**In vitro** effect of thymosin-alpha1 and interferon-alpha on Th1 and Th2 cytokine synthesis in patients with eAg-negative chronic hepatitis B

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**SUMMARY.** Thymosin alpha-1 (Tα1) has been shown to be effective in chronic hepatitis B treatment. This study investigated the effect of Tα1 and interferon-alpha (IFNα) on cytokine production by peripheral blood mononuclear cells (PBMCs) of 12 patients with eAg-negative chronic hepatitis B (HBV). We evaluated the effect of incubation with Tα1, IFNα or both on the synthesis of T-helper 1 (Th1) cytokines [interleukin-2 (IL-2), IFNγ] and Th2 cytokines (IL-4, IL-10) and of antiviral protein 2′,5′-oligoadenylate synthetase (2′,5′-OAS) in patients and in a group of 10 healthy controls. Concerning Th1 profile, controls showed lower IL-2 synthesis than HBV patients. In HBV setting, IFNα/Tα1 combination was able to increase IL-2 production significantly, when compared with baseline condition. About the Th2-cytokines, controls showed statistically lower synthesis of IL-4 and higher production of IL-10, than HBV patients. In these latter, IFNz increased the synthesis of IL-10 compared with baseline. Interestingly, both Tα1 alone and the IFNz/Tα1 combination reversed this effect. Finally, compared with baseline, the synthesis of 2′,5′-OAS was significantly higher in the presence of Tα1 and IFNz alone, and in the presence of IFNz/Tα1 association, while no differences were found between controls and HBV patients. In conclusion, in PBMCs from eAg-negative HBV patients, Tα1 alone was able to increase the antiviral protein synthesis, while in association with IFNz, it stimulated the IL-2 synthesis and inhibited the IFN-induced IL-10 production. These results need further investigations, but reinforce the idea of an immunotherapeutic approach for chronic hepatitis B.

**Keywords**: hepatitis B, immune response, thymosin alpha-1, T-helper 1, T-helper 2.

**INTRODUCTION**

Hepatitis B virus (HBV) is one of major causes of chronic liver disease worldwide [1]. Chronic hepatitis B can range from a persistent asymptomatic carrier state to a more or less progressive chronic infection, potentially evolving to cirrhosis, hepatocellular carcinoma and liver failure [2]. HBV is not a cytopathic virus and liver injury is mainly mediated by the host immune response against virus-infected liver cells and by the production of cytokines. However, the pathogenetic mechanisms responsible for both viral persistence and variable course of infection have only partly been clarified.

It has been shown that a vigorous, polyclonal and multispecific cytotoxic and T-helper (Th) cell response to HBV is readily detectable in the peripheral blood of patients with self-limited hepatitis B, but is weak, antigenically restricted or undetectable in patients with chronic infection [3]. Thus, this T-cell response is believed to play a pivotal role in the outcome of HBV.

Disturbed functions of CD4 Th lymphocytes in the course of chronic hepatitis B have been well documented [4]. Human CD4 T cells are known to be functionally heterogeneous, containing two distinct Th subsets: Th1-producing interferon-gamma (IFNγ) and interleukin (IL)-2, and Th2-releasing IL-4, IL-5 and IL-10 [5,6]. In immunopathogenesis of chronic hepatitis B, it has been suggested that a preferential shift towards a Th1-polarized phenotype could be associated with a spontaneous viral clearance or with a successful control of infection [7], while an early commitment towards a Th2 synthesis plays a significant role in disease progression [8]. On the other hand, some authors also showed that the Th1 cytokine profile correlates with...
hepatic inflammatory activity and liver damage progression [9].

Thymosin alpha-1 (Tα1) is a synthetic polypeptide of thymic origin that stimulates the maturation of thymocytes, the differentiation into active T cells and restores T-cell function by T-cell-mediated antibody production [10–13]. Tα1 concentration has been found to be low in patients with chronic HBV [14], and clinical trials with Tα1 indicates that it could be at least as effective as IFNα [15,16].

The aim of the present study was to evaluate the ‘in vitro’ effect of IFNα, Tα1, and of their association on the production of Th1 (IL-2, IFNγ) and Th2 cytokines (IL-4, IL-10), as well as the synthesis of an antiviral protein, the 2′,5′-oligoadenylate synthetase (2′,5′-OAS), by peripheral blood mononuclear cells (PBMCs) of patients with eAg-negative chronic HBV infection.

PATIENTS AND METHODS

Patients

Twelve patients [male/female: 9/3; median age (years): 44.5, range: 19–62] with HBcAg-negative chronic hepatitis B were enrolled in this study. All patients were hepatitis B surface antigen-positive and hepatitis B e antigen-negative for at least 1 year and had detectable serum HBV-DNA within the last 3 months before starting the study. Chronic hepatitis B was confirmed by histological evaluation in all cases. No patient had compensated liver disease, detectable antibodies against hepatitis C, D or human immunodeficiency viruses; none had evidence of hepatocellular carcinoma or had received any anti-viral drug.

Ten healthy subjects [male/female: 6/4; median age (years): 38, range: 28–52], seronegative for any HBV serological markers, without a clinical history of hepatitis and without symptoms or signs of liver disease were studied as controls. Their demographic characteristics were not significantly different from those of patients. All subjects gave written informed consent to the study that conformed to the Helsinki declaration.

Methods

Venous blood samples were collected into heparinized tubes and immediately processed.

PBMCs were obtained by centrifugation of blood over Ficoll solution (Sigma-Aldrich, Milan Italy) and washed three times in RPMI1640 medium containing 10% heat inactivated fetal calf serum (Gibco Invitrogen, Milan, Italy), 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin and 10 mM HEPES (Gibco Invitrogen). The cells were resuspended at the concentration of 1 × 10⁶ PBMC/mL and were then subjected to four different experimental conditions, all in the presence of Concanavalin A (ConA) at 10 mg/mL (Sigma); as previously reported [17,18], ConA was used as a polyclonal stimulus for triggering cytokine production.

The incubation conditions were as follows:
1. culture medium alone (baseline condition);
2. culture medium containing 500 U/mL of IFNα (Wellferon, Glaxo Wellcome; Verona, Italy);
3. culture medium containing 10 μg/mL of Tα1 (SciClone Pharmaceuticals, San Mateo, CA, USA);
4. culture medium containing IFNα and Tα1 together.

Peripheral blood mononuclear cells of healthy controls were cultured only in the presence of culture medium alone without addition of IFNα and/or Tα1. The cells were cultured for 24 h at 37 °C in atmosphere containing 5% CO₂, and then harvested for cytokine analysis: after centrifugation at 500 × g, the supernatants were collected and stored at –80 °C for subsequent test.

The cytokine production was assessed by enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA), using the supernatants harvested from cell cultures. Optical density was determined by plate photometer at 450 nm, and corrected by subtraction of readings at 540 nm. The values of patients were obtained by interpolation with the standard curve. Both the patients and the standard samples were assessed in duplicate.

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 (Elken Chemical, Tokyo, Japan). The sensitivity of the method was 10–810 pmol/dL. Serum HBV markers were tested by Chemiluminescent Microparticles Immunoassay (CMIIA; Architect® Abbott Laboratories, Aprilia, Italy). Serum HBV-DNA was measured quantitatively by real-time PCR assay (Affigene® HBV tender; Alfa Wassermann, Bologna, Italy). The limit of detection for this test was 50 IU/mL.

Nucleotide polymorphisms at nucleotide (nt)1762 and nt1764 in the basal core promoter region and at codon 28 in the precore region were identified by line probe assay Innolipa HBV PreCore (Innogenetics Srl, Pomezia, Italy). Viral genotype was assessed by Trugene® HBV Genotyping Kit (Bayer Diagnostic, Tarrytown, NY, USA).

Statistical analysis

The data are expressed as median (range). The statistical analysis was performed by non-parametric test; in particular, comparison between the different experimental conditions was analysed by Wilcoxon’s test for paired data and correlations between variables were evaluated by 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.

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RESULTS

The main biochemical and virological characteristics of patients are listed in Table 1.

**Th1-cytokines**

At baseline, the concentration of IL-2 of HBV-infected patients was significantly higher when compared with healthy controls \[435 \text{ (75–1828) vs } 86 \text{ (25–139) pg/mL, } P = 0.000\] (Fig. 1a). Concerning the HBV patients, compared with the baseline condition, the addition of IFNz or Tz1 to the culture medium did not significantly modify the IL-2 production (Fig. 1b). Interestingly, the association IFNz/Tz1 induced a significant increase in IL-2 when compared both with baseline \[1081 \text{ (239–2930) vs } 435 \text{ (75–1828) pg/mL, } P = 0.02\] and with Tz1 alone \[1081 \text{ (239–2930) vs } 652.5 \text{ (100–2195) pg/mL, } P = 0.01\].

The IFNγ concentrations (Fig. 2a) did not show any significant differences between patients and controls at the baseline conditions \[2877 \text{ (1567–6738) vs } 2957 \text{ (170–8650) pg/mL, } P = 0.6\]. In HBV patients, the IFNγ level in the different stimulate conditions was not significantly different with respect to baseline. However, the association IFNz/Tz1 was able to increase IFNγ synthesis significantly compared with Tz1 alone \[3220 \text{ (1165–9130) vs } 3000 \text{ (315–7780) pg/mL, } P = 0.03\] (Fig. 2b). No correlation was found between HBV-DNA serum levels and ALT levels and Th1-cytokines both in baseline and in stimulated cultures.

**Th2-cytokines**

At baseline, compared with controls, the IL-4 levels were significantly higher in patients \[0 \text{ (0–83) vs } 41 \text{ (0–207) pg/mL, } P = 0.01\] (Fig. 3a), while the IL-10 levels were significantly lower \[1051 \text{ (295–3150) vs } 2666 \text{ (439–4118) pg/mL, } P = 0.003\] (Fig. 4a). As far as HBV patients are concerned, IL-4 production (Fig. 3b) in the presence of IFNz was higher when compared with all the other experimental conditions, even if the increase reached statistical significance only when compared with the association IFNz/Tz1 \[167 \text{ (0–238) vs } 15 \text{ (0–132) pg/mL, } P = 0.03\], Regarding IL-10 production (Fig. 4b), the addition of IFNz alone resulted in a significant increase in comparison with all the other experimental conditions: baseline \[1705 \text{ (570–4530) vs

### Table 1 Biochemical and virological characteristics of patients

<table>
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<tr>
<th>Case no.</th>
<th>Sex</th>
<th>Age</th>
<th>GPT (U/L)</th>
<th>HBV-DNA (IU/mL)</th>
<th>Nucleotide at 1762/1764</th>
<th>Nucleotide at 1896</th>
<th>Genotype</th>
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<tr>
<td>1</td>
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<td>47</td>
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<td>1 831 823</td>
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<td>A</td>
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<td>9960</td>
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1051.5 (295–3150) pg/mL, \( P = 0.003 \), Tα1 alone [1705 (570–4530) vs 656.5 (280–2060) pg/mL, \( P = 0.003 \)], and IFNα/Tα1 together [1705 (570–4530) vs 982.5 (458–23205) pg/mL, \( P = 0.003 \)]. Finally, the IL-10 concentration in the presence of Tα1 alone was found significantly lower than baseline condition [656.5 (280–2060) vs 1051.5 (295–3150) pg/mL, \( P = 0.02 \)].

No correlation was found between HBV-DNA serum levels and alanine aminotransferase (ALT) levels and Th2-cytokines both in baseline and stimulated cultures.

**DISCUSSION**

As HBV is a non-cytopathic virus, the immune response against virus-infected liver cells and the production of inflammatory cytokines are thought to be responsible for both liver disease and viral clearance [19]. It has been shown that individuals with self-limited HBV infection mount a vigorous polyclonal Th and cytotoxic (CTL) response to multiple viral epitopes; these responses can be persistently found following viral clearance [20–23]. On the contrary, these responses are usually weak or absent in patients who fail to clear the virus and become chronically infected [3,4,24]. Furthermore, a consistent lymphocyte infiltrate with Th-1 profile has been found in the liver of patients chronically infected by HBV, suggesting a pathogenetic role in liver injury [25]. Although these data are still controversial, there is general agreement about the concept that hyporesponsiveness of HBV-specific T cells is an important determinant of virus persistence in chronic HBV infection. Thus, therapeutic strategies should aim to correct this deficiency [26].

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**Fig. 2** (a) Interferon-gamma synthesis (pg/mL) by PBMCs from healthy controls and HBV patients at the baseline condition (baseline). The red line is the median of values. (b) Interferon-gamma synthesis (pg/mL) by PBMCs from HBV patients stimulated by ConA and incubated with Tα1, IFNα or both. The red line is the median of values.

**Fig. 3** (a) Interleukin-4 synthesis (pg/mL) by PBMCs from healthy controls and HBV patients at the baseline condition (baseline). The red line is the median of values. (b) Interleukin-4 synthesis (pg/mL) by PBMCs from HBV patients stimulated by ConA and incubated with Tα1, IFNα or both. The red line is the median of values.
Thymosin-α1 is an immunoregulatory agent capable of triggering maturational events in lymphocytes, to augment T-cell function, to promote reconstitution of immune defects, to amplify the expression of class I HLA and to increase natural killer cell activity [11–13,27]. In PBMCs of patients with chronic hepatitis C, Tα1 has been shown to enhance the Th1 immune response [17].

Data of trials with Tα1 in chronic hepatitis B indicate that it may be at least as effective as IFNα [15,16]. Interestingly, Tα1 is better tolerated than IFNα and seems to induce a gradual and sustained increase in virological response overtime after discontinuation of treatment [15,28,29]. We performed this study to evaluate the effect of Tα1, alone or in association with IFNα on cytokine and antiviral protein 2’5’-OAS synthesis from PBMCs of patients with chronic hepatitis B.

Despite the limited number of patients, this study demonstrated that PBMCs of patients with chronic HBV infection produce more IL-2 than those of healthy controls. On the contrary, IFNγ production was not different between patients and controls. This finding could simply reflect the absence of stimulation of the immune system in healthy subjects, rather than a Th1 polarization in HBV patients, as previously reported [30]. A predominant shift of the immune response towards a Th1 profile has been suggested to be crucial for the eradication of HBV, while a prevalent Th2 response seems to favour viral persistence [21,31]. Therefore, it is possible to speculate that the use of immunomodulatory agent capable of reinforcing the Th1 response could be advantageous to control HBV infection. Our in vitro data show that Tα1 alone, as well as IFNα alone are not able to modify either IL-2 or IFNγ production compared with baseline condition. However, in association, they are able to induce a significant increase in IL-2 not only compared to baseline but also compared to Tα1 alone. As we have not studied the local cytokine production in HBV-infected liver samples, this IL-2 increased production needs further investigations. IL-2 is a pleiotropic cytokine with several antiviral and immunomodulatory proprieties that have been demonstrated to downregulate hepatocellular HBV gene expression by an indirect, noncytopathic process [32]. Thus, we can hypothesize that the association between IFNα and Tα1 could have a role in controlling HBV chronic infection.

As far as the Th2 cytokines are concerned, at baseline, the behaviours in patients and controls were completely opposed and more difficult to explain. In particular, the higher value of IL-4 concentration in patients is not unexpected as it...
reflects the Th2 polarization in chronic infection, as previously described [9,33]. On the other hand, our finding of lower levels of IL-10 in HBV patients is in contrast to previous data [30]. These discrepancies could be partially because of different experimental design and different study population. IL-10 is a potent immunosuppressor that has been recently shown to play a key role in T-cell exhaustion and viral persistence in animal models of viral infection [34].

In HBV patients, Hyodo et al. [30] found a large number of IL-10-secreting cells after stimulation of PBMCs with HBeAg, while no specific stimulation was performed in our study. Furthermore, it should be pointed out that our study sample included only patients with precore mutant infection (HBeAg-negative, HBV-DNA-positive). It is well known that HBeAg is responsible for immunotolerance and T cell anergy [35]. We can speculate that in our patients the precore mutation could have some influence in IL-10 synthesis, even though further studies are needed in this setting.

Our results show that IL-4 production by PBMCs of HBV patients was not modified by the different experimental condition compared to baseline. Nevertheless, IFNz alone significantly increased IL-10 synthesis, while Tz1 induced a significant decrease with respect to baseline. Incubation of Tz1 in combination with IFNz was able to reverse the IFN-induced increase of IL-10. Finally, we confirmed that Tz1 amplifies the synthesis of IFN-induced antiviral protein 2,5-OAS [17].

In summary, Tz1 alone and IFNz alone were found to reduce and increase respectively the synthesis of IL-10 by PBMCs of patients with HBeAg-negative chronic HBV infection. Furthermore, both drugs alone or in association significantly increased the antiviral protein 2,5-OAS production. On the other hand, the association Tz1 plus IFNz induced a significant increase in IL-2 and blocked the IL-10 increase induced by IFNz. If this profile could be effectively advantageous in the treatment of chronic hepatitis B, more information is needed about intrahepatic environment. It is well known that nucleoside/nucleotide analogues, widely used for treatment of chronic HBV, are able to achieve the suppression of viral replication, but at the same time, they are inadequate to induce recovery of the immune system [36]. In this view, compounds able to modulate the host immune response, should be further investigated to boost or increase HBV-immune response.

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REFERENCES


