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Association of cytokine gene polymorphisms and serum concentrations with the outcome of chronic hepatitis B

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ABSTRACT

Objective: The present paper investigated possible correlations between the clinical presentation of hepatitis B and the TNF-α −308G/A, IFN-γ +874A/T, TGF-beta1 −509C/T, and IL-10 −1081A/G polymorphisms and associated serum levels of these cytokines.
Methods: Fifty-three hepatitis patients were selected and divided into two groups: *A* – inactive ($n = 30$) and *B* – chronic hepatitis/cirrhosis ($n = 23$). The control group consisted of 100 subjects who were positive for anti-HBc and anti-HBs. The serum concentrations of the cytokines were determined by immunoenzymatic assays. The polymorphisms of the cytokines genes were assessed by PCR and PCR-SSP.
Results: The mean serum levels of IFN-γ of the control group were significantly higher than those of groups *A* and *B*, whereas the mean levels TGF-beta1 were significantly higher in groups *A* and *B* in comparison with the control. In the case of IL-10, the mean serum level recorded in the control group was significantly higher than that of group *B*. The TNF-α −308AG genotype was considerably more frequent in group *B* (43.3%) than the control (14.4%).
Conclusion: Higher serum levels of IFN-γ and TGF-beta1 were associated with chronic hepatitis B, and lower serum levels of IL-10 were found in patients with the active disease. Furthermore the presence of allele A of the TNF-α −308 polymorphism suggest a risk of the progressive disease.

1. Introduction

Worldwide, it is estimated that over 2 billion people have had contact with the hepatitis B virus (HBV), and that 350–400 million are chronically infected, of which approximate 15–40% will evolve to decompensation. An estimated 500,000–1 million deaths are attributed to this infection each year, resulting from complications caused by cirrhosis or hepatocellular carcinomas (HCC) [1].

Once infected with HBV, the subject may have an acute self-limited infection or evolve to chronicity. The chronic form of hepatitis B is characterized by several phases, including immunotolerance, chronic hepatitis with positive or negative HBeAg, as well as the chronic disease carrier [2–4]. This complex clinical framework is the result of the interaction of factors inherent to the host and the virus, as well as environmental conditions, but is related primarily to the age and immunological status of the infected person [3,5–7].

The understanding of the complex virus–host interaction, which results in a variety of clinical manifestations, necessarily requires a good working knowledge of the immunopathogenesis of the disease and the importance of the immunological profile of the host and its cytokine secretion pattern [8,9].

The proinflammatory cytokines TNF-α and IFN-γ are critical to viral clearance in the acute phase [9–11], while in the chronic hepatitis phase, the production and function of these cytokines are altered by the immune deficiency of the host and the viral escape mechanism [12–14]. Chronic HBV patients are assumed to have a predominance of the Th2 response over that of Th1, resulting in an increase in the production of anti-inflammatory cytokines, such as IL-10 [13,15]. The persistent activation of the star cells in the liver stimulates the continuous synthesis of TGF-beta1 by these cells, which has a restorative function, and the synthesis of collagen, which has an antagonistic effect to the action of the inflammatory cytokines [16–19].

A number of studies have attempted to associate specific mutations in the cytokine genes with a given pattern of secretion [20,21], as well as their influence on the evolution of chronic hepatitis B [19,22–25]. The present study describes the serum levels associated with the polymorphisms in the TNF-α −308G/A, IFN-γ +874T/A, TGF-β1 −509C/T and IL-10 −1082A/G genes in patients with chronic hepatitis B, in order to identify specific immunogenetic markers associated with chronic HBV infection.

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2. Materials and methods

2.1. Populations examined

Following the approval of the project by the research ethics committee, 53 chronic (HBC) hepatitis B patients (36 males and 17 females) were selected from those being treated at a renowned hepatology clinic in the northern Brazilian city of Belém (Pará state) between January 2007 and December 2008. The patients were divided into two groups: (*A*) inactive carriers, defined by the presence of HBeAg negative, viral load lower than 2000 UI/mL, and normal levels of alanine aminotransferase; and (*B*) those with chronic hepatitis with cirrhosis or not (HBeAg negative or positive, high viral load, liver biopsy with inflammatory activity or fibrosis higher or equal to #2 on the scale METAVIR, and high levels of alanine aminotransferase). The control group comprised 97 healthy people (39 males and 58 females) with natural immunity to HBV, positive anti-HBc IgG and anti-HBs, and negative anti-HCV and anti-HIV. The members of both groups were resident in the northern Brazilian city of Belém (capital of the State of Pará) and were of similar ethnic origin. All patients were clinically evaluated and submitted to biochemical tests, ultrasound, endoscopy, and, if possible, liver biopsy. Patients co-infected with HCV, HDV, and HIV were excluded from this analysis.

2.2. Ethical considerations

The individuals were briefed about the project and those who accepted were given an inform consent to sign. The present work was submitted and approved by the Ethics Committee of the Santa Casa de Misericórdia do Pará Foundation and followed the Brazilian Guidelines and Regulatory Standards for Research Involving Human Subjects (Resolutions 196/1996 and 347/2005 of the Brazilian National Health Council).

2.3. Serology and viral load

Serological analyses (HBsAg, anti-HBc IgG and IgM, HBeAg, anti-HBe, and anti-HBs) were conducted using an enzyme immunoassay kit (Organon Teknika®, Netherlands; Abbott®, USA; Ortho Clinical Diagnostics®, Germany) according to the manufacturers' instructions. Viremias were quantified by the hybridization method using the AMPLICOR (Roche®, USA) commercial kit, which has detection limit of 60 UI/mL.

2.4. Cytokine serum levels

Plasma levels of TNF-α, INF-γ, TGF-β1, and IL-10 were quantified at the ICB/UFPA Virology Laboratory of Virology using an enzyme immunoassay (Human ELISA Ready-SET-Go, EBioscience, Inc. California, San Diego, USA). This test was based on the use of a specific monoclonal antibody for each of the markers mentioned above, according to their specific protocols.

2.5. Evaluation of the polymorphisms of the cytokine genes

The polymorphisms of the TNF-α, INF-γ, TGF-β1, and IL-10 genes were evaluated by PCR gene amplification followed by the analysis of restriction fragment length polymorphisms (RFLPs) or the amplification of specific alleles (Table 1). The DNA was extracted from peripheral blood leukocytes, using the Puregene kit from Gentra Systems, Inc., USA. The amplified products were electrophoresed (100 V/45 min) in 3% agarose gel with TAE 1× buffer (TAE 40× stock – TrisBase 1.6 M, 0.8 M Na acetate and EDTA-Na_2 40 mM/1000 mL deionized water), containing 5 μL of ethidium bromide (10 mg/mL), and visualized by transillumination with a source of ultraviolet light.

2.6. Statistical analysis

The parametric data were analyzed using a one or two criteria Analysis of Variance (ANOVA) and Student's *t*. Nonparametric data were analyzed using the Chi-square (χ^2) test. Association between variables was evaluated with use of Spearman's correlation coefficient. A multivariate Hotelling approach was used in the ANOVAs. A significance level of $p = 0.05$ (5%) was used in all analyses.

3. Results

The distribution of genotype and allele frequencies showed no statistically significant differences when the groups were compared, but the distribution of cytokine alleles and genotypes indicates an association between chronic infection with HBV and the mutant allele of the TNF-α gene (Table 2).

The mean serum level of TNF-α in patients with chronic hepatitis B was 20.04 ± 9.44 pg/mL, while that of the control group was 21.44 ± 15.79 pg/mL ($p = 0.574$). Similar mean values ($p > 0.05$) were also recorded in groups *A* (20.55 ± 9.99 pg/mL) and *B* (19.34 ± 8.81 pg/mL) – see Fig. 1.

The mean serum level of IFN-γ in the patients with chronic hepatitis B was significantly lower ($p < 0.0001$) than that of the control group, with mean values of 94.69 ± 33.14 pg/mL and 136.78 ± 84.37 pg/mL, respectively. The mean level recorded in the control group is also significantly higher ($p < 0.01$) than the mean recorded in either group *A* (87.44 ± 25.11 pg/mL) or *B* (104.57 ± 40.22 pg/mL) – see Fig. 1.

Table 1
List of the primer sequences and methods used for investigating the TNF, IFN, TGF-β1 and IL-10 gene polymorphisms.

Polymorphism	Method	Primer	Alleles
TNF-α −308G > A	RFLP (NcoI)	TNF-α FW 5′-AGG CAA TAG GTT TTG AGG GCC AT-3′ TNF-α R 5′-TCC TCC CTG CTC CGA TTC ATT CG-3′	G: 87 pb 20 pb A: 107 pb
IFN-γ +874T > A	SSP-PCR	IFN-γ PC 5′-TCA ACA AAG CTG ATA CTC CA-3′ IFN-γ T 5′-TTC TTA CAA CAC AAA ATC AAA TCT-3′ IFN-γ A 5′-TTC TTA CAA CAC AAA ATC AAA TCA-3	T: 262 pb A: 262 pb
TGF-β1 −509C > T	RFLP (DdeI)	TGF-β1FW 5′-GGA GAG CAA TTC TTA TAG GTG -3′ TGF-β1 R 5′-TAG GAG AAG GAG GGT CTG TC-3′	C: 74 pb 46 pb T: 120 pb
IL-10 −1082A > G	RFLP (MnlI)	IL-10 FW 5′- TCT GAA GAA GTC CTG ATG TC-3′ IL-10 R 5′- CTC TTA CCT ATC CCT ACT TCC-3′	A: 125 pb 65 pb G: 93 pb 65 pb 32 pb

Table 2
Distribution of cytokine alleles and genotypes in hepatitis B patients and controls.

Genotype	Control group *N* (%)	Chronic hepatitis B *N* (%)	Group *A* *N* (%)	Group *B* *N* (%)	Odds ratio	*p*
TNF-alpha −308						0.019[i]
GG	82 (84.5)	41 (77.3)	23 (76.6)	12 (51.2)		
GA	14 (14.4)	10 (18.9)	06 (20.0)	10 (43.3)		
AA	01 (1.1)	02 (3.8)	01 (3.4)	01 (4.5)		0.001[j]
G	178 (91.7)	92 (86.8)	52 (86.7)	34 (73.9)	2.587[a]	
A	16 (8.3)	14 (13.2)	08 (13.3)	12 (26.1)	2.294[b]	
INF-gamma +874						0.364[k]
TT	09 (9.4)	07 (13.2)	04 (13.3)	03 (13.0)		
TA	37 (28.1)	19 (35.8)	14 (46.7)	05 (21.8)		
AA	51 (52.5)	27 (51.0)	12 (40.0)	15 (65.2)		
T	55 (28.4)	33 (31.1)	22 (36.7)	11 (23.9)	0.875[c]	0.372[l]
A	139 (71.6)	73 (68.9)	38 (63.3)	35 (76.1)	0.533[d]	
TGF-beta1 −309						0.569[m]
CC	24 (24.7)	17 (32.0)	09 (30.0)	08 (34.7)		
CT	39 (40.2)	19 (36.0)	09 (30.0)	10 (43.4)		
TT	34 (35.1)	17 (32.0)	12 (40.0)	05 (21.9)		
C	87 (44.8)	53 (50.0)	27 (45.0)	26 (56.2)	0.813[e]	0.346[n]
T	107 (55.2)	53 (50.0)	33 (55.0)	20 (43.8)	0.629[f]	
IL-10 −1082						0.866[o]
AA	47 (48.4)	27 (50.9)	17 (56.7)	10 (43.5)		
AG	41 (42.3)	20 (37.7)	10 (33.3)	10 (43.5)		
GG	09 (9.3)	06 (11.4)	03 (10.0)	03 (13.0)		
A	135 (69.6)	74 (69.8)	44 (73.3)	30 (65.2)	0.982[g]	0.665[p]
G	59 (31.4)	32 (30.2)	16 (26.7)	16 (34.8)	1.466[h]	

N = chromosome number; group *A* = inactive carrier; group *B* = chronic hepatitis with or without cirrhosis.
[a] Control vs. chronic hepatitis B ($p = 0.011$, IC 95% = 1.277–5.241).
[b] Group *A* vs. group *B* ($p = 0.157$, IC 95% = 0.849–6.196).
[c] Control vs. chronic hepatitis B ($p = 0.709$, IC 95% = 0.522–1.466).
[d] Group *A* vs. group *B* ($p = 0.109$, IC 95% = 0.265–1.071).
[e] Control vs. chronic hepatitis B ($p = 0.462$, IC 95% = 0.506–1.306).
[f] Group *A* vs. group *B* ($p = 0.327$, IC 95% = 0.290–1.364).
[g] Control vs. chronic hepatitis B ($p = 0.982$, IC 95% = 0.586–1.644).
[h] Group *A* vs. group *B* ($p = 0.491$, IC 95% = 0.637–3.377).
[i] $\chi 2 = 11.703$ group *B* vs. control.
[j] $\chi^2 = 11.332$ group *B* vs. control.
[k] $\chi^2 = 4.322$.
[l] $\chi^2 = 1.975$.
[m] $\chi^2 = 2.929$.
[n] $\chi^2 = 2.121$.
[o] $\chi^2 = 1.268$.
[p] $\chi^2 = 0.813$.

Very significantly higher serum levels of TGF-β1 were recorded in the patients (1163.00 ± 1084.11 pg/mL) in comparison with the control group, 422.39 ± 210.54 pg/mL ($p < 0.0001$). Similar differences were observed between the control group and the values recorded for groups *A* (1201.77 ± 1207.58 pg/mL) and *B* (1108.09 ± 777.44 pg/mL). On the other hand, serum levels of IL-10 were significantly higher in the control group (39.83 ± 15.82 pg/mL) in comparison with the patients in general (32.97 ± 10.77 pg/mL), and those of groups *A* (30.35 ± 8.21 pg/mL) and *B* (34.49 ± 11.07 pg/mL).

Correlation analysis between serum levels of cytokines with the viral load showed no association. Similar results were obtained from the comparison between the SNPs and viremia levels (data not shown).

4. Discussion

The present study recorded a significantly higher prevalence of chronic hepatitis B in males and also older individuals in comparison with those with spontaneous resolution. This is consistent with the findings of previous studies, which have shown a three- to sixfold propensity in males for the development of the chronic condition, which may be explained by the theoretically protective effects of estradiol, which induces the production of IFN-γ. This hormone supports the seroconversion of antibodies to anti-HBs and anti-HBe in acute cases, as well as providing an antioxidant effect in the chronic phase, which may lead to a more benign clinical progression in premenopausal women [26].

The distribution of TNF-α genotypes in the study groups was similar to that recorded in previous studies in Asia and Brazil [22,27]. The present study identified a 2.6-fold higher risk of developing the chronic disease in individuals with the A allele. Jeng et al. [28] also recorded a fourfold risk of chronic HBV in carriers with HCC.

The analysis of the −308G/A intergroup of the TNF-α polymorphism indicated a significant increase in the GA genotype in patients with the active disease (group *B*) in comparison with the control group. This mutation has been related to more aggressive development of the disease [29].

The mutant IFN-γ +874T/A allele was relatively frequent in all groups, indicating a lack of any systematic relationship with the risk of chronic HBV infection. These findings contrast with those from other studies [19,30,31] in which the +874AA genotype was associated with an increased risk of chronic HBV and the A allele with increased susceptibility to infection. However, conflicting results have been obtained from other studies [22,27], and the exact effect of this mutation remains unclear.

The +874T > A mutation induces a reduction in the secretion of IFN-γ [32], which is fundamental to the innate and adaptive immune responses of the organism against HBV. Decreased levels of IFN-γ are found in chronic HBV carriers in relation to acute

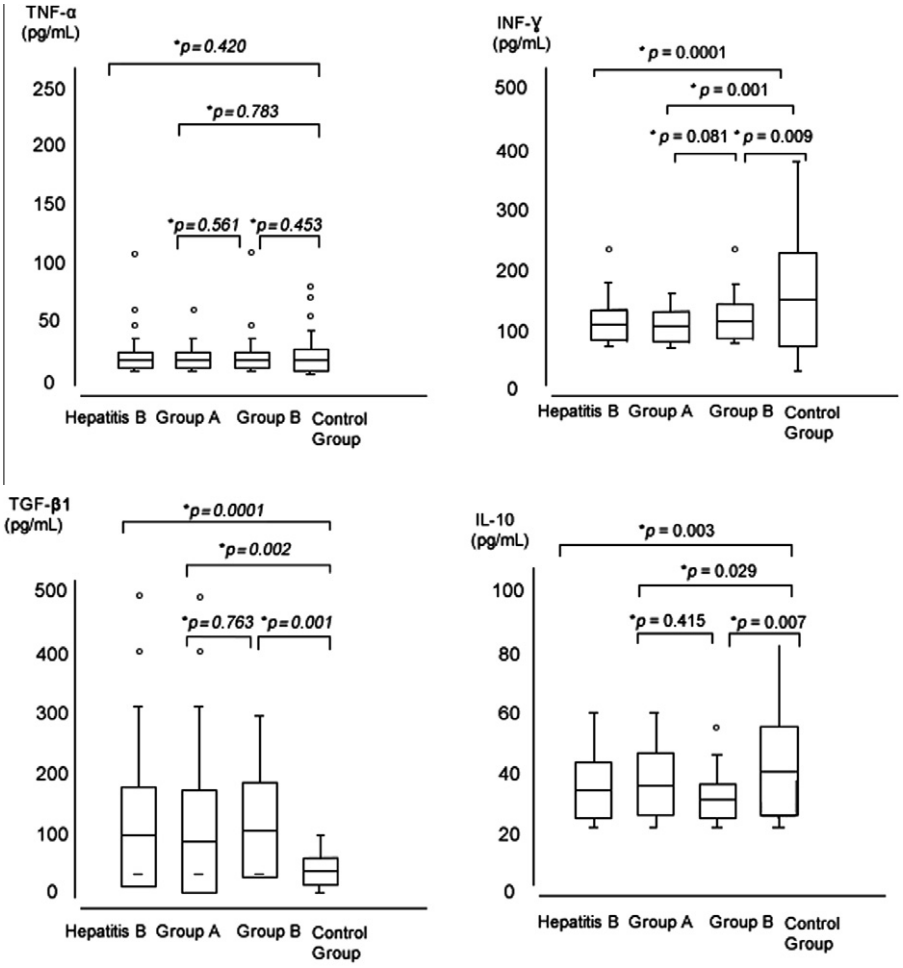

Fig. 1. Serum levels of TNF-α, INF-γ, TGF-β1 and IL-10 in patients with chronic hepatitis B (all patients), inactive carriers (group *A*), chronic patients (group *B*), and the control group. *Probabilities for Student's *t*.

self-limited patients [9,13,14]. In the present study, this pattern was confirmed by the significantly lower serum levels of IFN-γ recorded in inactive carriers (infected or replicants) in comparison with the control group.

In the case of the TGF-β1 −509C/T polymorphism, in which the mutation of C to T increases the synthesis of the cytokine, a predominance of the CT genotype was recorded in both patients and controls, and no statistical difference was found in the frequency of this genotype in groups *A* and *B*. In their study of patients with chronic hepatitis B, Migita et al. [22] recorded a frequency of 52.1% for the CT genotype in the infected group, a value similar to that recorded in the present study, although in the earlier study, the principal objective was the evaluation of the risk of the development of neoplasia in patients with the mutant T allele, which was recorded in 85.4% of the HCC cases, and in 68.1% of patients without HCC.

In the case of another anti-inflammatory cytokine, IL-10, the present study recorded similar frequencies for the IL-10 −1082A/G polymorphism in both patient and control groups, with a predominance of the AA genotype. An Asian study [30] reported higher frequencies of the AG genotype in both controls and chronic patients, but a higher risk of chronic HBV and progression to cirrhosis in patients with the AA genotype.

Unexpectedly, serum levels of IL-10 were significantly higher in the patients – especially those with the more active form of the disease – in comparison with the control group. The IL-10 cytokine is known to be produced by LTh2, which inhibits the action of IFN-γ and controls the suppression of fibrogenesis by stellate cells mediated by the action of TGF-β1 [33]. In chronic HBV carriers, there is a predominance of the Th2 response, which hinders Th1-dependent viral clearance. There is believed to be a concomitant increase in the action of Treg cells (CD25+), which would increase the production of IL-10 and suppress the Th1 response stimulated by HBcAg [15].

The lower serum levels of this cytokine observed in HBV patients is similar to the pattern recorded in chronic HCV infection, in which plasma concentrations of IL-10 were significantly lower in infected patients than in uninfected individuals [34,35]. In the present study, a possible explanation for the elevated levels of IL-10 found in the control group is the fact that the levels were measured only in the serum and not the tissue, with no other assessment of the variants of this locus, such as the influence of different genotypes on the pattern of cytokine secretion. Sample size may have been an additional problem.

The balance between the responses of the Th1 and Th2 cells is known to be essential to the adequate functioning of the system and viral shedding, which does not occur in patients with chronic HBV [9,13]. The data available at the present time indicate the importance of the interaction of three factors in the epidemiology of hepatitis B – the characteristics of the virus, the host, and the environment. Given this, an integrated approach will be essential to a better understanding of the phenomenon.

Advances in all field of research, including immunology and human genetics, will undoubtedly contribute to the eventual

elucidation of this phenomenon, but it is important to maintain a holistic view of the human being as an organism, and the social and environmental contexts of this organism. Future studies with larger samples and a more ample approach to the problem will be needed to ratify the results of the present study, and contribute to the resolution of the many as yet unanswered questions remaining with regard to the immunopathogenesis of chronic HBV infection.

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