T Helper Type 1/T Helper Type 17-Related Cytokines in Chronic Hepatitis C Patients before and after Interferon and Ribavirin Therapy

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Key Words

Cytokine · Interferon- γ · Interleukin-17 · Hepatitis C virus · Interferon therapy · Ribavirin therapy

Abstract

Objective: This study examined the T helper (Th) 1/Th17-related cytokines, interferon (IFN)- γ and interleukin (IL)-17 in the serum of biopsy-proven chronic hepatitis C patients before and after IFN and ribavirin therapy to address whether or not viral clearance is related to Th1/Th17 cytokines. **Subjects and Methods:** The serum levels of IFN- γ and IL-17 were assayed by ELISA on 26 patients with chronic hepatitis C virus (HCV) infection before the start and 3 months after treatment with pegylated IFN- α plus ribavirin and compared with sera from 15 normal control subjects. **Results:** IFN- γ and IL-17 levels are higher in the serum of patients with chronic hepatitis than in normal controls and these elevated levels were not directly correlated ($r = -0.01$, $p = 0.96$ for IFN- γ and $r = -0.08$, $p = 0.66$ for IL-17) to the viremic state of the HCV infection. In contrast to IL-17, IFN- γ showed significant reduction after 12 weeks of treatment with pegylated IFN plus ribavirin. However, IFN- γ and IL-17 serum levels were not significantly ($p = 0.19$ and $p = 0.70$, respectively) different among responders and nonresponders for pegylated IFN

plus ribavirin therapy. **Conclusion:** Our findings suggest that the combined treatment with pegylated IFN- α and ribavirin downmodulates the secretion of key cytokine IFN- γ as early as 12 weeks after treatment in infected patients. These findings could encourage new exciting possibilities for immune-based interventions with the aim of restoring functional antiviral T cell responses combined with improved viral clearance.

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Introduction

The adaptive effector CD4⁺ T helper (Th)-mediated immune response is highly heterogeneous, based on the development of distinct subsets that are characterized by different profiles of cytokine production. Initially, two polarized forms of Th effectors, type 1 (Th1) or type 2 (Th2), were identified in both mice and humans [1]. Th1 cells produce interferon (IFN)- γ and are mainly devoted to protection against intracellular microbes, whereas Th2 cells produce interleukin (IL)-4, IL-5, IL-10, and IL-13 and are involved in the protection against extracellular infectious agents. More recently, a novel subset of Th, which is distinct from Th1 and Th2, and is called Th17,

has been described [2]. Th17 cells are characterized by the ability to secrete IL-17A, IL-17F, IL-21, IL-22, and IL-6 [3]. The ubiquitous expression of IL-17 receptors means that it can act on a broad range of cellular targets. IL-17A and IL-17F function primarily as pro-inflammatory mediators, inducing local production of IL-6, prostaglandin E₂, and nitric oxide, and specifically recruiting neutrophils [4]. Th17 cells have been shown to have a protective function in certain bacterial infections [5]. The production of IL-17 has been reported in patients with human immunodeficiency virus infection [6], herpes simplex virus [7] and respiratory syncytial virus infections [8] in rodents. Annunziato et al. [9] identified a subset of cells sharing features of both Th1 and Th17; hence a possible flexibility between Th1 and Th17 cells was suggested.

Hepatitis C virus (HCV) is an enveloped, approximately 9.6-kb, positive-sense, single-stranded RNA virus and is classified in the family of Flaviviridae. Over 170 million individuals worldwide are infected with HCV [10]. Chronic HCV infection is responsible for inflammation of the liver, and approximately 20% of patients progress to liver cirrhosis with an increased risk for the development of hepatocellular carcinoma [10]. Currently, a combination therapy including pegylated IFN- α plus ribavirin represents the standard treatment regimen for patients with chronic HCV infection [11]. Early antiviral response to therapy appears to be predictive of response to treatment [12]. Spontaneous clearance of HCV is associated with a vigorous and robust response of CD4⁺ and CD8⁺ T cells [13], and several studies have indicated an important role for T cells in antiviral therapy-induced clearance [14]. Rowan et al. [15] reported that specific Th17 cells were induced in HCV-infected patients, and demonstrated that transforming growth factor (TGF)- β and IL-10, which are induced by the HCV nonstructural protein 4, suppress Th1 and Th17 responses in HCV-infected patients. This study aimed to assess Th1/Th17-related cytokines (IFN- γ /IL-17) in the serum of patients with chronic hepatitis C infection before and after pegylated IFN- α plus ribavirin therapy compared to normal controls.

Subjects and Methods

Twenty-six patients [male: 17, female: 10, duration of hepatic illness: 32 ± 17 months (range 20–55)] with chronic hepatitis C were selected from those attending the treatment center of viral hepatitis in Ismailia Hospital, Egypt. A diagnosis of chronic hepatitis was made if the following criteria were present after a 6-month follow-up: significant and persistent symptoms; fluctu-

ating or persistently elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (>1.5-fold of normal levels), normal serum albumin and prothrombin time; ultrasonography revealing an enlarged bright texture of the liver $\pm$ portal tract thickening and $\pm$ normal spleen. Liver biopsy was performed to assess hepatic injury using the histological activity index as modified by Ishak [16]. The treatment protocol included: 180 μ g of pegylated IFN- α given subcutaneously once a week plus ribavirin; the dose of ribavirin was adjusted (1,000–1,200 mg/day) given orally according to the body weight. Patients with a history of hepatotoxic drug, alcohol intake, and previous IFN therapy, or who were co-infected with hepatitis B virus were excluded. Fifteen healthy subjects (male: 11, female: 4) were included. Both patients and controls were age and sex matched. The study was approved by the institutional review board of Suez Canal University. Written informed consent was obtained from all participants.

Venous blood samples (7 ml in EDTA and plain tubes) were drawn from each subject for PCR and cytokine testing; serum samples were stored at -60°C until the time of assay. All samples were subjected to the following routine laboratory investigations: complete blood count (CELL-DYN 1700), liver function tests including ALT, AST, total bilirubin, direct bilirubin and serum albumin (HITACHI 912 automatic analyzer, Roche Diagnostics, Mannheim, Germany), α -fetoprotein (VIDAS autoanalyzer, bioMérieux, France), and viral hepatitis markers [HCV antibodies and HBsAg (MEIA, Abbott Laboratories)]. Quantitative HCV real-time PCR was performed for the patients before the start of pegylated IFN- α plus ribavirin treatment and at week 12 of treatment to detect the early virological response using a light cycler-RNA master hybridization probe system in accordance with the manufacturer's instruction (Roche Diagnostics). The lower detection limit was <1,000 IU/ml.

Serum levels of IFN- γ and IL-17 were assessed using a commercially available sandwich enzyme immunoassay kit (Quantikine R&D system, USA). The minimum detection limits were 8 pg/ml for IFN- γ and 15 pg/ml for IL-17.

Statistical analysis was performed using SPSS program version 11. Data are presented as means $\pm$ standard deviation. Independent sample (Student's) t test, paired sample t test and Pearson correlation analysis were used as appropriate. Statistical significance was considered at the 5% level of probability ($p < 0.05$).

Results

Clinical, histological and laboratory parameters of HCV-infected patients and controls enrolled in this study are listed in table 1. Data analysis demonstrated statistically significant differences in platelet counts, ALT, AST, and α -fetoprotein between the HCV-infected patients and controls ($p = 0.030$, $p = 0.003$, $p = 0.001$, $p = 0.012$, respectively).

In HCV-infected patients, prior to starting antiviral treatment, the mean baseline level of IFN- γ was 51 ± 20 pg/ml (range 34–135), whereas control subjects' mean level was 40 ± 4.3 pg/ml (range 30–47). The mean value

of IL-17 in HCV-infected patients was 66 ± 28 pg/ml (range 39–180), whereas for control subjects, the mean level was 43 ± 7.0 pg/ml (range 34–64). The differences between IFN- γ and IL-17 levels for HCV patients and control subjects were statistically significant ($p = 0.014$ and $p = 0.001$, respectively).

Three months after starting treatment with pegylated IFN- α plus ribavirin, the mean level of IFN- γ was 45 ± 10 pg/ml (range 32–78), and the mean level of IL-17 was 69 ± 36 pg/ml (range 42–240). A statistically significant decrease ($p = 0.03$) was obtained between the mean values of IFN- γ before and after treatment but not for IL-17 ($p = 0.45$). The levels of IFN- γ and IL-17 among responders ($n = 19$) versus nonresponders ($n = 7$) for pegylated IFN plus ribavirin therapy did not show any significant differences ($p = 0.19$ and $p = 0.70$, respectively).

The correlations between serum IFN- γ levels and viremia ($r = -0.01$, $p = 0.96$), grading scores ($r = -0.27$, $p = 0.17$), and fibrosis scores ($r = -0.01$, $p = 0.94$) were not significant. Nonsignificant correlations were also detected between the serum levels of IL-17 and viremia ($r = -0.08$, $p = 0.66$), fibrosis scores ($r = -0.19$, $p = 0.34$), and grading scores ($r = -0.41$, $p = 0.23$). A significant positive correlation between serum levels of IFN- γ and IL-17 were detected in controls and HCV-infected patients before and after therapy ($r = 0.57$, $p = 0.027$; $r = 0.70$, $p = 0.001$ and $r = 0.61$, $p = 0.001$, respectively) (fig. 1).

Discussion

Higher levels of Th1 (IFN- γ) and Th 17 (IL-17) were detected in patients compared to controls as shown previously [17]. Both specific Th17 and Th1 cells are induced against the core protein in HCV infection [15, 18].

In chronic HCV infection, a weak CD4⁺/CD8⁺ T cell response is observed. This is explained on the basis of the tolerogenic potential of the liver, which may be caused by either a primary T cell failure in which the dendritic cells fail to stimulate the antigen-specific T cells, or T cell exhaustion in which virus-specific T cells become depleted by a continuous high viral load [19].

Loss of functional properties of antiviral T cells comprises a continuum of severity, ranging from mild to severe deficiencies. Mild deficiencies include the failure to produce certain cytokines while the more severe ones include deficiency in cytokine production [20] and inhibition of intrahepatic IFN- γ production by HCV NS5A as seen in transgenic mice [21]. Defective IFN- γ production by peripheral blood mononuclear cells from HCV-infected

Fig. 1. Scatter diagram showing a significant positive correlation between serum levels of IFN- γ and IL-17 in chronic HCV-infected patients after pegylated IFN plus ribavirin therapy.

Table 1. Clinical, histological and laboratory parameters in chronic hepatitis C patients and controls

Variables	Chronic HCV (n = 26)	Control (n = 15)
Risk factors, n (%)		
Blood transfusion	3 (10)	–
Antibilharzial antibody	20 (74)	–
Previous surgery	3 (10)	–
Histology		
Grading score	2.8 ± 1.3	–
Fibrosis score	5.5 ± 2.0	–
Liver function		
ALT, U/l	$46 \pm 22^*$	17 ± 5
AST, U/l	$47 \pm 37^*$	22 ± 5
T-bilirubin, mg/dl	0.6 ± 0.3	0.6 ± 0.2
D-bilirubin, mg/dl	0.2 ± 0.1	0.1 ± 0.1
Albumin, g/dl	4.2 ± 0.6	4.1 ± 0.7
α -fetoprotein, IU/ml	$9.7 \pm 9.0^*$	4.8 ± 2.1
Viral load		
Mean, IU/ml $\times 10^3$	$1,041 \pm 1,883$	–
Minimum, IU/ml	16,347	
Maximum, IU/ml $\times 10^3$	9,300,000	
Hematological parameters		
Hemoglobin, g/dl	14.0 ± 1.4	14.5 ± 1.0
WBC count, $\times 10^3/\mu\text{l}$	6.4 ± 1.9	6.5 ± 1.4
Platelet count, $\times 10^3/\mu\text{l}$	$200 \pm 78^*$	250 ± 64

WBC = White blood cells. * $p < 0.05$. Figures are means $\pm$ SD, unless indicated otherwise.

ed patients in response to nonstructural protein 4 is another example of the severe deficiencies [22].

Hepatitis C also induces immunosuppressive cytokines IL-10 and TGF- β , which are able to inhibit Th17 and Th1. Neutralization of TGF- β has been shown to increase IL-17 production in response to hepatitis C non-structural protein 4 [15].

On the other hand, some studies demonstrated that inducing elevated levels of IL-17-producing CD4+ and/or CD8+ T cells may facilitate viral persistence in the host. Hou et al. [23] reported that Th17 cells promote persistent viral infection in a model of chronic virus infection and IL-17 upregulates anti-apoptotic molecules and consequently increases persistent infection by enhancing the survival of virus-infected cells and blocking target cell destruction by cytotoxic T cells. Also, Oyoshi et al. [24] reported that IL-17 contributes to viral replication in a model of disseminated vaccinia infection rather than providing host defense against viral infection.

Contrary to our results, the serum levels of IFN- γ in healthy volunteers were similar to levels observed in HCV-infected Polish patients [25], and chronic active hepatitis B patients [26]. This difference may be attributed to the extent of histological liver damage, the activity of the infectious process, assessed by its severity and duration, as well as the difference in the prevalent viral genotype. R-Viso et al. [27] reported significantly lower expression of IFN- γ in the biopsy specimens of HCV-infected cases and liver donor controls assessed by immunohistochemistry; this variation may be attributed to the use of different methodology and liver biopsy specimens rather than serum samples.

In the present study, after pegylated IFN- α plus ribavirin treatment, we observed a significant decrease in the serum levels of IFN- γ , but the levels of IL-17 did not show any statistically significant difference after treatment. This may be explained by the effect of IFN therapy, which induced cytokine shifts. Wang et al. [28] reported that IFN- β reduces the expression of Th1-induced cytokines such as IFN and IL-12 while enhancing anti-inflammatory Th2 responses in patients with multiple sclerosis.

In the current study, we did not find any significant differences in the levels of IFN- γ and IL-17 among responders versus nonresponders for pegylated IFN plus ribavirin therapy. Different results were reported by Perrella et al. [29] who found that rapid virological clearance of HCV-RNA in peripheral blood mononuclear cells with a restored and improved HCV-specific IFN- γ response was significantly higher in those with a sustained virological response. This discrepancy may be attributed to

factors such as the prevalent genotype of the virus as well as the method of assessment of IFN- γ ; we assessed IFN- γ in the serum whereas they assessed it intracellularly.

Serum assessment of cytokines has a number of positive aspects, not the least of which is the feasibility of assaying many samples quickly; it also reflects the systemic effects of cytokines and cytokine production by both immune and non-blood cells. However, as a major function of most cytokines is to act in intercellular communication, cytokine levels in serum and plasma provide at best an indirect measure of this key cytokine function [30].

Cytometric, cellular cytokine assessment, although directly reflecting the cytokine function, is laborious and time consuming. Jason et al. [30] examined the relationships between identical serum and cellular cytokines, and concluded that the relationship, although weak, is in a positive direction.

In the present study, a significant positive correlation between serum levels of IFN- γ and IL-17 was detected in normal controls and chronic HCV patients before and after therapy. This finding may be attributed to the developmental relationship between Th17 and Th1 cells. Furthermore, Romagnani [31] observed the presence in the circulation of Th cells that can produce IL-17 and IFN- γ , as well as the flexibility of human Th17 clones to produce IFN- γ in addition to IL-17 in response to IL-12, suggesting that there may be a developmental relationship between Th17 and Th1 cells, at least in humans.

Conclusion

The combined treatment with pegylated IFN- α and ribavirin downmodulated the secretion of key cytokine IFN- γ as early as 12 weeks after treatment in the infected host. These results provide an opportunity to evaluate the impact of treatment with pegylated IFN- α and ribavirin on prevention of the immune-driven tissue damage, the hepatic inflammation, and progression to liver cirrhosis in infected patients. However, more work is needed to evaluate the influence of other factors in the response to the treatment.

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