Journal of Viral Hepatitis, 2001, **8**, 194–201

In vitro effect of thymosin-α1 and interferon-α on Th1 and Th2 cytokine synthesis in patients with chronic hepatitis C

P. Andreone,[1] C. Cursaro,[1] A. Gramenzi,[1] M. Margotti,[1] E. Ferri,[1] S. Talarico,[1] M. Biselli,[1] F. Felline,[1] C. Tuthill,[2] E. Martins,[2] G. Gasbarrini[3] and M. Bernardi[1] [1]*Semeiotica Medica, Dipartimento di Medicina Interna, Cardioangiologia ed Epatologia, Università di Bologna; Bologna, Italy,* [2]*SciClone Pharmaceuticals, Inc., San Mateo, California, USA and* [3]*Clinica Medica, Università Cattolica del Sacro Cuore, Roma, Italy*

Received 18 August 2000; accepted for publication 2 December 2000

SUMMARY. Current evidence suggests that increased expression of Th1-associated cytokines is important for immune-mediated eradication of hepatitis C infection, while an increase in Th2-associated cytokines is associated with persistence of infection. In this study we evaluated the effects of thymosin-α1 (TA1), a naturally occurring thymic peptide, and interferon-α (IFN-α) on cytokine production in peripheral blood mononuclear cells from untreated patients with chronic hepatitis C. We examined the effect of incubation with TA1, IFN-α, or both, on production of Th1-associated cytokines (IL-2, IFN-γ), Th2-associated cytokines (IL-4, IL-10), and synthesis of the antiviral protein 2′,5′-oligoadenylate synthetase. TA1 treatment induced a significant increase in production of IL-2 and 2′,5′-oligoadenylate synthetase. Smaller increases were also seen after treatment with IFN-α, while incubation with TA1 and IFN-α together led to an additive or synergistic effect. Incubation with TA1 resulted in a decrease in IL-4 and IL-10, whereas IFN-α increased these cytokines. The addition of TA1 to IFN-α significantly reversed this IFN-α-induced increase. Hence, TA1 treatment could benefit patients with hepatitis C infection by increasing the Th1-type response, fundamental for sustained clearance of hepatitis C; and by decreasing the Th2-type response, associated with persistence of viraemia.

Keywords: cytokines, hepatitis C, immune response, interferon-α, Th1, thymosin-α1.

INTRODUCTION

Infection with the hepatitis C virus (HCV) is a significant public health problem worldwide, in both frequency and severity. Up to 85% of patients infected with HCV go on to chronic infection, with a high incidence of cirrhosis and hepatocellular carcinoma [1]. Recent studies suggest that the immune response plays a determining role, in both the pathogenesis of liver damage and elimination of the virus during the course of the infection. The mechanisms responsible for chronic persistence of the virus are still poorly defined, although the high degree of viral variability suggests that mutations in immunogenic regions of HCV antigens might be the primary strategy adopted by the virus to evade immune surveillance [2–4].

One of the mechanisms involved in the immune response during viral infection is cytokine synthesis by monocytes and T lymphocytes (CD4 or CD8). Based on the range of cytokines synthesized and on the function they perform in the immune response, the T lymphocytes are classified as Th1 cells, characterized by the production of interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumour necrosis factor-alpha (TNF-α), or Th2 cells, which produce interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), and interleukin-10 (IL-10) [5]. The Th1 cytokine response seems to be particularly important for the effective eradication of infections caused by intracellular pathogens, including viruses, while a Th2 response may be associated with persistence of these infections [6].

Thymosin-α1 (TA1) is a synthetically produced thymic peptide that has been shown to enhance the response of T lymphocytes and stimulate the production of endogenous interleukins and interferons [7,8]. Following favourable clinical results obtained from the treatment of chronic hepatitis caused by the hepatitis B virus (HBV) [9–12] TA1

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; IFN-α, interferon-α; IL, interleukin; 2′,5′-OAS, 2′,5′-oligoadenylate synthetase; PBMC, peripheral blood mononuclear cells; TA1, thymosin-α1.

Correspondence: Dr Pietro Andreone, Semeiotica Medica, Policlinico S. Orsola, Via Massarenti, 9-40138 Bologna, Italy.
E-mail: andreone@med.unibo.it

Part of this study was supported by ARME (Associazione per la Ricerca sulle Malattie Epatiche).

has also been tried against chronic infections due to HCV. In a double-blind clinical study, TA1 monotherapy, compared with a placebo, did not show significant biochemical or virological effectiveness [13], but in multiple studies in combination with interferon-α (IFN-α) it significantly increased the therapeutic response rate to hepatitis C in comparison to treatment with IFN-α alone [14–16]. TA1 has a number of immunomodulating activities, centred primarily on augmentation of T-cell function, and specifically on the generation of a Th1 profile. For example, TA1 increases production of IFN-α, IL-2, IL-3 and expression of IL-2 receptor following activation by mitogens or antigens [17–19]. TA1 stimulates thymopoiesis in a human co-culture system by increasing the number of thymocytes and expanding $CD44^{+}25^{+}3^{-}$ and $CD3^{+}4^{+}$ T-cells [20], and antagonizes dexamethasone-induced apoptosis in thymocytes *in vitro* in a dose-dependent fashion [21]. TA1 enhances production of CD3, CD4 and CD8 cells in patients with chronic hepatitis B [9] or cancer [22], mice infected with influenza A virus [23] or aged mice immunodepressed by hydrocortisone treatment [24].

The purpose of this study was to evaluate the *in vitro* effects of IFN-α, TA1, and the combination of these compounds on the production of Th1 cytokines (IL-2, IFN-γ) and Th2 cytokines (IL-4, IL-10), as well as the synthesis of a protein with antiviral activity, 2′,5′-oligoadenylate synthetase (2′,5′-OAS), by peripheral blood mononuclear cells (PBMCs) obtained from patients with chronic HCV infection.

MATERIALS AND METHODS

Patients

PBMCs were obtained from eight patients (male/female: 7/1; average age: 32.5 years, range 21–61 years) with virological and histological evidence of chronic hepatitis caused by the HCV, who had never been treated with IFN-α. The patient characteristics are listed in Table 1. All patients tested negative for the hepatitis B surface antigen. Blood samples were obtained from each patient in the morning after 8-h fasting, and the samples were processed immediately. All patients had given informed consent for the study, in compliance with the Declaration of Helsinki.

Methods

The following determinations were carried out on all patients: HBsAg (AUSRIA 2, Abbott; Chicago, IL); anti-HCV antibody (Ortho Diagnostic Systems; Raritan, NJ); qualitative HCV-RNA by PCR using nested primers of the noncoding region 5′ of the viral genome (sensitivity about 10 molecules of HCV-RNA/mL); quantitative HCV-RNA by b-DNA (HCV-RNA, Chiron Corporation; Emeryville, CA; sensitivity limit of 200 000 copies of genome/mL); HCV genotype using Inno-LiPA test (HCV line probe assay, Innogenetics; Zwijndrecht, Belgium).

Preparation of cell cultures

Peripheral blood (15 mL) was drawn from each patient using a heparinized syringe (20 U/mL of blood with heparin sodium, Parke-Davis, Inc., Milan, Italy). All the samples were diluted 1:1 with physiological saline; each suspension was layered on Histopaque-1077 (Sigma; St Louis, MO), and then centrifuged at 1800 r.p.m. for 15 min at room temperature. The mononuclear cells at the interface between the plasma and the Histopaque were harvested and centrifuged for 10 min at 300 r.p.m. after the addition of 20 mL of RPMI containing 10% fetal calf serum (Gibco BRL, Life Technologies; European Division, Italy) and glutamine (200 mM, Gibco BRL, Life Technologies; European Division, Milan, Italy), and tested for viability with trypan blue. The pellets were diluted with 1 mL of culture medium and the mononuclear cells were counted in a Neubauer counting chamber with a reverse phase-contrast microscope (Zeiss, Munich, Germany). The cells were transferred to sterile test tubes at a concentration of 1×10^{6}/mL to obtain liquid cultures.

Table 1 Patient characteristics

Case	Sex	Age (years)	Duration of infection (years)	Source of infection	ALT (U/L)	AST (U/L)	Viral load (genomes/mL)	Genotype
1	F	50	20	Unknown	65	40	1.0×10^{6}	1b
2	M	36	9	IVDA	274	62	6.6×10^{6}	1b
3	M	61	8	Unknown	153	87	5.94×10^{6}	1b
4	M	23	8	IVDA	219	93	2.5×10^{6}	4
5	M	25	7	IVDA	217	79	12.37×10^{6}	3a
6	M	33	13	IVDA	142	60	3.8×10^{6}	3a
7	M	21	5	IVDA	107	34	0.23×10^{6}	3a
8	M	32	2	Unknown	94	38	15.06×10^{6}	1b

IVDA, intravenous drug abuse; ALT, normal values: 0–40 U/L; AST, normal values: 0–37 U/L.

The cells from each patient were subjected to eight different experimental conditions; four in the presence and four in the absence of concanavalin A (ConA) at 10 mg/mL (Sigma). The incubation conditions were as follows: culture medium alone (RPMI); culture medium containing 500 U/mL of IFN-α (Wellferon; Glaxo Wellcome, Verona, Italy); culture medium containing 10 μg/mL TA1 (SciClone Pharmaceuticals, San Mateo, CA); and culture medium containing IFN-α and TA1 together. The cell cultures were set for 24 h at 37 °C in a moist chamber with an atmosphere containing 5% carbon dioxide. After 24 h, the supernatants were harvested, centrifuged at 3000 r.p.m. for 10 min, separated from the cells, divided into aliquots, and immediately preserved at −80 °C for subsequent testing.

The doses of TA1 (10 μg/mL) and IFN-α (500 U/mL) were chosen based on the results of previous studies demonstrating that those doses were able to stimulate the synthesis of IL2 and 2′,5′-OAS [25].

The production of IL-2, IFN-γ, IL-4, and IL-10 was determined by immunoenzymatic assays (for IFN-γ, IL-4, and IL-10: R & D Systems; Minneapolis, MN; for IL-2: Endogen; Woburn, MA) using the supernatants harvested from the cell cultures stimulated with ConA. The limit of sensitivity of the assays were 6 pg/mL for IL-2, 3 pg/mL for IFN-γ and IL-10, and 1.5 pg/mL for IL-4.

The activity of 2′,5′-OAS was determined in the supernatants of the cell cultures incubated in the absence of ConA, using a radioimmunological assay (Eiken Chemical Co., Ltd.; Tokyo, Japan). The sensitivity of the method was 10 pmol/dL.

Statistical analysis

The data, expressed as mean ± standard error of the mean (M ± SEM) were analysed using nonparametric statistical tests. In particular, the comparison between the average concentration of cytokines was evaluated by Wilcoxon's test for paired data. Linear regression for the relationship between variables was calculated using Spearman's correlation coefficient. A value of $P < 0.05$ was considered statistically significant. Data processing was carried out with the SPSS statistical package for Windows.

RESULTS

Th1 cytokines

The production of IL-2 and IFN-γ by PBMCs under the various experimental conditions is shown in Figs 1 and 2, respectively. The addition of IFN-α to the culture significantly increased the synthesis of IL-2, as compared with the baseline conditions of ConA stimulation (185 ± 30 pg/mL vs. 100 ± 36 pg/mL, $P = 0.01$). This increase was even greater in the presence of TA1 (643 ± 121 pg/mL vs. 100 ± 36 pg/mL, $P = 0.01$) and in the presence of IFN-α plus TA1 (801 ± 141 pg/mL vs. 100 ± 36 pg/mL, $P = 0.01$). Compared with treatment with IFN-α alone, production of IL-2 in the presence of TA1 or IFN-α plus TA1 was significantly higher ($P = 0.01$ in both cases). The increase observed in the presence of IFN-α plus TA1 as compared with TA1 alone was additively and statistically significant ($P = 0.025$).

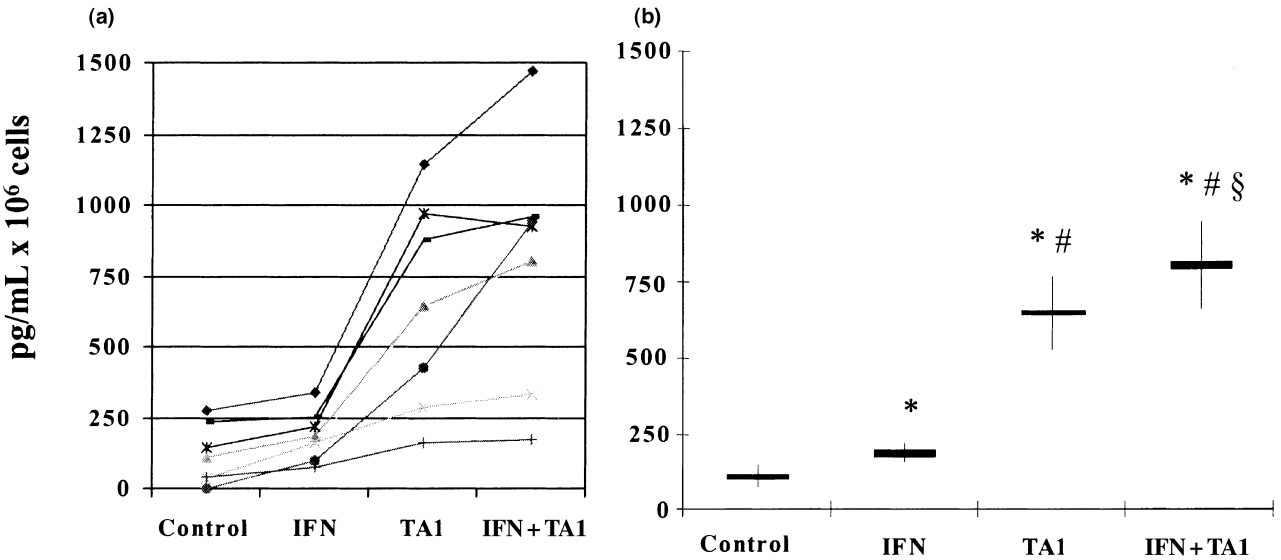

Fig. 1 Interleukin-2 synthesis in peripheral blood mononuclear cells stimulated by ConA and incubated with TA1, IFN-α, or both. (a) Individual data. (b) Mean + standard error of the mean. *$P = 0.01$ vs. control; #$P = 0.01$ vs. IFN-α; §$P = 0.025$ vs. TA1.

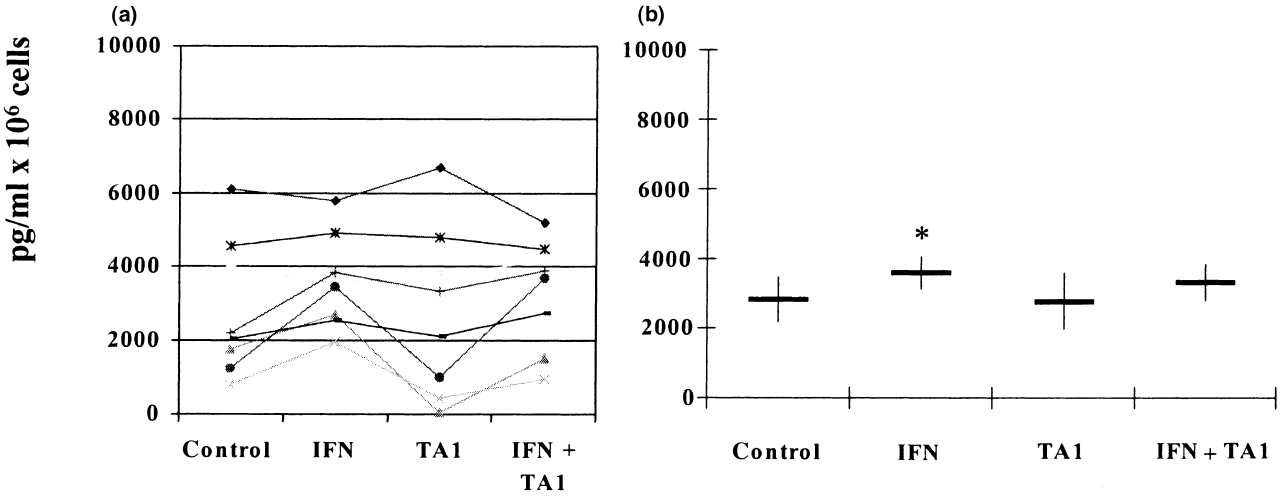

Fig. 2 IFN-γ synthesis in peripheral blood mononuclear cells stimulated by ConA and incubated with TA1, IFN-α, or both. (a) Individual data. (b) Mean ± standard error of the mean. *$P = 0.05$ vs. control.

Treatment of cells with IFN-α brought about an increase in the synthesis of IFN-γ as compared with the baseline conditions (3590 ± 450 pg/mL vs. 2824 ± 655 pg/mL, $P = 0.05$), but there was no significant change in the synthesis of IFN-γ in the presence of TA1 or in the presence of IFN-α plus TA1 as compared with the baseline conditions (2774 ± 815 pg/mL and 3327 ± 521 pg/mL vs. 2824 ± 655 pg/mL, P = ns).

No correlation was found between age, ALT levels or HCV-RNA titres and either baseline or stimulated Th1 cytokines.

Th2 cytokines

Figures 3 and 4 show the changes in the synthesis of IL-4 and IL-10, respectively. Treatment of cells with IFN-α significantly increased the synthesis of IL-4 as compared with baseline conditions of ConA stimulation (117 ± 17 pg/mL vs. 89 ± 17 pg/mL, $P = 0.01$). On the other hand, in the presence of either TA1 or IFN-α plus TA1, there was a significant reduction in the synthesis of IL-4 in comparison with baseline conditions (21 ± 13 pg/mL and 19 ± 7 pg/mL vs. 89 ± 49 pg/mL, $P = 0.01$ in both cases) and in comparison to the presence of IFN-α alone ($P = 0.01$ in both cases).

The production of IL-10 was similar to that of IL-4, where IFN-α significantly increased synthesis as compared with the baseline level (1749 ± 209 pg/mL vs. 1098 ± 200 pg/mL, $P = 0.01$), while both TA1 alone or IFN-α plus TA1 caused a significant reduction in synthesis compared with baseline conditions (297 ± 42 pg/mL and 349 ± 69 pg/mL vs.

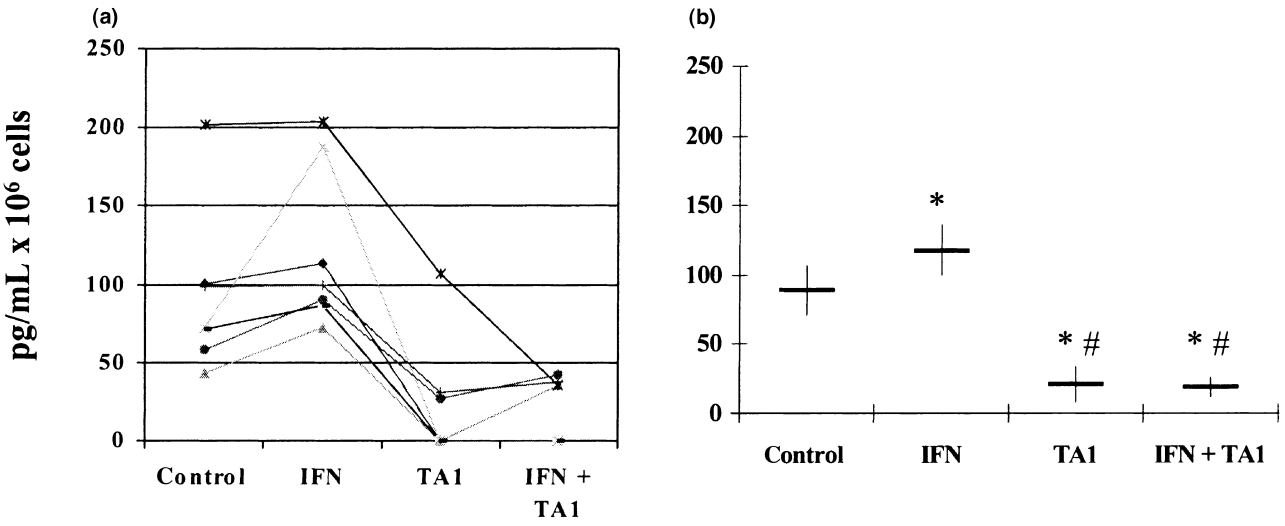

Fig. 3 Interleukin-4 synthesis in peripheral blood mononuclear cells stimulated by ConA and incubated with TA1, IFN-α, or both. (a) Individual data. (b) Mean ± standard error of the mean. *$P = 0.01$ vs. control; #$P = 0.01$ vs. IFN-α.

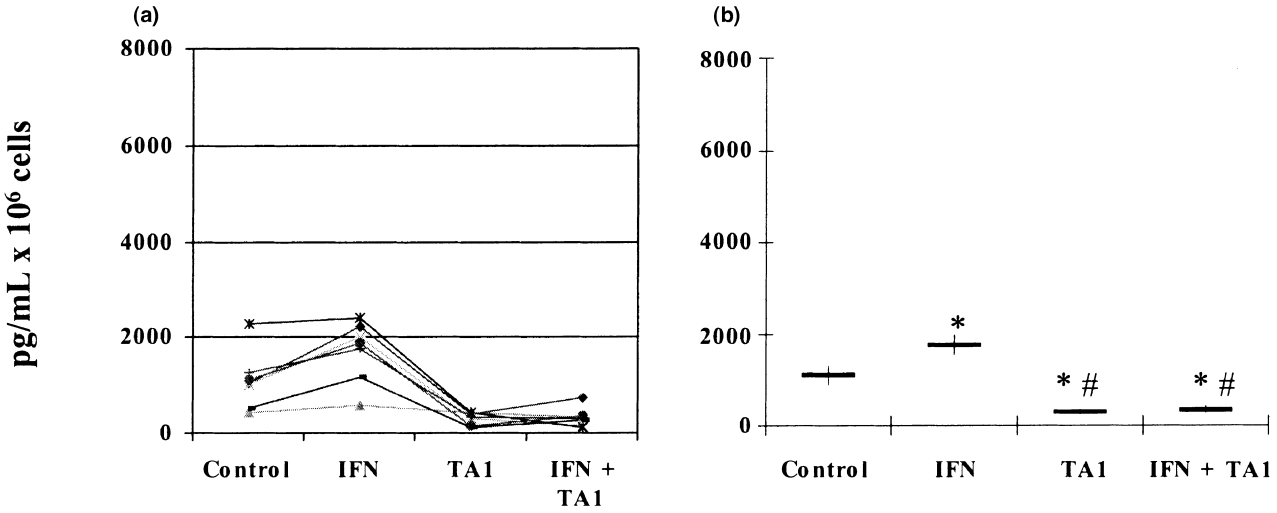

Fig. 4 Interleukin-10 synthesis in peripheral blood mononuclear cells stimulated by conA and incubated with TA1, IFN-α, or both. (a) Individual data. (b) Mean ± standard error of the mean. *$P = 0.01$ vs. control; #$P = 0.01$ vs. IFN-α.

1098 ± 200 pg/mL, $P = 0.01$ for both) and compared with IFN-α ($P = 0.01$ in both cases).

No correlation was found between age, ALT levels or HCV-RNA titres and either baseline or stimulated Th2 cytokines.

Synthesis of the antiviral protein 2′,5′-OAS

Figure 5 shows the results of the synthesis of 2′,5′-OAS. The synthesis of this antiviral protein was significantly increased in the presence of IFN-α as compared with baseline conditions (1056 ± 571 pmol/mL vs. 97 ± 45 pmol/mL, $P = 0.01$). In the presence of TA1 the average increase was greater than that observed in the presence of IFN-α, however, it was not statistically significant with respect to the baseline value (1309 ± 1142 pmol/mL vs. 97 ± 45 pmol/mL, P = ns). The increase observed in the presence of IFN-α plus TA1 was significantly higher as compared with the baseline (8955 ± 4114 pmol/mL vs. 97 ± 45 pmol/mL, $P = 0.01$) and with respect to the presence of IFN-α alone ($P = 0.01$) or TA1 alone ($P = 0.01$), reflecting a possible synergistic effect of the two drugs.

No correlation was found between age, ALT levels or HCV-RNA titres and either baseline or stimulated 2′,5′-OAS.

DISCUSSION

The mechanisms by which HCV evades surveillance by the host's immune system and produces a chronic infection are

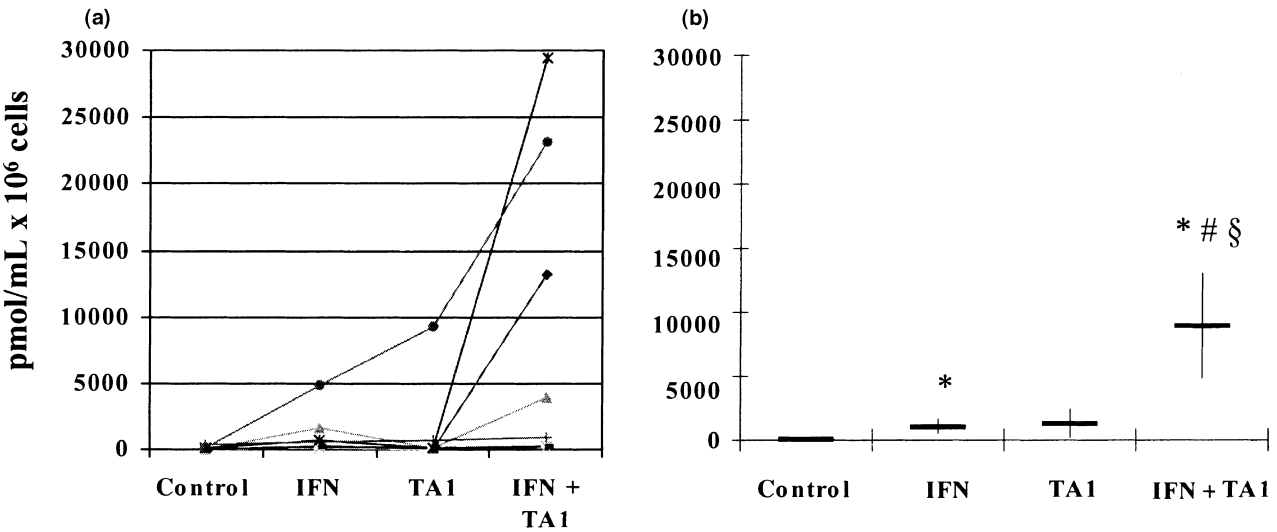

Fig. 5 2′,5′-Oligoadenylate synthetase synthesis in peripheral blood mononuclear cells incubated with TA1, IFN-α, or both. (a) Individual data. (b) Mean ± standard error of the mean. *$P = 0.01$ vs. control; #$P = 0.01$ vs. IFN-α; §$P = 0.01$ vs. TA1.

not fully understood. A Th1 response appears to be associated with a vigorous antiviral response, while a polarization towards Th2 has been suggested to be correlated with decreased severity of hepatic damage [26,27]. The evaluation of Th1/Th2 response profile in patients with chronic HCV infection has yielded conflicting results. Some studies have found an activation of all T lymphocyte responses in serum, with high levels of IL-2, IL-4, IL-10, TNF-α, and INF-γ as compared with normal controls [28–29]. Conversely, Cacciarelli *et al.* [30] have shown that during the course of chronic infection with HCV, elevation of the Th2-associated cytokine levels was markedly higher than that of the Th1-associated cytokines. Reiser *et al.* [31] have also shown that some patients with chronic HCV infection have high levels of IL-4 and IL-10. Recently, we found high serum levels of soluble CD30, a glycoprotein preferentially expressed and released by T lymphocytes producing Th2 cytokines in patients, with chronic hepatitis C and there was a correlation with histological activity of the disease [32]. Taken together, these data suggest that the release of Th2 cytokines could be involved in the virus' mechanism of avoiding immune surveillance.

The present study demonstrated that TA1 induces a significant increase in the production of the Th1 cytokine IL-2 and, to a lesser extent, of the antiviral protein 2′,5′-OAS by PBMCs from patients with chronic HCV infection. An increase in these parameters was also seen after treatment of these cells with IFN-α, but the increase due to TA1 was significantly greater. In fact, incubation of the PBMCs with a combination of IFN-α and TA1 leads to an additive or even synergistic effect on synthesis of IL-2 and 2′-5′ OAS.

Many effects of TA1 appear to be synergistic with those of Th1 cytokines, and the peptide may work best in combination with other immunomodulators. For example, TA1 enhanced the effects of IFN-α on the natural killer (NK)-cell activity of lymphocytes from mice [33], as well as from normal human donors [34] or HIV-infected patients [35]. TA1 also augments the effects of IL-2 in lymphocytes from both normal and immune-suppressed mice [36]. This additive or even synergistic effect seen upon combination of treatment with TA1 and IFN-α was also seen in the present study.

Response to treatment by HCV-infected patients with IFN-α has been correlated with a Th1 type of T-cell response, while non-responders produce more Th2 cytokines [37]. Treatment of PBMCs from patients with HCV with IFN-α has previously been shown to result in an increase in both Th1 and Th2-associated cytokines, which would be less than optimal for clearing an HCV infection [38]. In the present study, incubation of PBMC from HCV-infected patients with TA1 resulted in a decrease in the Th2 cytokines IL-4 and IL-10. Conversely, incubation with IFN-α increased these Th2 cytokines. In previous studies, the addition of ribavirin to IFN-α appeared to inhibit the production of Th2 cytokines [38]. This is similar to the results seen in the present study with TA1 treatment where incubation with TA1 in combination with IFN-α blocked the PBMC counter-regulatory response resulting in a decrease of the IFN-α-induced production of IL-4 and IL-10. However, TA1 immune effects are broader than those of ribavirin, as TA1 also increases the production and maturation of CD4 and CD8 T-cells [20–22]. Therefore, TA1 may benefit patients with HCV infection twofold: first, by blocking the IFN-α-induced Th2 response and, second, by increasing T-cell subsets fundamental for sustained clearance of HCV.

Recent studies have shown that the clearance of HCV infection after IFN-α treatment can be described as a two-phase process [39]; the initial IFN-α dose-dependent decline appears to be related to prevention of virion production or release while the second, slower phase appears to be related to clearance of infected cells by the immune system. Although the first phase of HCV-RNA decline is seen in the majority of IFN-treated patients, viraemia will continue to decline in the second phase only in responders. Thus, an effective HCV-specific immune response appears to be necessary for sustained HCV clearance. Therefore, the addition of an immunomodulatory therapy, such as TA1, could positively influence the second step of HCV clearance by enhancing the host Th1 response. The resulting effect would be an increase in the response rate as compared with IFN-α monotherapy.

Three studies have investigated the therapeutic effect of TA1 in combination with IFN-α for treatment of chronic hepatitis C. These are a phase 3 study in the USA where patients were treated for 6 months [16] and two phase 2 studies in Italy where patients were treated for 6 months or 12 months [14,15]. These studies have been analysed independently, using both pooled and meta-analysis techniques [40]. Pooled intent-to-treat analysis revealed an end-of-treatment biochemical response of 45% in the TA1 plus IFN-α combination treatment group compared with 22% in the IFN-α monotherapy group ($P = 0.0096$), while a sustained biochemical response was observed in 22% of patients treated with TA1 plus IFN-α compared with 9% in the IFN-α monotherapy group. Meta-analysis of the virological response data indicated that TA1 plus IFN-α combination therapy was significantly superior to IFN monotherapy. The response rates obtained with the combination TA1 plus IFN-α are similar to those obtained with the combination IFN-α plus ribavirin [41,42], which is the suggested firstline treatment for chronic hepatitis caused by HCV. However, IFN-α plus ribavirin induces a long-term response in only 28% of patients infected with genotype 1 [41], while the association of TA1 with IFN-α has been shown to achieve sustained clearance of HCV-RNA in 39% of treated patients with genotype 1 [15]. Moreover, in all studies [16] TA1 has been well tolerated and was not associated with any significant side-effects, while ribavirin adds toxicity to the side-effect profile of IFN-α and the rate of discontinuation of the combined treatment is about 20% [43–46].

The data from the present study, although derived from a small number of patients, provides a mechanism to explain the beneficial effect of the combination of TA1 plus IFN-α in chronic hepatitis C. Treatment of PBMCs from patients with chronic hepatitis C with TA1 leads to an increase in Th1 cytokines with a simultaneous decrease in Th2 cytokines; an even greater response including generation of the antiviral protein 2′,5′-OAS is seen upon treatment with both TA1 with IFN-α. Further studies are needed to evaluate the *in vitro* and *in vivo* effects of different pharmacological doses of TA1 in association with IFN-α.

In conclusion, combination treatment of hepatitis C with IFN-α and TA1 could be ideal, as it stimulates Th1 helper cell subsets without a concomitant stimulation of Th2. The association of TA1 plus interferon is of interest also because it is very well tolerated and not associated with any significant side-effects.

REFERENCES

1 Alberti A, Chemello L, Benvegnu L. Natural history of hepatitis C. *J Hepatol* 1999; 31 (Suppl. 1): 17–24.

2 Minutello MA, Pileri P, Unutmaz D *et al.* Compartmentalization of T lymphocytes to the site of disease: intrahepatic $CD4^+$ T cells specific for the protein NS4 of hepatitis C virus in patients with chronic hepatitis C. *J Exp Med* 1993; 178: 17–25.

3 Koziel MJ, Dudley D, Wong JT *et al.* Intrahepatic cytotoxic T lymphocytes specific for hepatitis C virus in persons with chronic hepatitis. *J Immunol* 1992; 149: 3339–3344.

4 Koziel MJ, Wong DK, Dudley D, Houghton M, Walker BD. Hepatitis C virus-specific cytolytic T lymphocyte and T helper cell responses in seronegative persons. *J Infect Dis* 1997; 176: 859–866.

5 Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986; 136: 2348–2357.

6 Koziel MJ. Cytokines in viral hepatitis. *Semin Liver Dis* 1999; 19: 157–169.

7 Gramenzi A, Cursaro C, Andreone P, Bernardi M. Thymalfasin. Clinical pharmacology and antiviral applications. *Bio Drugs* 1998; 9: 477–486.

8 Oates KK, Goldstein AL. Thymosin. In: DeVita VT, Hellman S and Rosenberg SA, eds. *Biological Therapy of Cancer.* Philadelphia: JB Lippincott, 1995: 841–852.

9 Mutchnick MG, Appelman HD, Chung HT *et al.* Thymosin treatment of chronic hepatitis B: a placebo-controlled pilot trial. *Hepatology* 1991; 14: 409–415.

10 Andreone P, Cursaro C, Gramenzi A *et al.* A randomized controlled trial of thymosin alpha 1 versus interferon alpha treatment in patients with hepatitis B e antigen antibody – and hepatitis B virus DNA – positive chronic hepatitis B. *Hepatology* 1996; 24: 774–777.

11 Rasi G, Mutchnick MG, Di Virgilio D *et al.* Combination low-dose lymphoblastoid interferon and thymosin alpha 1 therapy in the treatment of chronic hepatitis B. *J Viral Hep* 1996; 3: 191–196.

12 Chien RN, Liaw YF, Chen TC, Yeh CT, Sheen IS. Efficacy of thymosin alpha1 in patients with chronic hepatitis B: a randomized, controlled trial. *Hepatology* 1998; 27: 1383–1387.

13 Andreone P, Cursaro C, Gramenzi A *et al.* A double-blind, placebo-controlled, pilot trial of thymosin alpha 1 for the treatment of chronic hepatitis C. *Liver* 1996; 16: 207–210.

14 Moscarella S, Buzzelli G, Monti M *et al.* Treatment with interferon-alpha and thymosin alpha 1 of naive patients affected by chronic hepatitis C. *4th International Meeting on Hepatitis C Virus and Related Viruses.* Kyoto, Japan. 1997.

15 Rasi G, DiVirgilio D, Mutchinick MG *et al.* Combination thymosin a1 and lymphoblastoid interferon treatment in chronic hepatitis C. *Gut* 1996; 39: 670–683.

16 Sherman KE, Sjogren M, Creager RL *et al.* Combination therapy with thymosin alpha1 and interferon for the treatment of chronic hepatitis C infection: a randomized, placebo- controlled double-blind trial. *Hepatology* 1998; 27: 1128–1135.

17 Sztein M, Serrate S. Characterization of the immunoregulatory properties of thymosin alpha 1 on interleukin-2 production and interleukin-2 receptor expression in normal human lymphocytes. *Int J Immunopharmacol* 1989; 11: 789–800.

18 Svedersky L, Hui A, May L, McKay P, Stebbing N. Induction and augmentation of mitogen-induced immune interferon production in human peripheral blood lymphocytes by Nα-desacetylthymosin alpha 1. *Eur J Immunol* 1982; 12: 244–247.

19 Leichtling KD, Serrate SA, Sztein MB. Thymosin alpha 1 modulates the expression of high affinity interleukin-2 receptors on normal human lymphocytes. *Int J Immunopharmacol* 1990; 12: 19–29.

20 Knutsen AP, Freeman JJ, Mueller KR, Roodman ST, Bouhasin JD. Thymosin-alpha1 stimulates maturation of $CD34^+$ stem cells into $CD3^+4^+$ cells in an in vitro thymic epithelia organ coculture model. *Int J Immunopharmacol* 1999; 21: 15–26.

21 Baumann CA, Badamchian M, Goldstein AL. Thymosin alpha 1 antagonizes dexamethasone and CD3-induced apoptosis of $CD4^+CD8^+$ thymocytes through the activation of cAMP and protein kinase C dependent second messenger pathways. *Mech Ageing Dev* 1997; 94: 85–101.

22 Salvati F, Rasi G, Portalone L, Antilli A, Garaci E. Combined treatment with thymosin alpha 1 and low-dose interferon-alpha after ifosfamide in non-small cell lung cancer: a phase II controlled trial. *Anticancer Res* 1996; 16: 1001–1004.

23 D'Agostini C, Palamara AT, Favalli C *et al.* Efficacy of combination therapy with amantadine, thymosin alpha 1 and alpha/beta interferon in mice infected with influenza A virus. *Int J Immunopharmacol* 1996; 18: 95–102.

24 Hadden JW, Saha A, Sosa M, Hadden EM. Immunotherapy with natural interleukins and/or thymosin alpha 1 potently augments T-lymphocyte responses of hydrocortisone-treated aged mice. *Int J Immunopharmacol* 1995; 17: 821–828.

25 Kouttab N, Goldstein A, Lu M, Lu L, Campbell B, Maizel A. Production of human B and T cell growth factors is enhanced by thymic hormones. *Immunopharmacology* 1988; 16: 97–105.

26 McCaughan G, Napoli J, McGuinness P. T1 versus T2 cytokine response in chronic HCV: implications for mechanisms of liver injury. *Viral Hep Rev* 1997; 3: 129–142.
27 Andreone P, Gramenzi A, Cursaro C, Felline F, Gasbarrini G, Bernardi M. Interaction between non-steroidal anti-inflammatory drugs and interferon alpha system: mechanisms and basis for the clinical use in the treatment of chronic viral hepatitis. In: Dianzani F and Gentilini P, eds. *New Trends in Hepatology*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 1996: 306–314.
28 Tilg H, Wilmer A, Vogel W *et al.* Serum levels of cytokines in chronic liver diseases. *Gastroenterology* 1992; 103: 264–274.
29 Lim H, Nelson D, Fang J. The tumor necrosis factor-alpha system in chronic hepatitis C. *Hepatology* 1994; 20: 251A.
30 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.
31 Reiser M, Marousis C, Nelson D. Th2 cytokine profile in chronic hepatitis C. *Gastroenterology* 1995; 108: A1154.
32 Foschi F, Gramenzi A, Castelli E *et al.* Soluble CD30 serum levels in HCV-positive chronic active hepatitis: a surrogate marker of disease activity? *Cytokine* 2000; 12: 815–818.
33 Favalli C, Jezzi T, Mastino A, Rinaldi-Garaci C, Riccardi C, Garaci E. Modulation of natural killer activity by thymosin alpha 1 and interferon. *Cancer Immunol Immunother* 1985; 20: 189–192.
34 Serrate S, Schulof R, Leondaridis L, Goldstein AL, Sztein MB. Modulation of human natural killer cell cytotoxic activity, lymphokine production, and interleukin 2 receptor expression by thymic hormones. *J Immunol* 1987; 139: 2338–2343.
35 Garaci E, Rocchi G, Perroni L *et al.* Combination treatment with zidovudine, thymosin alpha 1, and interferon-alpha in human immunodeficiency virus infection. *Int J Clin Lab Res* 1994; 24: 23–28.
36 Mastino A, Favalli C, Grelli S, Innocenti F, Garaci E. Thymosin alpha 1 potentiates interleukin 2-induced cytotoxic activity in mice. *Cell Immunol* 1991; 133: 196–205.
37 Tsai S, Liaw Y-F, Chen M-H, Huang C-Y, Kuo G. Detection of type-2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *Hepatology* 1997; 25: 449–458.
38 Martin J, Navas S, Quiroga J, Pardo M, Carreno V. Effects of the ribavirin-interferon alpha combination on cultured peripheral blood mononuclear cells from chronic hepatitis C patients. *Cytokine* 1998; 10: 635–644.
39 Neumann A, Lam N, Dahari H *et al.* Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon alpha therapy. *Science* 1998; 282: 103–197.
40 Sherman K, Sherman S. Interferon plus thymosin a-1 treatment of chronic hepatitis C infection: a meta-analysis. In: Schinazi R, Sommadossi J and Thomas H, eds. *Therapies for Viral Hepatitis.* London: International Medical Press, 1998: 379–383.
41 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1485–1492.
42 Poynard T, Marcellin P, Lee SS *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; 352: 1426–1432.
43 Davis GL, Esteban-Mur R, Rustgi V *et al.* Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1493–1499.
44 Bodenheimer HC Jr, Lindsay KL, Davis GL, Lewis JH, Thung SN, Seeff LB. Tolerance and efficacy of oral ribavirin treatment of chronic hepatitis C. A multicenter trial. *Hepatology* 1997; 26: 473–477.
45 Pol S, Zylberberg H, Fontaine H, Brechot C. Treatment of chronic hepatitis C in special groups. *J Hepatol* 1999; 31 (Suppl. 1): 205–209.
46 Fiel MI, Schiano TD, Guido M *et al.* Increased hepatic iron deposition resulting from treatment of chronic hepatitis C with ribavirin. *Am J Clin Pathol* 2000; 113: 35–39.