be detected in supernatants from unstimulated PBMC in any of the three groups studied. After Sendai virus stimulation IFN-α protein increased to similar values in all groups (17 600 ± 2223; 12 616 ± 1572 and 19 348 ± 4766 pg/mL in healthy controls, HCV-RNA positive and HCV-RNA negative patients, respectively).

Expression of IFN-α subtypes in PBMC

The IFN-α cDNAs generated by RT-PCR were cloned and sequenced. In unstimulated PBMC, we studied 75 clones from eight healthy subjects and 83 clones from eight patients with chronic hepatitis C. We found a broad spectrum of IFN-α subtypes in both healthy controls and HCV-RNA positive patients. IFN-α5, IFN-α1 and IFN-α21 were the main subtypes, comprising nearly 80% of total IFN-α in unstimulated PBMC from healthy subjects. In patients with HCV infection, as compared to normal controls, we found a significant increase in the expression of IFN-α5 ($P < 0.05$) which represented 65% of all sequenced clones (Table 3).

Five patients and five controls were also studied after Sendai virus stimulation. As shown in Table 4, after Sendai virus stimulation, a mixture of different IFN-α subtypes were expressed in both patients and controls. IFN-α1, IFN-α8,

Table 2 IRF-1, IRF-2 and IFN-α mRNA levels (as a ratio to β-actin) in unstimulated and Sendai virus stimulated peripheral blood mononuclear cells (PBMC) in healthy controls, in patients with chronic hepatitis C and positive HCV-RNA in serum (HCV-RNA(+)) and patients who cleared HCV-RNA (HCV-RNA (−)) after interferon therapy

	IRF-1		IRF-2		IFN-α	
	Unstimulated PBMC	Stimulated PBMC	Unstimulated PBMC	Stimulated PBMC	Unstimulated PBMC	Stimulated PBMC
Healthy Controls	0.04 ± 0.01[a]	0.13 ± 0.01*	0.67 ± 0.06	1.16 ± 0.16*	Nd[b]	1.45 ± 0.12*
HCV-RNA (+)	0.08 ± 0.01	0.18 ± 0.01*	0.87 ± 0.07	1.61 ± 0.12*	Nd	1.45 ± 0.16*
HCV-RNA (−)	0.05 ± 0.01	0.13 ± 0.01*	0.85 ± 0.1	1.56 ± 0.16*	Nd	1.73 ± 0.17*

[a] Means ± SD. [b] Nd, no detectable (mRNA of IFN-α was no detectable using 20 cycles of PCR amplification, which is the number of cycles used to quantify IFN-α mRNA in Sendai virus stimulated PBMC to avoid interferences with the plateau effect. IFN-α mRNA in unstimulated PBMC was detectable after 30 cycles of amplification).
* $P < 0.05$ Sendai virus stimulated vs. unstimulated PBMC.

Table 3 IFN-α subtypes in unstimulated peripheral blood mononuclear cells from healthy controls and patients with chronic hepatitis C

	Tested clones	α1	α2	α4	α5	α7	α8	α10	α14	α16	α17	α21	α22
C1	12	0	0	0	8	0	1	0	0	0	0	3	0
C2	8	0	2	0	4	0	0	1	0	1	0	0	0
C3	8	2	0	0	3	0	0	0	0	0	0	2	1
C4	7	0	1	1	1	1	1	2	0	0	0	0	0
C5	12	0	1	0	8	0	0	0	0	0	0	3	0
C6	9	2	0	0	3	0	0	0	0	0	0	4	0
C7	8	0	0	0	8	0	0	0	0	0	0	0	0
C8	11	10	0	0	0	0	1	0	0	0	0	0	0
Total	75	14	4	1	35	1	3	3	0	1	0	12	1
P1	10	2	0	0	7	0	1	0	0	0	0	0	0
P2	12	2	0	0	6	2	0	0	0	0	0	2	0
P3	11	0	1	0	10	0	0	0	0	0	0	0	0
P4	10	0	0	0	8	0	0	0	0	0	0	2	0
P5	11	2	0	0	7	0	2	0	0	0	0	0	0
P6	9	0	0	0	7	1	0	0	0	0	0	1	0
P7	9	1	0	0	5	0	0	0	0	0	0	3	0
P8	11	2	0	2	4	0	0	1	0	1	0	0	1
Total	83	9	1	2	54*	3	3	1	0	1	0	8	1

C1–8 are samples from eight different normal controls. P1–8 are samples from eight different HCV-RNA (+) chronic hepatitis C patients.
* $P < 0.05$ vs. healthy controls.

IFN-α2 and IFN-α5 were the major subtypes expressed in healthy controls, comprising 82.8% of total IFN. IFN-α1, IFN-α8 and IFN-α2 were the major subtypes expressed in HCV-RNA positive patients, comprising 79% of total IFN. IFN-α8 was the only IFN-α subtype which increased significantly in both, healthy controls ($P < 0.05$) and HCV-RNA positive patients ($P < 0.05$). The expression of IFN-α5 subtype decreased significantly after Sendai virus stimulation in HCV positive patients ($P < 0.05$). This subtype also tended to decrease after Sendai virus infection in healthy controls but in these subjects the differences were not statistically significant.

DISCUSSION

Both the activation of the interferon system and the specific antiviral T-cell immunity, measured in peripheral blood mononuclear cells, appear to be important in the control of

Table 4 IFN-α subtypes in peripheral blood mononuclear cells from normal controls (C) and patients with chronic hepatitis C (P) before and after Sendai virus stimulation

	Tested clones	α1	α2	α4	α5	α7	α8	α10	α14	α16	α17	α21	α22
C1	12*-10†	0–4	0–1	0–0	8–0	0–0	1–2	0–3	0–0	0–0	0–0	3–0	0–0
C2	8–12	0–6	2–3	0–0	4–1	0–0	0–2	1–0	0–0	1–0	0–0	0–0	0–0
C3	8–8	2–1	0–0	0–0	3–2	0–0	0–2	0–1	0–0	0–2	0–0	2–0	1–0
C4	7–11	0–2	1–0	1–0	1–3	1–0	1–4	2–0	0–1	0–0	0–0	0–1	0–0
C5	12–11	0–3	1–3	0–0	8–1	0–0	0–3	0–0	0–0	0–0	0–0	3–1	0–0
Total	47–52	2–16	4–7	1–0	24–7	1–0	2–13‡	3–4	0–1	1–2	0–0	8–2	1–0
P1	10–12	2–6	0–1	0–0	7–2	0–1	1–2	0–0	0–0	0–0	0–0	0–0	0–0
P2	12–9	2–1	0–2	0–0	6–1	2–0	0–3	0–0	0–0	0–1	0–1	2–0	0–0
P3	11–12	0–2	1–2	0–0	10–0	0–2	0–4	0–1	0–0	0–0	0–0	0–1	0–0
P4	10–12	0–6	0–2	0–0	8–1	0–0	0–3	0–0	0–0	0–0	0–0	2–0	0–0
P5	11–12	2–5	0–0	0–0	7–0	0–0	2–6	0–1	0–0	0–0	0–0	0–0	0–0
Total	54–57	6–20	1–7	0–0	38–4‡	2–3	3–18‡	0–2	0–0	0–1	0–1	4–1	0–0

C1–5 are samples from five different normal controls. P1–5 are samples from five different HCV-RNA (+) chronic hepatitis C patients.

*Number of cDNA clones before Sendai virus stimulation. †Number of cDNA clones after Sendai virus stimulation. ‡$P < 0.05$ Sendai virus stimulated vs. unstimulated.

HCV infection [7,36]. This report shows that the IFN system is activated in mononuclear cells from HCV-infected patients. Previously we have observed increased values of IFN-α and IFN-β mRNA in PBMC from these individuals [14]. Now we show the presence of a significant elevation of the IRF-1 and IRF-1/IRF-2 ratio in PBMC from patients with chronic hepatitis C. IRF-1 and IRF-2 have been identified as regulators of interferon and interferon-inducible genes. High levels of IFN-inducible genes such as 2′,5′-oligoadenylate synthetase and Mx proteins have been found in PBMC from HCV infected individuals [37–39]. Similarly, high level of expression of interferon-inducible genes have been detected in the liver from chimpanzees during the acute phase of hepatitis C [40]. Although IRF-1 and IRF-2 are considered to be crucial in providing protection against several viral infections [41,42], we found no correlation between IRF levels and viral load or the activity of the liver disease. Recently, a more relevant role for other members of the IRF family, such as IRF-3 and IRF-7, has been described in viral infections [43,44]. IRF-1 is stimulated not only by viral infection of the cells but also by cytokines such as interferon-gamma and IL-12 [45,46]. Since IFN-γ is elevated in HCV infection [47,48] this might contribute to the high levels of IRF-1 seen in this disease.

To further analyse whether the interferon machinery of PBMC responded normally to stimulation in patients with chronic hepatitis C, we investigated the changes in IFN-α and IRFs transcripts and the production of IFN-α protein after *in vitro* stimulation of PBMC with Sendai virus. We found that HCV infection did not result in disturbed inducibility of IRFs genes nor of IFN-α production. These findings are in contrast with those reported in patients with HBV or HIV infections where the IFN system is impaired in PBMC [49–50].

Very few studies have explored the role of the different IFN-α subtypes in viral infections in humans. In 1987, Tovey *et al.* using RNA blot hybridization showed that IFN-α could be detected in the apparent absence of induction [51]. Another study performed by Greenway and coworkers detected IFN-α in the cytoplasm of PBMC, in the absence of viral stimulation, by immunofluorescence using polyclonal antipeptide antisera [27]. A similar finding was reported by Brandt *et al.* using the polymerase chain reaction with universal primers and with selected subtype specific primers [26]. Lallemand *et al.* found that IFN-α5 was the main subtype expressed in PBMC using PCR technique followed by direct dideoxy sequencing [28]. The availability of automatic sequencing after cloning the RT-PCR product, allowed us to perform a more complete analysis of the IFN-α subtypes expressed in normal PBMC. We have found that IFN-α5, IFN-α1 and IFN-α21 are the main IFN-α subtypes constitutively expressed in unstimulated PBMC from healthy controls. The constitutive expression of IFN-α has been related to the maintenance of HLA proteins on the cell surface [28]. In chronically HCV-infected cells we found a significant and selective increase of IFN-α5 subtype.

Interestingly, IFN-α5 which is the only subtype expressed in normal liver tissue [14] and the main subtype in normal pancreas [52], is reduced in the liver from HCV infected patients [14]. In HCV infection the liver is the main site of viral replication and low levels of IFN-α5 may favour persistent HCV infection of this organ. PBMC (with higher

levels of IFN-α5 transcripts) may act as a reservoir for HCV but these cells allow HCV replication at levels considerably lower than in the liver [2]. Further studies using quantitative PCR specific for IFN-α5 are needed to determine whether the levels of this IFN-α subtype can correlate with the replicative activity of the virus.

In contrast to what happens in natural HCV infection, *in vitro* infection with Sendai virus selectively upregulates IFN-α8 in both normal subjects and HCV infected patients cells. It has been reported that monocytes and the so called 'natural IFN-producing cells' (NIPC), which are thought to be dendritic cells, are the two major IFN-α producing cells in PBMC [53]. IFN-α production in response to Sendai virus occurs predominantly in monocytes, whereas most enveloped viruses stimulate NIPC [54]. IFN-α produced by virus-activated IFN-producing cells contributes to the differentiation of these cells into dendritic cells [55]. If the different IFN subtype expression we detected reflects selective IFN-α production by different PBMC subpopulations and whether it has a role in the altered function of dendritic cells in patients with HCV [56] needs to be studied.

In summary, the interferon system is activated in PBMC from subjects with chronic HCV infection. The response of PBMC to *in vitro* stimulation with Sendai virus is preserved in chronic hepatitis C. While Sendai virus induces preferentially IFN-α8 in both patients and healthy controls, HCV infection of PBMC is associated with IFN-α5 subtype expression. The role of IFN-α5 in the control of HCV infection should be investigated.

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REFERENCES

1 Gil B, Qian C, Riezu-Boj JI, Civeira MP, Prieto J. Hepatic and extrahepatic HCV RNA strands in chronic hepatitis C. different patterns of response to interferon treatment. *Hepatology* 1993; 18: 1050–1054.
2 Qian C, Camps J, Maluenda MD, Civeira MP, Prieto J. Replication of hepatitis C virus in peripheral blood mononuclear cells. Effect of alpha-interferon therapy. *J Hepatol* 1992; 16: 380–383.
3 Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997; 349: 825–832.
4 Fattovich G, Giustina G, Degos F *et al.* Effectiveness of interferon alfa on incidence of hepatocellular carcinoma and decompensation in cirrhosis type C. European Concerted Action on Viral Hepatitis (EUROHEP). *J Hepatol* 1997; 27: 201–205.
5 Heim MH, Moradpour D, Blum HE. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. *J Virol* 1999; 73: 8469–8475.
6 Hiroishi K, Kita H, Kojima M *et al.* Cytotoxic T lymphocyte response and viral load in hepatitis C virus infection. *Hepatology* 1997; 25: 705–712.
7 Lasarte JJ, Garcia-Granero M, Lopez A *et al.* Cellular immunity to hepatitis C virus core protein and the response to interferon in patients with chronic hepatitis C. *Hepatology* 1998; 28: 815–822.
8 Davis GL. New schedules of interferon for chronic hepatitis C. *J Hepatol* 1999; 31: 227–231.
9 Staeheli P. Interferon-induced proteins and the antiviral state. *Adv Virus Res* 1990; 38: 147–200.
10 Samuel CE. Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. *Virology* 1991; 183: 1–11.
11 Sen GC, Ransohoff RM. Interferon-induced antiviral actions and their regulation. *Adv Virus Res* 1993; 42: 57–102.
12 Pestka S, Langer JA, Zoon KC, Samuel CE. Interferons and their actions. *Annu Rev Biochem* 1987; 56: 727–777.
13 Marrack P, Kappler J, Mitchell T. Type I interferons keep activated T cells alive. *J Exp Med* 1999; 189: 521–530.
14 Castelruiz Y, Larrea E, Boya P, Civeira MP, Prieto J. Interferon alfa subtypes and levels of type I interferons in the liver and peripheral mononuclear cells in patients with chronic hepatitis C and controls. *Hepatology* 1999; 29: 1900–1904.
15 Au WC, Su Y, Raj NB, Pitha PM. Virus-mediated induction of interferon A gene requires cooperation between multiple binding factors in the interferon alpha promoter region. *J Biol Chem* 1993; 268: 24032–24040.
16 Fujita T, Kimura Y, Miyamoto M, Barsoumian EL, Taniguchi T. Induction of endogenous IFN-alpha and IFN-beta genes by a regulatory transcription factor, IRF-1. *Nature* 1989; 337: 270–272.
17 Harada H, Willison K, Sakakibara J, Miyamoto M, Fujita T, Taniguchi T. Absence of the type I IFN system in EC cells: transcriptional activator (IRF-1) and repressor (IRF-2) genes are developmentally regulated. *Cell* 1990; 63: 303–312.
18 Houle JL, Santoro N. Analysis of human interferon-alpha gene promoters by multiple sequence alignment. *J Interferon Cytokine Res* 1996; 16: 93–98.
19 Miyamoto M, Fujita T, Kimura Y *et al.* Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. *Cell* 1988; 54: 903–913.
20 Weissmann C, Weber H. The interferon genes. *Prog Nucleic Acid Res Mol Biol* 1986; 33: 251–300.
21 Diaz MO, Pomykala HM, Bohlander SK *et al.* Structure of the human type-I interferon gene cluster determined from a YAC clone contig. *Genomics* 1994; 22: 540–552.
22 Hiscott J, Cantell K, Weissmann C. Differential expression of human interferon genes. *Nucleic Acids Res* 1984; 12: 3727–3746.
23 Foster GR, Rodrigues O, Ghouze F *et al.* Different relative activities of human cell-derived interferon-alpha subtypes: IFN-alpha 8 has very high antiviral potency. *J Interferon Cytokine Res* 1996; 16: 1027–1033.

24 Yeow WS, Lawson CM, Beilharz MW. Antiviral activities of individual murine IFN-alpha subtypes *in vivo*: intramuscular injection of IFN expression constructs reduces cytomegalovirus replication. *J Immunol* 1998; 160: 2932–2939.
25 Hibbert L, Foster GR. Human type I interferons differ greatly in their effects on the proliferation of primary B cells. *J Interferon Cytokine Res* 1999; 19: 309–318.
26 Brandt ER, Linnane AW, Devenish RJ. Expression of IFN A genes in subpopulations of peripheral blood cells. *Br J Haematol* 1994; 86: 717–725.
27 Greenway AL, Hertzog PJ, Devenish RJ, Linnane AW. Constitutive and virus-induced interferon production by peripheral blood leukocytes. *Exp Hematol* 1995; 23: 229–235.
28 Lallemand C, Lebon P, Rizza P, Blanchard B, Tovey MG. Constitutive expression of specific interferon isotypes in peripheral blood leukocytes from normal individuals and in promonocytic U937 cells. *J Leukoc Biol* 1996; 60: 137–146.
29 Nyman TA, Tolo H, Parkkinen J, Kalkkinen N. Identification of nine interferon-alpha subtypes produced by Sendai virus-induced human peripheral blood leucocytes. *Biochem J* 1998; 329: 295–302.
30 Larrea E, Garcia N, Qian C, Civeira MP, Prieto J. Tumor necrosis factor alpha gene expression and the response to interferon in chronic hepatitis C. *Hepatology* 1996; 23: 210–217.
31 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987; 162: 156–159.
32 Goeddel DV, Leung DW, Dull TJ *et al.* The structure of eight distinct cloned human leukocyte interferon cDNAs. *Nature* 1981; 290: 20–26.
33 Ng SY, Gunning P, Eddy R *et al.* Evolution of the functional human beta-actin gene and its multi-pseudogene family: conservation of noncoding regions and chromosomal dispersion of pseudogenes. *Mol Cell Biol* 1985; 5: 2720–2732.
34 Maruyama M, Fujita T, Taniguchi T. Sequence of a cDNA coding for human IRF-1. *Nucleic Acids Res* 1989; 17: 3292.
35 Itoh S, Harada H, Fujita T, Mimura T, Taniguchi T. Sequence of a cDNA coding for human IRF-2. *Nucleic Acids Res* 1989; 17: 8372.
36 Diepolder HM, Zachoval R, Hoffmann RM *et al.* Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* 1995; 346: 1006–1007.
37 Solinas A, Cossu P, Poddighe P *et al.* Changes of serum 2′,5′-oligoadenylate synthetase activity during interferon treatment of chronic hepatitis C. *Liver* 1993; 13: 253–258.
38 Pawlotsky JM, Hovanessian A, Roudot-Thoraval F *et al.* Activity of the interferon-induced 2′,5′-oligoadenylate synthetase in patients with chronic hepatitis C. *J Interferon Cytokine Res* 1995; 15: 857–862.
39 Fernandez M, Quiroga JA, Martin J *et al. In vivo* and *in vitro* induction of MxA protein in peripheral blood mononuclear cells from patients chronically infected with hepatitis C virus. *J Infect Dis* 1999; 180: 262–267.
40 Kato T, Esumi M, Yamashita S, Abe K, Shikata T. Interferon-inducible gene expression in chimpanzee liver infected with hepatitis C virus. *Virology* 1992; 190: 856–860.
41 Dutia BM, Allen DJ, Dyson H, Nash AA. Type I interferons and IRF-1 play a critical role in the control of a gamma-herpesvirus infection. *Virology* 1999; 261: 173–179.
42 Grieder FB, Vogel SN. Role of interferon and interferon regulatory factors in early protection against Venezuelan equine encephalitis virus infection. *Virology* 1999; 257: 106–118.
43 Au WC, Moore PA, LaFleur DW, Tombal B, Pitha PM. Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. *J Biol Chem* 1998; 273: 29210–29217.
44 Juang Y, Lowther W, Kellum M *et al.* Primary activation of interferon A and interferon B gene transcription by interferon regulatory factor 3. *Proc Natl Acad Sci USA* 1998; 95: 9837–9842.
45 Lee KY, Anderson E, Madani K, Rosen GD. Loss of STAT1 expression confers resistance to IFN-gamma-induced apoptosis in ME180 cells. *FEBS Lett* 1999; 459: 323–326.
46 Galon J, Sudarshan C, Ito S, Finbloom D, O'Shea JJ. IL-12 induces IFN regulating factor-1 (IRF-1) gene expression in human NK and T cells. *J Immunol* 1999; 162: 7256–7262.
47 Cacciarelli TV, Martinez OM, Gish RG, Villanueva JC, Krams SM. Immunoregulatory cytokines in chronic hepatitis C virus infection: pre- and posttreatment with interferon alfa. *Hepatology* 1996; 24: 6–9.
48 Schweyer S, Mihm S, Radzun HJ, Hartmann H, Fayyazi A. Liver infiltrating T lymphocytes express interferon gamma and inducible nitric oxide synthase in chronic hepatitis C virus infection. *Gut* 2000; 46: 255–259.
49 Abb J, Zachoval R, Eisenburg J, Pape GR, Zachoval V, Deinhardt F. Production of interferon alpha and interferon gamma by peripheral blood leukocytes from patients with chronic hepatitis B virus infection. *J Med Virol* 1985; 16: 171–176.
50 Chehadeh W, Chabou S, Fontier C *et al.* In HIV-1-infected patients, plasma levels of HIV-1 RNA are inversely correlated with IFN-alpha responsiveness of whole-blood cultures to sendai virus. *J Clin Virol* 2000; 16: 123–128.
51 Tovey MG, Streuli M, Gresser I *et al.* Interferon messenger RNA is produced constitutively in the organs of normal individuals. *Proc Natl Acad Sci USA* 1987; 84: 5038–5042.
52 Huang X, Yuang J, Goddard A *et al.* Interferon expression in the pancreases of patients with type I diabetes. *Diabetes* 1995; 44: 658–664.
53 Siegal FP, Kadowaki N, Shodell M *et al.* The nature of the principal type 1 interferon-producing cells in human blood. *Science* 1999; 284: 1835–1837.
54 Payvandi F, Amrute S, Fitzgerald-Bocarsly P. Exogenous and endogenous IL-10 regulate IFN-alpha production by peripheral blood mononuclear cells in response to viral stimulation. *J Immunol* 1998; 160: 5861–5868.
55 Kadowaki N, Antonenko S, Lau JY, Liu YJ. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. *J Exp Med* 2000; 192: 219–226.
56 Kanto T, Hayashi N, Takehara T *et al.* Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J Immunol* 1999; 162: 5584–5591.