

Tumor Necrosis Factor α Gene Expression and the Response to Interferon in Chronic Hepatitis C

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Tumor necrosis factor α (TNF- α) is a cytokine with pleiotropic properties that is induced in a variety of pathological situations including viral infections. In this work, we analyzed the expression of TNF- α gene in patients with chronic hepatitis C. Serum TNF- α levels were found to be elevated in all chronic hepatitis C patients including those cases presenting sustained biochemical remission of the disease after interferon therapy. Untreated patients with chronic hepatitis C showed increased TNF- α messenger RNA (mRNA) levels in the liver and mononuclear cells as compared with healthy controls. After completion of treatment with interferon, patients experiencing sustained complete response showed values of TNF- α mRNA, both in the liver and in peripheral mononuclear cells, within the normal range, significantly lower than patients who did not respond to interferon and than those with complete response who relapsed after interferon withdrawal. Pretreatment values of TNF- α mRNA were lower in long-term responders to interferon than in cases who failed to respond to the treatment. Values of TNF- α mRNA in the liver or in mononuclear cells were higher in specimens with positive hepatitis C virus (HCV) RNA than in those samples where the virus was undetectable. Neither the intensity of the liver damage nor the amount of HCV RNA in serum or in cells showed correlation with the levels of TNF- α transcripts in peripheral mononuclear cells but it was found that high TNF- α values were associated with genotype 1b. In conclusion, there is an enhanced expression of TNF- α in HCV infection. High levels of this cytokine may play a role in the resistance to interferon therapy. (HEPATOLOGY 1996;23:210-217.)

Tumor necrosis factor α (TNF- α) is a cytokine, produced primarily by activated monocytes and lymphocytes, that possesses pleiotropic properties.^{1,2} It participates in the induction of the immune response to

infectious agents and has been shown to exert direct antiviral effects.³⁻⁵ Although low levels of TNF- α can contribute to cell protection, excessive amounts may cause cell damage.^{2,6}

The biosynthesis of TNF- α is tightly regulated by transcriptional and post-transcriptional mechanisms.¹ A variety of viruses have been shown to induce the expression of TNF- α *in vitro*.⁶⁻⁹ The accumulation of TNF- α messenger RNA (mRNA) that occur in virus-infected cells^{6,8,9} may result from both increased transcription and stabilization of the transcripts.⁸ Accumulation of TNF- α mRNA primes the cell for enhanced release of bioactive TNF- α in response to other inducers such as cytokines, C5a, or bacterial products.^{1,8}

TNF- α has been involved in the pathogenesis of a diversity of liver conditions including viral hepatitis.¹⁰⁻¹⁸ Increased production of TNF- α by peripheral blood mononuclear cells (PBMC) has been observed in fulminant viral hepatitis,¹⁷ in chronic hepatitis B and in chronic non-A non-B hepatitis.^{16,18} Recently, raised serum TNF- α levels have been shown in chronic hepatitis C virus (HCV) infection.¹⁹

HCV is a single-stranded RNA virus that infects both the liver and lymphoid cells.²⁰⁻²² HCV infection has a strong tendency to chronicity and constitutes a very common cause of chronic liver disease in the western world. Thus far, interferon alfa (IFN- α) is the most effective antiviral agent in chronic hepatitis C. However, only about 50% of the treated patients respond to IFN- α therapy with normalization of serum transaminases, and half of these patients relapse after interruption of the treatment.²³

Little is known about the mechanisms determining the severity of the liver damage and the response to IFN- α in HCV infection. In this paper, we have studied the levels of TNF- α in serum, TNF- α transcripts in the liver and PBMC in patients with chronic hepatitis C and the relationship between this cytokine and the response to IFN- α treatment. Our results indicate that the presence of HCV RNA in the liver or in PBMC is associated with increased TNF- α gene expression and that enhanced TNF- α synthesis may contribute to cause refractoriness to the therapeutic actions of IFN- α . No relationship was observed between TNF- α mRNA values and the activity of the liver disease.

PATIENTS AND METHODS

Patients. Serum TNF- α concentration was determined in 17 healthy controls (7 male and 10 female; age range, 25 to

Abbreviations: TNF- α , tumor necrosis factor α ; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cells; HCV, hepatitis C virus; IFN- α , interferon alfa; UT, untreated group; SR, sustained response; Rr, response with relapse; NR, no response; ALT, alanine aminotransferase; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcriptase-polymerase chain reaction; cDNA, complementary DNA.

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50 years) and 47 patients with chronic hepatitis C (27 male and 20 female; age range, 21 to 75 years). This group of patients included 16 untreated patients and 31 patients who were studied after completion of treatment with IFN- α (3 MU daily for 2 months and 3 MU 3 times a week for 8 to 10 months); of these, 15 did not respond to the therapy and 16 exhibited sustained biochemical remission of the disease for more than 6 months after stopping IFN- α administration. In addition we analyzed TNF- α mRNA levels in 71 PBMC samples from 61 patients with chronic hepatitis C (44 male and 17 female; age range, 21 to 72 years) and 17 healthy controls (11 male and 6 female; age range, 25 to 38 years). Liver biopsy results showed chronic active hepatitis in all of them, and cirrhosis was present in 4 cases. Histological activity index in liver samples was determined according to Knodell's scoring system.²⁴ Twenty patients had not received treatment (untreated group [UT]), and 36 were studied at the end of 10 to 12 months of IFN- α therapy. These patients were classified according to the response to IFN- α into three groups: sustained response (SR), response with relapse (Rr), and no response (NR). The SR group (n = 12) included all patients with normal alanine aminotransferase (ALT) values at the end of therapy with persisting normal values during more than 1 year of follow-up. The NR group (n = 11) included those patients with elevated ALT levels at the end of therapy. The Rr group (n = 13) included all patients with normal ALT levels at the end of treatment in whom serum ALT levels increased after interferon withdrawal. In 5 patients, the study was performed on two occasions, before initiation of the treatment with IFN- α and at the end of the therapy. All these cases showed a sustained response to the treatment. Fifteen additional patients showing persistent normal serum ALT levels after IFN- α therapy were studied 4 to 15 months after completion of the treatment (these cases are referred to as the SR-PT group). Five of these patients had also been studied at the end of treatment.

Samples from liver biopsies from 35 patients (26 males and 9 females; age range, 16 to 71 years) with chronic hepatitis C (4 with cirrhosis) were studied. This group of patients (of whom 14 also had PBMC analyzed) included 10 UT and 25 treated subjects in whom liver biopsy specimens were obtained at the end of 12 months of therapy (8 NR, 9 SR, and 8 Rr). Normal liver samples were obtained from 6 controls (1 male and 5 female; age range, 41 to 66 years) at laparotomy (in 5 cases because of gallstones and in 1 because of colon cancer without apparent liver metastasis).

All patients included in the present study were anti-HCV-positive using enzyme-linked immunosorbent assay (ELISA) (Ortho Diagnostic Systems, second-generation, Raritan, NJ) and all of them were or had been (before treatment) serum HCV RNA positive using the reverse transcription polymerase chain reaction. Other causes of chronic hepatitis were excluded.

Preparation of PBMC, Serum, and Liver Samples. For isolation of PBMC, fresh blood diluted with 1 volume of 0.9% NaCl was overlaid on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) with a density of 1.077 g/mL and centrifuged 600g for 30 minutes. The mononuclear cell layer was harvested at the interphase and washed twice in 0.9% NaCl. PBMC were lysed in 4 mol/L guanidinium thiocyanate and stored at -80°C until extraction of total RNA, which was performed according to the method of Chomczynski and Sacchi.²⁵

To obtain serum samples, venous blood was collected into sterile vacuum blood collection tubes and then centrifuged. Serum was stored at -70°C until used.

Liver specimens were obtained with Tru-Cut biopsy nee-

dles (Baxter, Deerfield, IL). One third of the specimen was immediately frozen in liquid nitrogen and stored at -80°C until the extraction of RNA, and the remaining was used for histological study.

Analysis of Serum TNF- α Levels. To analyze the serum concentration of TNF- α we used an ELISA (Cytoscreen; Biosource International, Camarillo, CA) using specific anti-human TNF- α antibodies. The assay was performed following the instructions of the manufacturers. The sensitivity limit of the assay was 1 pg/mL.

Analysis of TNF- α mRNA Levels. To analyze TNF- α mRNA levels we used a procedure based on the reverse transcription-polymerase chain reaction (RT-PCR). Either PBMC or liver total RNA (0.75 μg) was reverse-transcribed (60 minutes at 37°C) with 250 U of M-MuLV reverse transcriptase (BRL, Gaithersburg, MD) in 25 μL volume of $5\times$ RT buffer (0.25 mol/L Tris-HCl pH 8.3, 0.375 mol/L KCl, 15 mmol/L MgCl_2), supplemented with 5 mmol/L dithiothreitol, 0.4 mmol/L deoxynucleoside triphosphate, ribonuclease inhibitor (30 U), and random hexamers (250 ng) (Boehringer Mannheim, Mannheim, Germany). After heating (95°C , 5 min) and quick-chilling on ice, 6.7- or 10- μL aliquots of the complementary DNA (cDNA) pool (PBMC or liver respectively) were used for PCR amplification in 50 μL of $10\times$ PCR buffer (100 mmol/L Tris-HCl pH 9.3, 500 mmol/L KCl, and 1% Triton X-100) containing 0.02 mmol/L deoxynucleoside triphosphate, 50 $\mu\text{Ci}/\text{mL}$ of (α - ^{32}P)-deoxythymine triphosphate (Amersham, Buckinghamshire, England), upstream and downstream primers (40 ng each), 1.5 mmol/L MgCl_2 and 2 U of Taq DNA polymerase (Promega Corporation, MD). Blank reactions with no RNA were performed in all experiments. PCR amplifications of a fragment of β -actin cDNA (using 10 μL of the cDNA pool) were also performed as internal control for each sample. TNF- α cDNA fragments were amplified by 25 or 29 cycles (PBMC or liver, respectively) (94°C , 59°C , and 72°C , 1 minute each step), and β -actin was amplified by 20 cycles (94°C , 55°C , and 72°C , 1 minute each step) a protocol that avoided interference of the plateau effect. Oligonucleotides (5'-3') d(GTCAGATCATCTTCTCGAACC) and d(CAGATAGATGGGCTCATAACC) were the upstream and downstream primers, respectively, used for amplification of 360-base-pair (bp) fragment (nucleotides 315-673) from human TNF- α cDNA.²⁶ d(TCTACAATGAGCTGCGTGTG) and d(GGTGAGGATCTTCATGAGGT) were the primers used to amplify a 314-bp fragment from human β -actin cDNA that is located between nucleotides 1319-2079 in the reported human β -actin gene sequence.²⁷ After PCR amplifications, 20 μL aliquots of the PCR reactions were electrophoresed in 2% agarose gel and bands were visualized by ethidium bromide. 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 TNF- α mRNA were normalized to those of β -actin mRNA. Results are expressed as counts-per-minute (cpm) ratio of TNF- α to β -actin. When TNF- α mRNA values were normalized to those of β -actin mRNA, it was assumed that β -actin mRNA was constantly expressed in patients with chronic hepatitis C, both in patients who did not receive treatment and in those treated with IFN- α . To confirm this, we analyzed simultaneously 15 PBMC and 15 liver samples (5 healthy controls, 5 untreated patients, and 5 treated patients). No differences in the cpm values corresponding to β -actin mRNA were observed between the groups. In PBMC samples, cpm values were 14808.8 ± 1411.7 , 14238.2 ± 1508.8 , and 13453.8 ± 1038.3 in HC, UT, and treated patients, respectively; and in liver samples, cpm values were 8351.0 ± 626.0 , 9313.8

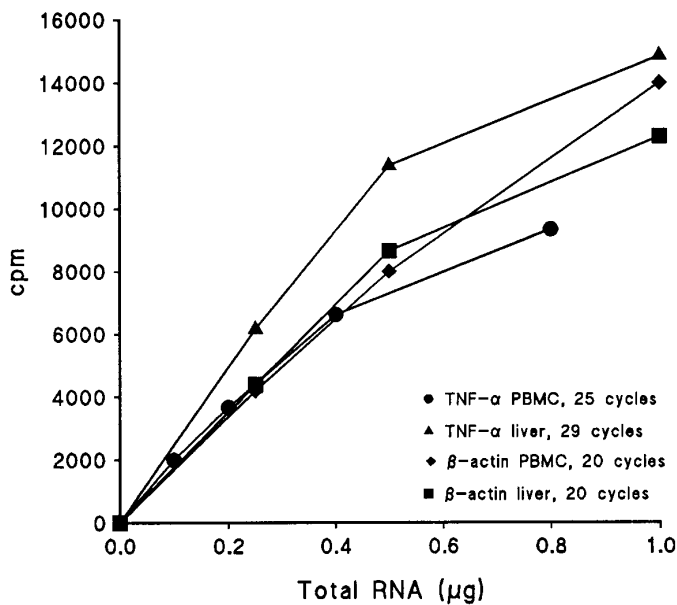


FIG 1. Relationship between input of total RNA in the reaction and cpm of the amplification band after RT-PCR of TNF- α and β -actin gene products in PBMC and in the liver.

± 1855 , and 7899.6 ± 744.0 in HC, UT, and treated patients, respectively.

Validation experiments of PCR assays using known quantities of total RNA (from 0 to 1 μ g) were performed (Fig. 1). The cpm values corresponding to the used amount of either TNF- α or β -actin mRNA (0.2 and 0.3 μ g for TNF- α in PBMC and liver, respectively, and 0.3 μ g for β -actin in both) were in the linear range of the curve. To analyze whether changes of TNF- α mRNA levels relative to those of β -actin resulted in changes of the ratio of TNF- α PCR product cpm/ β -actin PCR product cpm, we performed a RT-PCR with 0.1 or 0.3 μ g of total PBMC RNA from a healthy control (for TNF- α or β -actin, respectively) and added serial dilutions of known quantities of TNF- α riboprobe (from 25 pg to 0.19 pg). TNF- α riboprobe was obtained by run-off transcription of a plasmid which contained a 760-bp insert (nucleotides 156-916) from the human TNF- α cDNA.²⁶ As shown in Fig. 2, with increasing amounts of TNF- α mRNA used in the reaction, we obtained a progressive increase of the TNF- α / β -actin ratio (the increase was linear up to 1.56 pg of the TNF- α riboprobe).

The coefficient of interassay variation for TNF- α / β -actin was 13.58%. The identity of the PCR products from TNF- α cDNA amplification was further verified, first, by Southern blot analysis, using α -³²P-labelled *Pst*I/*Bam*HI fragment (1.6 kb-long) of TNF- α cDNA²⁶ as hybridization probe and, second, by digestion with *Bgl*II, *Pvu*II and *Eco*RI. The two former enzymes yielded the predicted restriction fragments while *Eco*RI did not digest the amplified PCR product (no restriction site for this endonuclease is present in the amplified region).

Detection and Quantification of HCV RNA. The presence of HCV RNA in liver and PBMC was studied by RT-PCR as previously described.²⁰ Briefly, 0.25 μ g of total RNA was reverse transcribed with M-MuLV reverse transcriptase (50 U) (BRL, Gaithersburg, MD) in 10- μ L volume of 5 \times RT buffer supplemented with 200 ng of the outer antisense primer. cDNA synthesis proceeded for 60 minutes at 37°C, and was stopped by heating at 95°C for 5 minutes followed by quick

chilling on ice. cDNA solution was used for the first PCR in 50 μ L of 10 \times PCR buffer, which also contained 200 ng of the outer sense primer and 2 U of Taq polymerase (Promega Corporation, MD). This first amplification was carried out by 35 cycles (94°C, 52°C, and 72°C, 1 minute each step except 7 minutes for final extension at 72°C). For the second PCR, 2 μ L of the first PCR product was amplified (30 cycles) using inner primers (200 ng each) and the same conditions as in the first PCR. After amplification, 15- μ L aliquots of the final PCR reaction were electrophoresed in 2% agarose gel and the bands were visualized by ethidium bromide staining. The sequences for oligonucleotide primers were chosen from five noncoding regions of the HCV genome.²⁸ The primers for HCV cDNA synthesis and PCR amplification were: the outer sense, GTATCTCGAGGCGACACTCCACCATAGAT, and outer antisense, ATACTCGAGGTGCACGGTCTACGAGACCT; and inner sense, CCACCATAGATCTCTCCCCTGT, and inner antisense, CACTCTCGAGCACCTATCAGGCAGT.^{28a} For HCV RNA analysis in serum, 50 μ L aliquots were extracted. HCV RNA was reverse transcribed with random primers and PCR was performed using the above conditions.

HCV RNA was quantified by a competitive PCR technique. Briefly, RNA from 200 μ L of serum or 1.5 μ g of total RNA from PBMC was reverse transcribed with random primers in

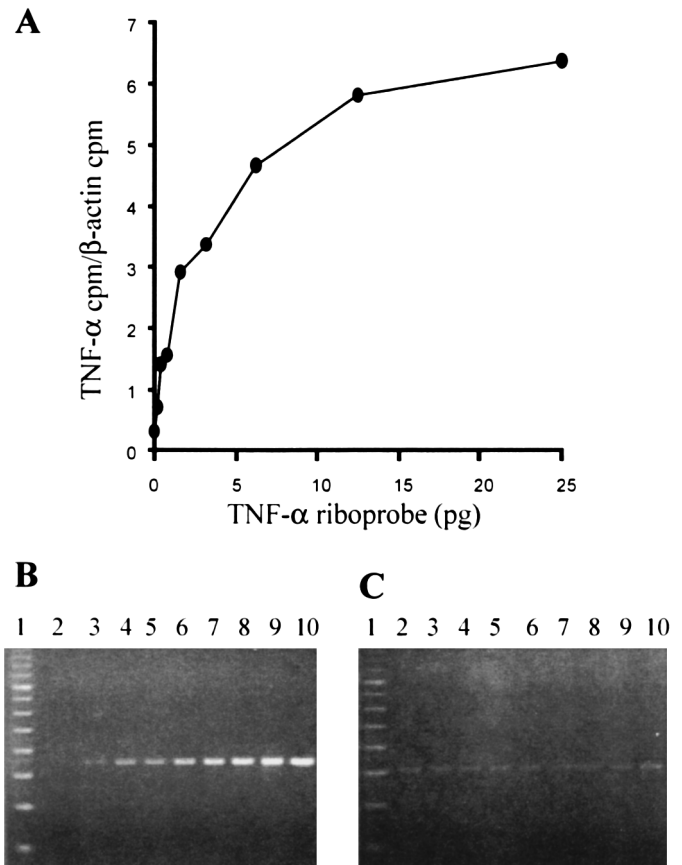


FIG 2. (A) Relationship between the amount of TNF- α riboprobe added to RNA extracted from normal PBMC and the ratio TNF- α PCR product cpm/ β -actin PCR product cpm. (B and C) The gel photographs show increasing intensity of the TNF- α amplification band (B) but constant intensity of the β -actin amplification band (C), when increasing amounts of a TNF- α riboprobe (0, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 pg, lines 2 to 10, respectively) are added to the RNA extracted from normal PBMC. Line 1, molecular weight.

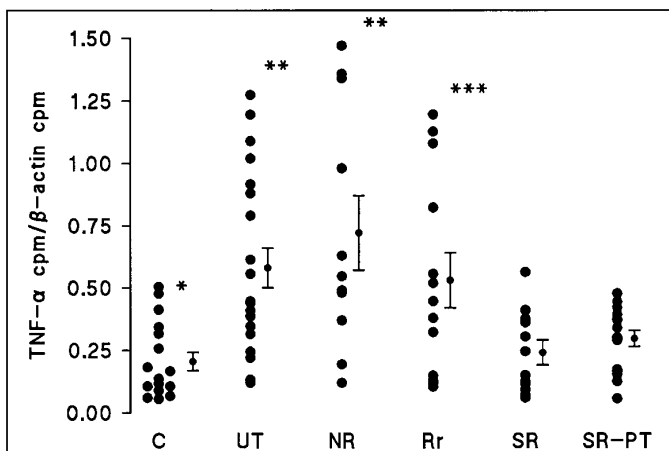


FIG 3. Transcriptional expression of TNF- α gene (as a ratio to that of β -actin) in PBMC in healthy controls (C) and patients with chronic hepatitis C without treatment (UT) or after 10 to 12 months of interferon alfa treatment. NR, patients with no response to interferon studied at the end of the treatment; Rr, patients with complete response but with post-therapy relapse studied at the end of the treatment; SR, patients with complete response without post-therapy relapse (sustained response) studied at the end of the treatment; SR-PT, patients with sustained response studied 4 to 15 months after completion of interferon treatment. * $P < .01$ vs. UT or NR or Rr; ** $P < .05$ vs. SR or SR-PT; *** $P < .05$ vs. SR.

40 μ L or 60 μ L of volume, respectively. After reverse transcription, 5 μ L of cDNA from serum or 10 μ L of cDNA from PBMC was mixed with serial dilutions of a competitor DNA (6×10^5 , 6×10^4 and 6×10^3 molecules for serum, and 3×10^5 , 6×10^4 , 3×10^4 , and 6×10^3 for PBMC) that differed from the cDNA of interest by a small deletion (45 bases). This competitor DNA was produced from cloned DNA from 5' noncoding region of one HCV-infected patient. Samples were amplified by nested PCR with two sets of primers (5'-CCTGTGAGGAACTACTGTCT-3' and 5'-CTATCAGGCAGT-ACCACAAG-3' for outer primers; 5'-ACTGTCTTCACGCAG-AAAGC-3' and 5'-GACCCAACACTACTCGGCTA-3' for inner primers). For both PCRs, 30 cycles were performed as follows: denaturation for 15 seconds at 95°C, annealing for 12 seconds at 52°C, and extension for 20 seconds at 72°C with a final extension for 1 minute at 72°C, in a Perkin Elmer (Norwalk, CT) Gene Amp PCR System 9600. First PCR were performed in 40- μ L mixtures and 2 μ L of the first PCR product was transferred to the second PCR mixture performed in 20 μ L, and containing 25 μ Ci/mL of (α - 32 P)-deoxycytidine triphosphate. After second PCR amplifications, 15- μ L aliquots of the PCR reactions were electrophoresed in 2.5% agarose gel, and bands were visualized by ethidium bromide. Equal-sized bands from cDNA and competitor DNA were excised and radioactivities were determined and compared. The cpm corresponding to competitor DNA was corrected by the number of cytosines in which competitor DNA differs from the studied cDNA. The copy number of HCV RNA in the samples is considered equal to the copy number of the competitor when the cpm of the studied cDNA/cpm of the competitor is equal to 1.

HCV Genotypes. Genotyping was performed by means of a hybridization procedure using specific probes for HCV genotypes 1a, 1b, 2a, 2b, and 3a, according to Simmonds et al.,²⁹ and the amplified nested PCR product of the HCV core region. Probes, primers, and technique were as described by Viazov et al.³⁰ with the following modifications: the primers for the

second PCR were 5'-labelled with digoxigenine, and hybridization was detected using an antidigoxigenine peroxidase-labeled antibody (Boehringer Mannheim).

Statistical Analysis. Results are presented as mean \pm SE. The statistical analysis was performed using nonparametric tests (Kruskal-Wallis and Mann-Whitney U tests). Pearson's correlation coefficients were evaluated by Student's t test.

RESULTS

TNF- α Gene Expression and the Response to Interferon Treatment. Serum TNF- α levels were higher in untreated patients with chronic hepatitis C (166.28 ± 33.21 pg/mL) and in those who failed to respond to IFN after 10 to 12 months of treatment (163.05 ± 36.72 pg/mL) than in healthy controls (19.28 ± 5.20 pg/mL; $P < .001$). Patients who made a complete and sustained biochemical response to IFN- α therapy also showed serum TNF- α levels (117.14 ± 24.46 pg/mL) significantly higher than control values ($P < .001$). Although in this group of patients serum TNF- α tended to be lower than in nonresponders to IFN- α , this difference did not attain statistical significance.

TNF- α mRNA levels in UT, NR, and Rr patients were higher than in healthy controls both in PBMC (Fig. 3) and in the liver (Fig. 4). In contrast, in SR patients, TNF- α mRNA values in PMBC and in liver tissue were similar to controls and significantly lower than in UT, NR, and Rr patients. No differences in hepatic and PBMC TNF- α mRNA levels were observed among these last three groups of patients. TNF- α gene expression in PBMC in SR-PT group was similar to that of the SR group (Fig. 3).

Fifteen patients from the UT group underwent IFN- α therapy. Of these, 5 experienced sustained response to the treatment, and 10 did not respond to IFN- α . Pretreatment levels of TNF- α mRNA in PBMC were significantly higher in the patients who failed to respond to IFN- α than in those patients who exhibited a sustained response to the therapy (0.75 ± 0.1 vs. 0.28

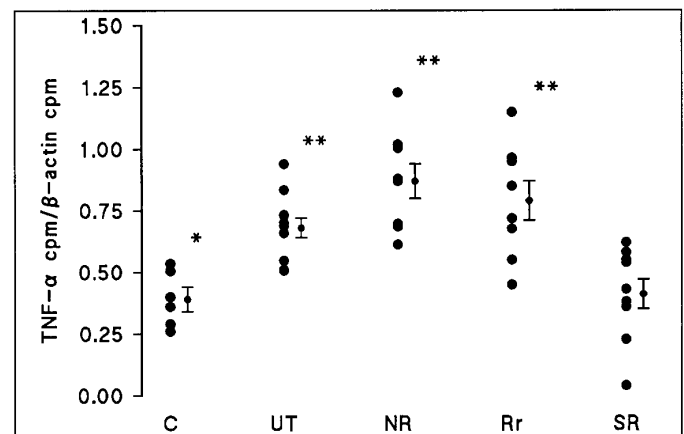


FIG 4. Transcriptional expression of TNF- α gene (as a ratio to that of β -actin) in the liver in patients with chronic hepatitis C. (C) normal liver. Other initials as in Fig. 2. * $P < .005$ vs. UT or NR or Rr; ** $P < .006$ vs. SR.

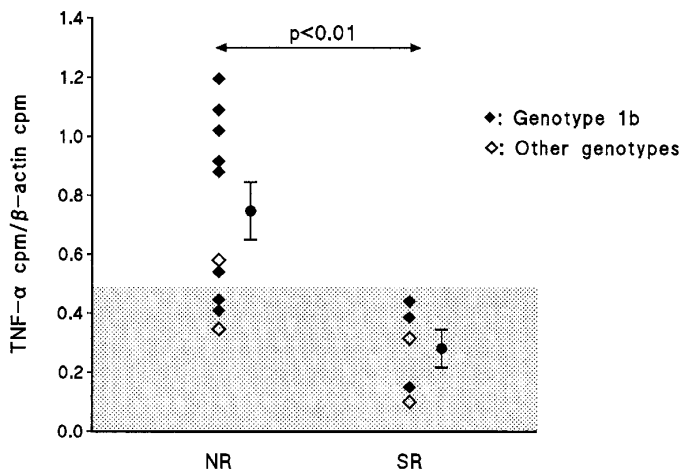


FIG 5. Pretreatment values of TNF- α mRNA (as a ratio to β -actin) in PBMC in patients with chronic hepatitis C; SR, patients who subsequently underwent interferon therapy and experienced a sustained complete response to the treatment, and NR patients who failed to respond to the therapy. Shaded area represents healthy control values (mean \pm 2SD).

\pm 0.06, $P < .01$) (Fig. 5). These data suggest that high pretreatment TNF- α mRNA values are associated with lack of response to IFN- α .

In five patients who presented sustained response to IFN- α , TNF- α mRNA levels were determined in PBMC before and at the end of the therapy. Of these cases, in one there was a substantial reduction in the levels of the cytokine transcripts (from 0.44 before treatment to 0.09 after treatment), in two there was a slight decrease (from 0.39 to 0.30 and from 0.13 to 0.06), and in the remaining two there was a slight increase (from 0.31 to 0.41 and from 0.13 to 0.24). Overall, IFN- α treatment did not induce significant changes in TNF- α mRNA values in this group of patients who already presented low basal levels of TNF- α transcripts.

HCV genotyping was performed in 18 UT patients and in 36 patients studied at the end of IFN- α therapy. In UT patients, genotype 1b was observed in 13, genotype 1a in 2 patients and genotype 3 in 3 patients. Among patients studied at the end of IFN- α therapy, genotype 1b was found in all NR subjects, in 11 of 13 Rr cases and in 8 of 12 SR patients; genotype 3 was found in 4 patients (2 Rr and 2 SR), and genotype 1a in 2 patients (both SR). When we analyzed the relationship between the transcriptional expression of TNF- α in PBMC and the HCV genotype of the patient, we observed that in both untreated patients (Fig. 5) and in the patients studied after IFN- α treatment (Fig. 6), high values of TNF- α transcripts (>0.60) were associated with genotype 1b. Also, as shown in Figs. 5 and 6, the majority of patients who failed to respond to IFN- α showed genotype 1b.

TNF- α Gene Expression and the Presence of HCV RNA. We studied the relationship between TNF- α gene expression and the presence of HCV RNA in the liver and in PBMC. As indicated in Table 1, in untreated patients, HCV RNA was detected in liver tissue in all

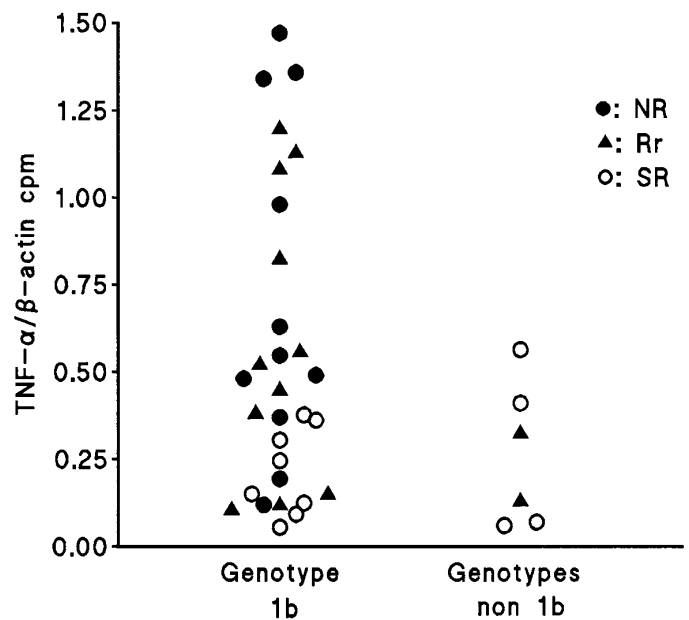


FIG 6. Levels of TNF- α mRNA (as a ratio to β -actin) in patients with chronic hepatitis C at the end of 10 to 12 months treatment with interferon. Patients were divided according to their HCV genotype. NR, no response to interferon; Rr, complete response with post-therapy relapse; SR, sustained complete response.

cases and in 19 of 20 PBMC samples. In patients who had received IFN- α treatment, HCV RNA was present in 19 of 51 PBMC samples and in 16 of 25 liver biopsy specimens (see Table 1).

As shown in Fig. 7, when the patients studied at the end of 10 to 12 months of treatment with IFN- α were divided according to the presence or absence of HCV RNA in the liver or in PBMC, it was shown that TNF- α mRNA values in the corresponding tissue were higher in those cases with detectable HCV RNA than in cases where HCV RNA was negative (0.70 ± 0.10 vs. 0.28 ± 0.03 , $P < .002$ in PBMC; 0.79 ± 0.06 vs. 0.49 ± 0.09 , $P < .05$ in liver).

TABLE 1. Presence of HCV RNA in Liver Samples and in PBMC From Patients With Chronic Hepatitis C

	Liver	PBMC
UT	9/9 [†]	19/20
NR*	7/8	9/11
Rr*	7/8	9/13
SR*	2/9	0/12
SR-PT	—	1/15

Abbreviations: UT, untreated patients; NR, patients who did not respond to the treatment; Rr, patients with complete response but with posttreatment relapse; SR, patients with complete response and sustained normal aminotransferases during posttherapy follow-up; SR-PT, patients with sustained response studied 4 to 12 months after completion of interferon treatment.

* Patients at the end of 10 to 12 months of treatment with interferon alfa.

[†] Number of positive cases/number of cases studied.

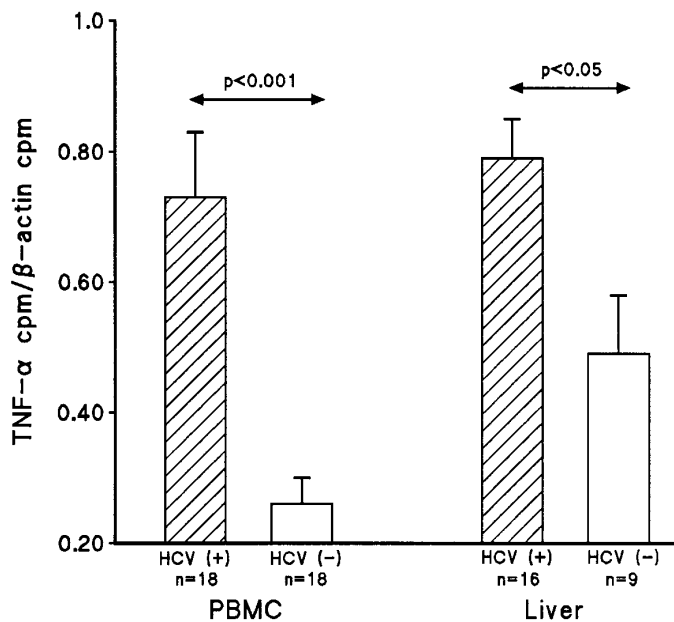


FIG 7. Levels of TNF- α mRNA (as a ratio to β -actin) in PBMC and in the liver in patients with chronic hepatitis C at the end of 10 to 12 months treatment with interferon. Patients were divided according to the presence (HCV+) or absence (HCV-) of HCV RNA in the corresponding specimen.

To evaluate whether TNF- α gene expression might be related to the quantity of the viral load, we quantified HCV RNA in serum in all of the 20 untreated patients and in PBMC in 15 of these subjects using a competitive PCR method. We found no correlation between the levels of TNF- α mRNA in PBMC and the levels of HCV RNA in serum ($P = .74$) or in PBMC ($P = .84$). Although the levels of HCV RNA before treatment tended to be higher in NR than in SR patients, the differences were not significant either in serum (3.4×10^7 copies/mL vs. 5.4×10^6 copies/mL; not significant [NS]) or in PBMC (9.6×10^4 copies/ μ g RNA vs. 6.3×10^4 copies/ μ g RNA; NS).

TNF- α Gene Expression and Liver Damage. TNF- α transcripts in the liver and in PBMC were similar in patients with and without cirrhosis (0.75 ± 0.1 vs. 0.67 ± 0.04 , and 0.31 ± 0.08 vs. 0.53 ± 0.06 , NS). We compared TNF- α mRNA values in liver samples from the eight Rr patients (all of them with low activity of the liver lesion at the end of the treatment) with those found in 10 untreated patients with high activity index in the liver biopsy specimens. In the former group the Knodell index in liver specimens obtained at the end of treatment was significantly lower than in the latter group of patients (6.75 ± 0.8 vs. 11.5 ± 0.9 ; $P < .005$), but hepatic TNF- α mRNA values were similar in both groups (0.79 ± 0.08 vs. 0.68 ± 0.04 ; NS). Furthermore, among untreated patients, there were five subjects with minimal changes in the liver biopsy results (mean histological activity index, 4); in these cases, TNF- α mRNA levels in PBMC were similar to those observed in untreated patients with active disease (mean histo-

logical activity index, 11.4) (0.44 ± 0.1 vs. 0.66 ± 0.1 ; NS).

Considering all patients, no correlation was observed between TNF- α mRNA values, measured in the liver or in PBMC and the levels of serum aminotransferases. Also, there was no correlation between TNF- α mRNA values and the Knodell's score of the liver biopsy results (similar results were obtained when total Knodell index or when only inflammatory parameters were considered).

DISCUSSION

This study and previous reports^{20,21} show that HCV can be observed not only in serum and hepatocytes but also in lymphoid cells, and our results indicate that HCV infection is associated with increased transcriptional expression of the TNF- α gene, in both the liver and PBMC and with high serum levels of TNF- α .

Induction of TNF- α gene by viruses has been shown to occur in a variety of *in vitro* models,^{4,6-9,31} as well as in diverse viral diseases, including HBV- and HCV-induced hepatitis.^{11,16-19,32} It has been recently shown that production of TNF- α in the liver takes place not only in nonparenchymal cells but also in hepatocytes.³³ The precise stimulus responsible for enhanced TNF- α gene expression in chronic viral hepatitis has not been defined. Although TNF- α may be generated during the inflammatory reaction that follows the immune recognition of viral antigens, the mere intracellular presence of viral compounds may also stimulate TNF- α gene expression as a built-in defense program of the cell to activate neighboring leukocytes or macrophages⁷ or to undergo apoptosis,³⁴ thus limiting the spread of infection.

In this study, we did not find a correlation between the activity of the liver disease and the level of TNF- α gene expression in the liver or PBMC. However, we observed a relationship between the presence of HCV RNA in the cells and the values of TNF- α transcripts. Thus, in UT patients TNF- α mRNA levels were shown to be increased in the liver and in PBMC, and HCV RNA was detectable in both sites; whereas in SR patients, HCV RNA was absent from both the liver and PBMC in the majority of cases, and TNF- α mRNA values were comparable to those observed in normal subjects. On the other hand, in NR subjects and in Rr patients, HCV RNA persists in the liver and lymphoid cells at the end of the treatment in most cases, and this is associated with an enhanced transcriptional expression of TNF- α in these two groups of patients.

The idea that the transcriptional expression of TNF- α is related more to the intracellular presence of the virus than to the activity of the liver disease is supported by the following observations. At the end of IFN- α therapy, serum aminotransferase levels were equally normal in Rr and SR patients, whereas TNF- α levels in PBMC and in the liver were significantly increased in the former group. The main difference between these two groups of patients was viral persistence in patients who eventually relapsed. On the other hand, UT patients showed a more active liver lesion (as estimated

by serum transaminase levels and histological activity index) than Rr patients, but both groups of patients had similarly increased TNF- α mRNA levels, and in both, HCV RNA was detectable in liver tissue and lymphoid cells.

Although the presence of the virus appears to determine an enhanced TNF- α gene expression, neither the level of viremia nor the quantity of HCV RNA in PBMC were shown to be related to the amount of TNF- α transcripts. Interestingly, the serum concentration of TNF- α was observed to be increased not only in untreated patients and in patients who did not respond to IFN- α treatment but also (although to a lesser extent) in those who exhibited sustained complete response to the therapy. Although, there is no clear explanation for this finding, it seems possible that persistent occult HCV infection in lymphoid organs might be responsible for increased TNF- α production (and perhaps delayed relapses) in patients with sustained complete response to IFN- α .

TNF- α has been shown to increase the production of oxygen-free radicals, such as superoxide anion and hydroxyl radical.¹ Through this effect, TNF- α can deplete the cells of reduced glutathion (GSH),³⁵ the main defense mechanism against oxidative stress.^{1,36} Oxygen radicals and oxidative stress in turn, can stimulate cells to produce TNF- α .³⁷ Oxygen-free radicals and GSH depletion can activate cell proteases involved in NF- κ B activation, a factor that increases TNF- α gene expression.³⁸ In chronic HCV infection, GSH concentration has been shown to be markedly reduced both in plasma and in PBMC.³⁹ Interestingly, GSH levels in PBMC correlated with the presence of HCV RNA in the cells and normalized when HCV RNA was cleared after IFN- α treatment.³⁹ Thus, it seems possible that oxidative stress might be involved in the induction of TNF- α in HCV infection and that TNF- α might contribute to the GSH depletion observed in chronic hepatitis C.

Notwithstanding the wide experience in the use of interferon treatment in chronic hepatitis C, little is known about the mechanisms determining the response, or the lack of response, to this agent in individual cases. In this study, pretreatment levels of TNF- α in PBMC were significantly higher in patients who failed to respond to IFN- α than in cases exhibiting sustained complete response to the therapy. Similar phenomenon have been described in chronic myelogenous leukemia, in which it has been observed that high pretreatment levels of TNF- α were associated with poor response to IFN- α therapy.⁴⁰ In hepatitis C, it is of interest that the highest values of TNF- α transcripts were observed in patients with genotype 1b, a genotype that is known to be associated with IFN- α resistance.⁴¹ Thus, in contrast with hepatitis B, for which TNF- α has been reported to exert a partial antiviral effect,^{42,43} this cytokine may favor viral persistence and the escape to interferon in HCV infection.

We have recently shown that administration of antioxidants, such as *N*-acetyl-cysteine, together with IFN- α can normalize serum aminotransferase levels in pa-

tients with chronic hepatitis C previously resistant to IFN- α alone.⁴⁴ From our data it could be speculated that high TNF- α levels produce oxidative stress, which in turn stimulates TNF- α synthesis and causes IFN- α resistance. This would explain the higher TNF- α values observed in patients insensitive to IFN- α treatment. Further studies are needed to determine whether pretreatment analysis of TNF- α gene expression can be useful to predict the response to IFN- α in chronic hepatitis C.

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