ORIGINAL ARTICLE

Antibody against Interferon-α 2b in Serum of the Patients with Chronic Hepatitis C and its Clinical Significance: A Clinical Trial

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Background and Aims: Hepatitis C virus (HCV) infection is treated with alpha interferon (IFN-α). The mechanisms by which IFN-α suppresses HCV replication are not well-known; only limited benefits are achieved with the therapy because the virus in turn, directs some mechanisms to resist the host IFN-α response. The present study was undertaken to determine the therapeutic effects of IFN-α 2b and to measure the serum level of antibody against IFN-α 2b (anti-IFN-α 2b) after treatment with IFN-α 2b in patients with chronic hepatitis C.

Methods: The levels of HCV antibody (anti-HCV), HCV-RNA and anti-IFN-α 2b immunoglobulin G (IgG) in serum of 94 patients with chronic hepatitis C who were treated with IFN-α 2b (three 3-MU injections per week for 3 months), were measured in different courses of treatment.

Results: The total negative rates for anti-HCV and HCV-RNA were 33% (31/94) and 38% (36/94) in patients treated with IFN-α 2b, respectively. The rates for those treated with routine medications were 10% (4/40), 15% (6/40), respectively (P<0.01). After the first, second and third course of treatment with IFN-α 2b, the negative rates of anti-HCV, HCV-RNA in serum were 0% (0/94), 15% (14/94) and 21% (20/94), 23% (22/94) and 33% (31/94), 38% (36/94), respectively. After the treatment with IFN-α 2b, the total positive rate of anti-IFN-α 2b IgG was 5% (5/94). There was no significant difference between the two treatment groups (P>0.05). Among them, after treatment with IFN-α 2b for three, six and 12 months, the positive rates of anti-IFN-α 2b IgG were 0% (0/94), 2% (2/94), 5% (5/94), respectively. The productive ability of anti-IFN-α2b IgG was similar during the different courses of treatment with IFN-α 2b (P>0.05).

Conclusions: IFN-α 2b has curative effect for patients with chronic hepatitis C and its therapeutic efficacy is better than that of routine treatments. Although IFN-α 2b has certain antigenicity, anti-IFN-α 2b IgG production is seen in only a few of patients during the course of treatment; the restrain effect of anti-IFN-α 2b IgG on the further treatment with IFN-α 2b is weak.

Keywords: Interferon-α 2b, Anti-IFN-α 2b, Hepatitis C, Treatment

Introduction

Viral hepatitis C is an important and common infectious disease in China. It is a health burden to our people and can become chronic easily. Conservative figures indicate that greater than 170 million people are persistently infected with hepatitis C virus (HCV) worldwide. Epidemiologic studies have identified the virus as a major cause of chronic hepatitis and liver disease in human. Infection with HCV is currently treated with alpha interferon (IFN-α). Although the treatment outcome is variable

among the six major HCV genotypes, only about half of all the treated patients respond to the therapy, suggesting that the virus encodes proteins that may directly or indirectly attenuate the antiviral actions of IFN-α. Although the mechanisms of IFN-α action against HCV replication have not yet been completely known, several recent studies suggest that that IFN-α has a better effect in the treatment of chronic hepatitis C. After treatment with IFN-α, the duplication of HCV in serum is restrained, its transaminase activity in serum is decreased, and the liver function of the patient is recovered [1-3]. At least three distinct cellular pathways of translational control are responsive to IFN-α and virus infection; those include the 2'-5' oligoadenylate synthetase (2'-5' OAS)/RNase L pathway, the protein kinase R (PKR)-eukaryotic initiation factor 2 alpha subunit (eIF2) pathway, and the P56-eIF3 pathway [4].

IFN-α 2b, a new antiviral medicine, has a better effect on immunoregulation, antiviral immunity and immune clearance of virus [5-11]. After subcutaneous administration, IFN-α specifically binds to high-affinity receptors at the surface of target cells. As IFN-α binds to surface receptors of immune cells, it has multiple immunomodulatory effects. IFN-α binding to various cells and induces numerous proteins and enzymatic pathways involved in establishing a non-virus specific antiviral state through distinct but complementary mechanisms. Specific IFN-α binding site to human hepatocytes and subsequent activation of IFN-α-induced genes leading to the establishment of an antiviral state have not yet been documented [12, 13]. We conducted this study to determine the curative effect of IFN-α 2b and to measure the level of antibody against IFN-α 2b (anti-IFN-α 2b) during the treatment with IFN-α 2b and its influence on the further treatment of patients with IFN-α 2b.

Materials and Methods

Materials

Ninety-four patients with chronic hepatitis C (66 men and 28 women), aged between 22 and 56 years (mean: 34.5), were selected from the Department of Infectious Diseases of the Second Miner's Hospital of Huainan, the Department of Infectious Diseases of the First People's Hospital of Huainan, the Department of Infectious Diseases of Chaoyang Hospital of Huainan from June 2000 to December 2001. The clinical diagnosis of chronic hepatitis C was based on the modified diagnosis criteria being affirmed on the Chinese Viral Hepatitis Conference in Xian (2000). The immunoglobulin G antibody of

HCV (anti-HCV IgG) or anti-HCV IgM in serum of patients was identified by enzyme-linked immunosorbent assay (ELISA); the HCV-RNA in serum was detected by reverse transcriptase polymerase chain reaction (RT-PCR). All the sera taken from patients with chronic hepatitis C had been found clear from co-infection with hepatitis A, B, D, E and human immunodeficiency virus (HIV) viruses. None of them had been treated with immunosuppressants for more than one year.

IFN-α 2b was produced by Anke Biology, High Science and Technology Company of Anhui Province. One course of the treatment consisted of three months; the total course of treatment was one year. Treatment with IFN-α 2b included three 3-MU intramuscular injection of the drug per week. Before and after the treatment, the level of transaminase, anti-HCV, HCV-RNA and anti-IFN-α 2b IgG in serum were measured. A group of 40 (27 male and 13 female) patients with chronic hepatitis C were served as the control group. They aged from 24 to 60 (mean: 36.2) years ad were treated with routine medications. The total of 20 normal blood donors were also included in the study as normal controls (14 men, and 6 women); they aged from 20 to 36 (mean: 24.3) year; their ALT was normal. They also had no active infection with hepatitis A, B, C, D, E, and had received no treatment with immunomodulator. All participants provided informed consent.

Reagent and instrument

The diagnostic ELISA kit for measurement of anti-IFN-α 2b was purchased from Hepatology Institute of Beijing Medical University, Centre of Study and Production of Diagnostic Reagent of Hepatitis of Beijing, No: 991215, 000620, 010310. The ELISA kits used for the quantitative measurement of anti-HCV was purchased from Huamei Biologic Engineering Company of Shanghai, No: 0000415. The diagnostic reagents for measurement of HCV-RNA with RT-PCR were purchased from Zhongya Gene Institute of Shanghai, No: 000520, 010114, 010722. The instruments of automatic enzyme-linked immune assay (EXL808) and automatic microplate washer (ELX-50) were purchased from Bio-Tek Co., USA. The quantitative PCR instrument (Takara-2000) was purchased from Takara Co., Japan.

Methods

Detection of anti-IFN-α 2b IgG in serum: Three mL of fasting venous blood was taken from patients with chronic hepatitis C, before and after

Table 1. The negative rate of anti-HCV, HCV-RNA in serum of patients with chronic hepatitis C before and after treatment with IFN-α 2b.

Group	n	Negative rate of anti-HCV %(n)		Negative rate of HCV-RNA %(n)	
		Before treatment	After treatment	Before treatment	After treatment
IFN-α 2b group	94	0 (0/94)	33 (31/94)*	0 (0/94)	38 (36/94)**
Three months	94	0 (0/94)	0 (0/94)	0 (0/94)	15 (14/94)
Six months	94	0 (0/94)	21 (20/94)	0 (0/94)	23 (22/94)
Twelve months	94	0 (0/94)	33 (31/94)	0 (0/94)	38 (36/94)
Routine treatment group	40	0 (0/40)	10 (4/40)*	0 (0/40)	15 (6/40)**

* χ^2 =7.6826, P<0.01

** χ^2 =7.0830, P<0.01

Table 2. The frequency of patients positive for anti-IFN-α 2b IgG.

Group	n	Negative rate of anti-HCV %(n)		Negative rate of HCV-RNA %(n)	
		number	%	number	%
IFN-α 2b group	94	0	0	5	5*
Three months	94	-	-	0	0**
Six months	94	-	-	2	2**
Twelve months	94	-	-	5	5**
Routine treatment group	40	0	0	0	0*

* χ^2 =1.6634, P>0.05

** χ^2 =5.5745, P>0.05

the treatment, and put in a sterile Eppendorf test tube. After the fresh serum of the patients being isolated by routine method, the anti-IFN-α 2b IgG in serum of the patients was measured. Polystyrene 96-well plates were coated with specific antigen of HCV, diluted in carbonate buffer, as capture antigens. The specific antibodies bound horseradish peroxidases were visualized with tetramethylbenzidine and H_2O_2 diluted in sodium acetate buffer at pH 6.0. The color reaction was stopped by addition of 1.2 mole of H_2SO_4; the absorbance was read at 450 nm. The concentration of antibodies against anti-IFN-α 2b was derived from the standard curves using standard samples in the kits. The controls of anti-IFN-α 2b with two pores of blank, two pores of negative, two pores of positive were made each time. The total optical density (OD) was detected by an automated enzyme-linked immune assay (EXL808) (450 nm) with every titer of OD being recorded for two times. A "positive" result was that the average titer of OD of sample (0.15 + the average titer of OD of "negative" control).

Detection of anti-HCV in serum: Before and after each course of the treatment, the venous blood (3 mL) of patients with chronic hepatitis C were collected in the morning and put in a sterile Eppendorf test tube. After the fresh serum of the patients was isolated by routine method, the anti-HCV in serum of patients was detected. Polystyrene 96-well plates were coated with different anti-HCV McAb, diluted in carbonate buffer, as capture antibodies. Bound horseradish peroxidase was visualized with tetramethylbenzidine and H_2O_2 diluted in sodium acetate buffer at pH 6.0. The color reaction was stopped by addition of 1.2 mole of H_2SO_4; the absorbance was read at 450 nm. The concentration of antibodies against HCV was derived from the standard curves using standard samples in the kits. The controls of anti-HCV with two pores of blank, two pores of negative, two pores of positive were made each time. Every titer was

Table 3. The results of anti-IFN-α 2bIgG in serum of the patients after treatment of IFN-α 2b with still anti-HCV (+) and HCV-RNA.

Group	N	anti-IFN-IgG	
		Positive number	Positive rate
Anti-HCV(+)?HCV-RNA(+) after treatment of α2b-IFN	63	5	7.94*
Anti-HCV(+)?HCV-RNA(+) after treatment of routine medicine	36	0	0.00*

vs routine medicine group, $^*\chi^2$ =1.5854, P>0.05
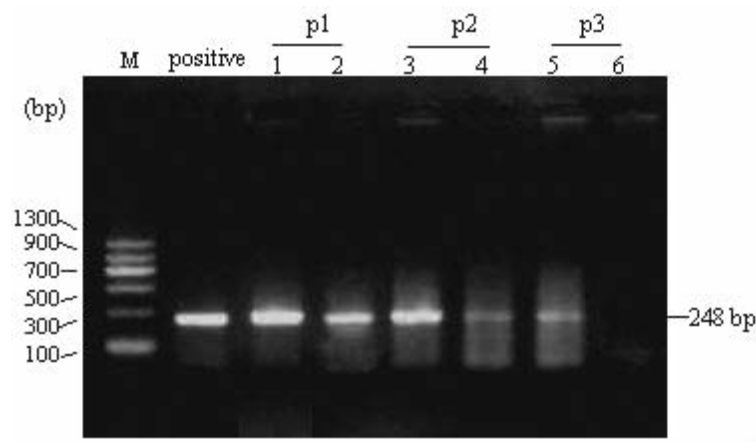
Figure 1. Expression of HCV-RNA before and after treatment with IFN-α 2b [Lanes 1,3,5 (before treatment) and lanes 2,4,6 (after treatment)].

measured twice by EXL808 (Bio-Teck) analysis instrument and the final average OD of titer was calculated. A "positive" result was that the average titer of OD of sample divided by the average titer of OD of "negative" control 2.1.

Detection of HCV-RNA in serum: The controls of HCV-RNA with one negative pore, and one positive pore were made for every detection. The HCV-RNA in serum was isolated with equal quantity routine extract liquid. The primers were selected from the 5'-non-coded region and part of C region of HCV. The sequence of primers of duplication was P9a (–) TCGCAAGCACCCTATCAG-GCAG, P9a (+) GGAACTACTGTCTTCACGCAGA respectively, which yielded a 248-bp specific PCR product. The reverse transcription parameters for HCV were preheated for 4 min at 94 °C, followed by 30 cycles of heating at 94 °C for 40 sec, 55 °C for 40 sec, 72 °C for 1 min, and a final elongation for 5 min at 72 °C, then cooling to 4 °C until the next step. The

cycling conditions for HCV cDNA were preheated for 4 min at 94 °C, followed by 35 cycles of heating at 94 °C for 50 sec, 55 °C for 40 sec, 72 °C for 90 sec, and a final elongation for 5 min at 72 °C, then cooling to 4 °C until electrophoresis. The duplication product was gel electrophoresed and stained with 2% ethidium bromide.

Statistical analysis

The data in our study were expressed as percentages. Differences between groups were assessed by χ^2 test. A P value <0.05 was considered statistically significant.

Results

After treatment with IFN-α 2b, the level of anti-HCV and HCV-RNA in serum of patients with chronic hepatitis C were obviously decreased. The negative rates of anti-HCV and HCV-RNA were all

higher in the IFN-α 2b-treated group than in those received the routine treatment (Table 1). No anti-IFN-α 2b IgG was detected in serum of normal blood donors and in patients with chronic hepatitis C before treatment with IFN-α 2b. However, anti-IFN-α 2b IgG were observed during the treatment with IFN-α 2b (Table 2). After treatment with IFN-α 2b, five patients positive for anti-IFN-α 2b IgG remained also positive for anti-HCV and HCV-RNA (Table 3). The level of HCV-RNA before and after the treatment was significantly different (Fig. 1).

Discussion

Interferons including IFN-α, IFN-β, and IFN-γ are potent biologically active proteins synthesised and secreted by somatic cells of all mammals. Among the three kinds of IFN, the biological effects of IFN-α against viruses are the highest; its principal effect is through preventing the duplication of virus in body. The antiviral activity of IFN is mediated via cell receptors and depends on the activation of signalling pathways, the expression of specific gene products, and the development of antiviral mechanisms. Sensitivity of cells to IFN-mediated antiviral activity is variable and depends on a number of factors including cell type, expression of IFN receptors and downstream effector response elements [14], neutralization antibody against IFN, effectiveness of antiviral mechanisms, the type of virus used to infect cells, *etc*. Although intracellular virus cannot be directly eliminated by IFN, protein kinase and 2',5'-oligo-adenylate synthetase (2', 5'-OAS) can be induced by IFN. A kind of endogenous endonuclease which can degrade virus-RNA is induced by 2', 5'-OAS and the necessary enzyme of ribosome can be inactivated by protein kinase so that the synthesis of protein decreases and the growth of virus is restrained. Detection of anti-HCV is the main method for the diagnosis of HCV infection and is regarded as one of the markers for following the treatment of hepatitis C. The specific core antigens—NS_3 and NS_4 antigen—are in the second generation diagnostic kits of anti-HCV. Therefore, different antibodies against antigens of HCV can be detected which makes the kits highly specific and sensitive [5, 15]. The level of HCV-RNA is an important index to evaluate the level of infection of the virus; it is one of direct evidences of HCV infection [6, 7]. The actual duplication level of HCV can be accurately determined by detection of anti-HCV and HCV-RNA in serum of patients.

Although the total curative effect of IFN is not enough high, it is still an effective antiviral medicine for the treatment of hepatitis C. It aims at HCV-RNA and interferes with the biosynthesis of HCV. Its curative effect is associated with the immune response of patients to the virus [8, 9]. After three courses of treatment of 94 patients with IFN-α 2b, the negative rates of anti-HCV and HCV-RNA in serum were 33%(31/94) and 38%(36/94). The curative effect of IFN-α 2b was batter than that of routine treatments (P<0.01). It is shown that IFN combines with IFN receptor (IFN-R) on the surface of the target cells infected by HCV, and a lot of antiviral protein, such as 2', 5'-OAS, and dsRNA-dependent protein kinase (DDPK) are induced so that HCV-RNA is decomposed, translation of HCV mRNA is restrained [10], and the duplication of HCV and single transmission of mRNA in the target cells are restrained. IFN has also immunoregulatory effects and can induce cellular immune response of HCV-CTL so that HCV particles are wiped out. The expression of HLA-antigens on surface of liver cells IFN can be induced so that the transmission effect of virus antigen and the eliminative effect of virus will all increase [11, 16, 17].

It was shown that the conjugated antibody and neutralizing antibody against IFN appear after two or three months of treatment and a high positive rate of anti-IFN is observed after treatment and persisted for three to six months. The highest and lowest type of anti-IFN in frequency was anti-IFN-α 2a and anti-IFN-α 1, respectively. The highest positive rate of anti-IFN was anti-IFN-α 2b [18, 19]. Anti-IFN was easily induced by a little dose and a long course of treatment. The positive rate of conjugated antibody against IFN was from 5% to 20%. Different levels of conjugated antibody had various clinical manifestations. The positive rate of neutralizing antibody of IFN is less than 5%. If the conjugated antibody against IFN was produced in early treatment, the curative effect of IFN must be decreased. The results of our study showed that little anti-IFN is produced in early treatment. However, the low level of anti-IFN-α 2b IgG could be produced in a few of patients with extending the treatment course; though its positive rate was only 5% (5/94), which is lower than that reported by Li, *et al.* [20]. The results showed that the antigenicity of IFN is weak so that only in a few patients a low level of anti-IFN response would be observed. IFN is still a safe and effective medicine for the treatment of the patients with chronic hepatitis C.

IFN are well-known to up-regulate various immune responses. It is thought that it does so

largely by increased expression of cell surface molecules [21]; for example, β2-microglobulin, class I MHC antigens, and IgG-Fc receptor (FcR) which involves in immune recognition. After treatment with IFN-α 2b for nine months, the positive rate of anti-HCV, and HCV-RNA in serum of patients with chronic hepatitis C was still 67% (63/94) and 62% (58/94), respectively. The results showed that the curative effect of IFN, low negative rate of anti-HCV, and HCV-RNA in serum were not satisfactory enough for most people. A possible reason is that after IFN entered the body, it would easily be decomposed by protease in kidney. The distribution of IFN is not even in different tissues—most of IFN is in blood, liver, kidney, and less is in other tissues. After HCV invades peripheral blood mononuclear cells (PBMC), the cellular immune response of the patient is obviously malfunctions and the antiviral response is restrained [22, 23]. In PBMC, some antigen synthesis of HCV is restrained by IFN and therefore cleaning of infected PBMCs from HCV becomes more difficult [7]. During the course of antiviral treatment, the immune system must be activated. The ratio of helper to suppressor T cells (T_h/T_s) and the level of interleukin-2-receptor (IL-2R) must be increased. Because of the disorder in cellular immune response in patients and lower level of membrane IL-2R, the antiviral effect of IFN would be limited to a certain extent. The data in our study confirmed that the positive rate of anti-IFN IgG in serum of patients with chronic hepatitis C who were treated with IFN-α 2b for three courses of treatment was only 8% (5/63). There was no statistically significant difference between the group of anti-HCV+, HCV-RNA+ treated with IFN-α 2b and those anti-HCV+, HCV-RNA+ treated with routine treatments (P>0.05). The possible reason was that IFN has a weak immunogenicity and a low level of anti-IFN-α 2b IgG can be induced during the treatment course. It is not confirmed that the low level of anti-IFN IgG is best direct and important reason for the therapy effect of IFN-α 2b with not enough satisfied for most people. During the treatment with IFN, little amount of anti-IFN IgG can neutralize even a high dose of IFN injected. Fever and general malaise can be induced by a high level of IFN. The cellular immune function of patients with chronic hepatitis C is damaged to a certain extent so that the induced reaction to IFN is light and the antiviral effect is weak [24-26]. The number of samples in our study was not enough and further studies on larger samples are needed so that the trend of anti-IFN IgG level can be identified in more detail. After treatment of patients with routine medications for nine months, few patients had positive anti-IFN IgG. The results showed that the anti-IFN IgG induced by IFN coming from autoimmune T lymphocytes was more difficult.

In conclusion, IFN-α 2b has a high curative effect for patients with chronic hepatitis C and with viremia. The negative rates of HCV-RNA and anti-HCV in serum of patients with chronic hepatitis C were higher after treatment with IFN with three 3-MU injections per week. If the course of treatment of IFN were long enough, the negative rates of HCV-RNA and anti-HCV in serum would be even higher. Although IFN-α 2b has certain antigenicity levels, the rate of anti-IFN-α 2b IgG production during the course of treatment and the restrain effect of anti-IFN-α 2b IgG on the treatment are low. If high level of anti-IFN-α 2b is produced during the treatment with IFN-α 2b, other effective antivirals should be used.

Acknowledgements

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