Association of cytokine genetic polymorphism with hepatites B infection evolution in adult patients

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The infection by the hepatitis B virus (HBV) has different forms of evolution, ranging from self-limited infection to chronic hepatic disease. The objective of this study was to evaluate the influence of cytokine genetic polymorphisms in the disease evolution. The patients were divided into two groups, one with chronic HBV (n = 30), and the other with self-limited infection (n = 41). The genotyping for TNF (–308), TGFB1 (+869, +915), IL-10 (1082, –819, and –592), IL-6 (–174), and IFNG (+874) was accomplished by the PCR-SSP (polymerase chain reaction with sequence specific primers technique using the One Lambda kit. Although no statistically significant differences were found between the groups, the combination of TNF –308GG and IFNG +874TA was found in a lower frequency in chronic patients than in individuals with self-limited infection (26.7 versus 46.3%; P = 0.079; OR = 0.40; IC95% = 0.14-1.11). In chronic patients with histological alterations it was not observed the genotype TGFB1+869 C/C, against 24.4% in the self limited infection group (100 versus 75.6%; P = 0.096; OR = 7.67; IC95% = 0.42-141.63). Further studies in other populations, and evaluation of a greater number of individuals could contribute for a better understanding of the cytokine genetic polymorphism influence in HBV infection evolution.

Key words: cytokine gene polymorphism - hepatitis B - genetic association

The infection by the hepatitis B virus (HBV) appears under different forms of evolution, ranging from the asymptomatic and self-limited infection to the chronic state, which can develop into chronic hepatitis, cirrhosis, and hepatic-cellular carcinoma (Thomas & Strickland 2000). It is estimated that the virus infected 350 million people in the whole world, forming a reservoir which facilitates the spreading of the disease (Kane 1995). Furthermore, in spite of the transmission reduction after the vaccination advent (Shih et al. 1999, Berlioz-Arthaud et al. 2003), the infection remains as an important cause of chronic hepatic disease (Thomas & Strickland 2000).

The Health Ministry estimates that 1% of the Brazilian population has chronic diseases related to HBV (Ministério da Saúde 2003, Ferreira 2004). The factors that lead a patient with acute infection by HBV to become persistently infected are not totally established. Environmental factors as well as factors innate to the virus do not completely explain the different forms of the HBV infection evolution (Chu & Lok 2002, Kao et al. 2002), bringing up a discussion of the implication of genetic factors in disease evolution (Thio et al. 1999, Thursz 2001).

The initial anti-HBV immune response promotes the killing of infected cells by the virus and the secretion of antiviral cytokines by cells of the innate immunity (Ferrari et al. 2003). However, the infection control is not reached at this stage of HBV response. A vigorous and polyclonal immune response of T cells is pointed out as fundamental for the virus elimination and disease resolution (Guidotti et al. 1999). The well-successful response to HBV is a result of both specific anti-virus cytotoxic T-cells activity, which eliminates the infected cells, and non-cytolitic mechanisms exerted through cytokines released by T and non-T cell infiltrates. The cytokines are able to suppress the viral expression and replication, mediating the infection control without causing death of the infected cell. The evolution of the infection to chronicity is associated to a weak or undetectable humoral immune response, and is identified by the persistency of HBV's surface antigen (HBsAg) in the circulation (Jung & Pape 2002, Ferrari et al. 2003).

Several studies have evaluated the influence of genetic factors in the cytokine production by cells of the immune system. The genetic polymorphism was pointed out because it correlates to both the transcription level and in vitro production of tumor necrosis factor alfa (TNF-α) (Kroeger et al. 1997, Westendorp et al. 1997, Hajeer & Hutchinson 2001). The genotypic variation of interferon gama (*IFNG*) (Pravica et al. 1999), interleukin 6 (*IL-6*) (Fishman et al. 1998), T cell growth factor beta (*TGFB1*) (Awad et al. 1998) and interleukin 10 (*IL-10*) (Crawley et al. 1999) were also shown to influence its production.

As cytokines play a fundamental role in the immune response to HBV, and HBV infection may present different forms of evolution (self-limited or persistent and progressive), we decided to evaluate the correlation of the *INFG*, *TNF*, *IL-6*, *IL-10*, and *TGFB1* genetic polymorphisms with the disease evolution. These cytokines can affect the infection control either by suppressing viral replication (INF-γ, TNF-α and IL-6) or inhibiting the T-cell activation (IL-10, TGF-β).

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PATIENTS AND METHODS

Patients - Thirty white Brazilian patients with chronic infection by HBV seen at Ambulatório de Doenças Infecciosas-Hepatite from Hospital Universitário Regional de Maringá (HURM) and at Ambulatório de Hepatite da Policlínica Zona Sul from Maringá municipality, Paraná, were enrolled in this study. All patients were serum positive for the HBV surface antigen (HBsAg) and 26 of them were serum positive in two or more tests performed in a time interval higher than six months. From the other four patients, in one of them the hepatic biopsy accomplished 14 months after the HBsAg test showed the presence of active chronic hepatitis in cirrhosis phase. The other three patients were negative for anti-HBs antibodies with intervals higher than six months after the HBsAg test and presented normal transaminase values in two or more evaluations.

A second group comprising 41 white Brazilian candidates for blood donation that showed positive results for the antibody against both the HBV core (anti-HBc) and the surface antigen of HBV (anti-HBs) was also enrolled. In this study, this group was named as self-limited infection group (Table I). All of the patients and blood donors gave the informed consent to participate in the study, which was approved by the Ethics Committee in Research with Human Beings of Maringá State University. All the participants of this study are white-Brazilians from Paraná, Brazil. Paraná's white population is predominantly of European origin (80.6%), with a small but significant contribution of African (12.5%) and Amerindians (7%) genes (Probst et al. 2000).

DNA extraction and genotyping of the cytokine genes - Genomic DNAs were extracted and purified from whole blood collected with ACD (acid citric dextrose) using EZ-DNA kit (Biological Industries, Israel), according to the manufacture's instructions.

The single nucleotide polymorphisms (SNPs) were analyzed for *TNF* (–308 G or A), *TGFB1* (+869 T or C, and +915 C or G), *IL-10* (–1082 A or G, –819 T or C, and –592 A or C), *IL-6* (–174 C or G), and *IFNG* (+874 T or A) by polymerase chain reaction (PCR) with sequence specific primers through the Cytokine Genotyping Primers Kit (One Lambda®, CA, US). All amplifications were performed according to the manufacturer's recommendations. The PCR products were then visualized by electrophoresis in 2% agarose gel stained with ethidium bromide.

Statistical analysis - The allelic and genotypic frequencies of each SNP were obtained by direct counting. The cytokine genetic polymorphism distribution of both HBV chronic patient group (CP) and self-limited infection group (SLI) were compared by Qui-square test and/or Fisher exact test with the aid of the Statistica 6.0 program. The *P* values smaller or equal to 0.05 were considered significant. For each comparison, it was calculated the value of OR (odds ratio), using Haldane's modifications of the Wolf method when necessary.

RESULTS

Tables II and III summarize, respectively, the cytokine genotype distribution and allelic frequencies of patients with either self-limited infection (n = 41), or with chronic hepatitis (n = 30).

The genotype and the allele frequencies between these groups showed no significant statistical differences for both *TNF* and *IFNG* polymorphisms. However, the genotypic combination of *TNF* –308 G/G with *IFNG* +874 T/A showed a lower frequency in chronic patients (25 versus 47.5%; *P* = 0.079; OR = 0.37; IC 95% = 0.13-1.01).

The *TGFB1* +869 and +915 genotypic and allelic frequencies between the groups with self-limited infection and chronic patients were not statistically significant. However, 11 of the 30 chronic hepatitis patients showed histological alterations confirmed by biopsy, and it was not observed the genotype *TGFB1*+869 C/C in this group, against 24.4% in the self limited infection group (Table IV).

The genotypic and allelic distribution related to the three SNPs of *IL–10* (–1082 A or G, –819 T or C, and –592 A or C) did not show any statistical difference among the studied groups.

Considering the genotype combinations, it was also verified a higher frequency of *TGFB1* +869 T/C with *IL-10* –819 C/C in chronic patients with histological alterations, as compared to the self-limited infection group (54.5% versus 24.4%; *P* = 0.073; OR = 3.72; IC 95% = 0.93-14.85).

The polymorphism of *IL-6* –174 did not show significant differences in relation to alleles, genotypes or phenotypic expression. Only 8.7% of individuals showed the C/C genotype.

TABLE I

Characterization of hepatites B virus chronic patient group (CP) and self-limited infection group (SLI)

Characteristics	CP (N = 30)	SLI (N = 41)
Age (average)	37 ± 11.65	39.5 ± 10.2
Sex (M/F)	18/12	20/21
Average patient follow up (months)	37.07 ± 31.11	-
ALT (average)	48.89 ± 59.53	-
AST (average)	32.43 ± 26.65	-
AgHBe (±)	5/23[a]	-
Anti-HBe (±)	24/4[a]	-

ALT: alanine aminotransferase; AST: serum aspartate transaminase; *a*: HBeAg and anti-HBe tests performed in 28 samples. Transaminase values reflect the period of sample's collection.

TABLE II

TNF, *TGFB1*, *IL-10*, *IL-6*, and *IFNG* genotype frequencies according to hepatitis B virus (HBV) infection evolution in adult individuals living at Maringá city, Paraná, Brazil

Polymorphism		HBV infection evolution						
		SLI (N = 41)		CP (N = 30)				
Gene	Genotype	n	%	n	%	*P*	OR	CI 95%
TNF	A/A	1	2.4	0	0	1.000	0.44	0.02-11.25
(-308)	A/G	7	17.1	9	30.0	0.198	2.08	0.67-6.43
	G/G	33	80.5	21	70.0	0.306	0.57	0.19-1.70
TGFB1	T/T G/G	14	34.2	10	33.3	0.943	0.96	0.36-2.61
(+869, +915)	T/C G/G	16	39.0	15	50.0	0.357	1.56	0.60-4.05
	T/C G/C	1	2.4	1	3.3	1.000	1.38	0.08-22.97
	C/C G/G	6	14.6	2	6.7	0.453	0.42	0.08-2.23
	T/T G/C	0	0	0	0	-	-	
	C/C G/C	4	9.8	2	6.7	1.000	0.66	0.11-3.87
	C/C C/C	0	0	0	0	-	-	-
	T/T C/C	0	0	0	0	-	-	-
	T/C C/C	0	0	0	0	-	-	-
IL-10	GCC/GCC	5	12.2	2	6.7	0.691	0.51	0.09-2.58
(-1082,-819,-592)	GCC/ACC	10	24.4	8	26.7	0.828	1.13	0.38-3.31
	GCC/ATA	10	24.4	8	26.7	0.828	1.13	0.38-3.31
	ACC/ACC	5	12.2	7	23.3	0.337	2.19	0.62-7.74
	ACC/ATA	8	19.5	4	13.3	0.541	0.63	0.17-2.34
	ATA/ATA	3	7.3	1	3.3	0.633	0.44	0.04-4.42
IL-6	G/G	20[a]	50.0	15	50.0	1.000	1.00	0.39-2.58
(-174)	G/C	18	45.0	14	46.7	0.890	1.07	0.41-2.77
	C/C	2	5.0	1	3.3	1.000	0.66	0.06-7.58
IFNG	T/T	3[a]	7.5	6	20.0	0.158	3.08	0.70-13.52
(+874)	T/A	23	57.5	12	40.0	0.147	0.49	0.19-1.29
	A/A	14	35.0	12	40.0	0.668	1.24	0.47-3.29

TNF: tumor necrosis factor; *TGFB1*: T cell growth factor B1; *IL*: interleukin; *INFG*: interferon gama; SLI: self-limited infection; CP: chronic patients; *a:*genotyping not accomplished in one sample; OR: odds ratio; CI: confidence interval.

TABLE III

TNF, *TGFB1*, *IL-10*, *IL-6*, and *IFNG* allele frequencies according to hepatites B virus (HBV) infection evolution in adult individuals living at Maringá city, Paraná, Brazil

Polymorphism		HBV infection evolution				
Gene	Allele	SLI (N = 40)	PC (N = 30)	*P*	OR	CI 95%
TNF(–308)	A	0.110	0.150	0.477	1.43	0.53-3.86
	G	0.890	0.850			
TGFB1(+869)	T	0.549	0.600	0.543	1.23	0.63-2.42
	C	0.451	0.400			
TGFB1(+915)	G	0.939	0.950	1.000	1.23	0.28-5.38
	C	0.061	0.050			
IL-10(–1082)	G	0.366	0.333	0.689	0.87	0.43-1.75
	A	0.634	0.667			
IL-10(–819)	C	0.707	0.767	0.430	1.36	0.63-2.92
	T	0.293	0.233			
IL-10(–592)	C	0.707	0.767	0.430	1.36	0.63-2.92
	A	0.293	0.233			
IL-6(–174)	G	0.725[a]	0.733	0.913	1.04	0.49-2.22
	C	0.275	0.267			
IFNG(+874)	T	0.637[a]	0.600	0.651	1.17	0.59-2.33
	A	0.363	0.400			

TNF: tumor necrosis factor; *TGFB1*: T cell growth factor B1; *IL*: interleukin; *INFG*: interferon gama; SLI: self-limited infection; CP: chronic patients; OR: odds ratio; CI: confidence interval; *a:* genotyping not accomplished in one sample.

TABLE IV

Genotype frequencies of *TGFB1*+869 according to HBV infection evolution in adult individuals living at Maringá city, Brazil

Genotype	CPHA		SLI				
	N = 11	%	N = 41	%	*P*	OR	95% CI
C/C	0	0	10	24.4	0.096	0.48	0.13-1.70
T/C	7	63.6	17	41.5	0.308	2.47	0.62-9.79
T/T	4	36.4	14	34.1	1.000	1.10	0.28-4.41

TGFB1: T cell growth factor B1; SLI: self-limited infection; CPHA: chronic patients with histological alterations; OR: odds ratio; CI: confidence interval.

DISCUSSION

Cytokines are soluble polypeptides that act in defense against viral infections, either directing the Th1/Th2 host's immune response predominant pattern, or directly inhibiting viral replication. Nucleotide variations in regulatory gene regions can result in two or more alleles for each cytokine locus, which in some cases manifest by the increase or decrease of cytokine gene transcription (Bidwell et al. 1998).

Studies on the cytokine gene polymorphisms points out to its role on the evolution of HBV infection, ranging from the pathogenesis of fulminating hepatitis (Leifeld et al. 2002) until its action in viral replication inhibition (Chen et al. 2005). Studies in HBV infected patients point out to the role of IFN-γ in the infection control. Suri et al. (2001), investigating the non-cytolitic activity over the patients' hepatocites with acute and chronic infection observed the action of IFN-γ as a viral replication inhibitor. Recently, Szkaradkiewicz et al. (2003) related the Th1 activity to self-limited hepatitis B recuperation. Nevertheless, the predominantly cyto-kine pattern found in chronic patients was related to an increase of IL-10 production. Hyodo et al. (2004) observed an increased production of this cytokine in response to the *core* antigen of HBV (AgHBc) in patients with chronic infection, suggesting that the excessive production of IL-10 may contribute to HBV persistency in these patients.

Considering the findings that the level of TNF-α was decreased in animal models that developed into chronicity of the infection by HBV (Hodgson & Michalak 2001), various studies have been carried out seeking to demonstrate the association of *TNF* genetic polymorphism and the infection evolution.

In a Korean population with chronic disease patients and individuals promptly recovered, Kim et al. (2003a) observed an association between the allele A in position –308 (G/A or A/A) with resolution of HBV infection. In accordance with these findings, a study with HBeAg positive mothers was carried out demonstrating a significantly higher frequency of both *TNF* 308GG and allele G in mothers with chronic infection (Kim et al. 2003b). Cheong et al. (2006) did not find associations of the *TNF* 308 polymorphism with either resolution or persistence of HBV infection. Nonetheless, he found that the haplotype –308 G/ –238 G was associated with HBV persistence. On the other hand, Xu et al. (2005) verified a higher frequency of both, *TNF* –308 GA and the allele A in chronic patients with severe disease in relation to controls, asymptomatic patients and patients with mild disease. Nevertheless, they did not see any difference between chronic patients, as a single group, and controls. They also found that the TNF-α serum level was significantly higher in G/A genotype patients with severe disease in relation to those with G/G genotype. These results point out to the contribution of TNF-α to the severity of HBV infection. The outcome of HBV infection was also evaluated in relation to the polymorphism of other positions at the TNF-α promoting region, where it was observed an association between –238 G/G SNP and chronic infection (Lu at al. 2004, Du et al. 2006).

We could not find the same relation when evaluating the *TNF* polymorphism by itself. However, it was observed a higher frequency of patients with self-limited infection with the combination that has been shown as lower production potential of TNF-α (*TNF* –308 G/G) (Kroeger et al. 1997), and intermediate production potential of INF-γ (*INF-γ* +874 T/A) (Pravica et al. 1999).

In the present study, the susceptibility to chronic infection by HBV was not associated to the genetic polymorphism of IL-10 or IFN-γ as for alleles or genotypes. Accordingly, a study conducted by Miyazoe et al. (2002) did not find a significant difference between HBV infected patients and health volunteers. However, the frequencies of the *IL-10* alleles T–819 and A–592 were significantly higher in asymptomatic patients when compared to patients with chronic progressive disease. Another study investigating the genetic capability of cytokine's production and susceptibility to chronic infection (Ben-Ari et al. 2003) did not show any association with IL-10 production potential as determined by polymorphisms at –1082, –819, and –592 positions. However, the majority of patients with chronic infection (65.2%) in this last study presented a genotype the authors related to INF-γ low level production potential in relation to control group (37.5%).

We also did not find any relation to *TGFB1* polymorphism. Nonetheless, 11 patients of the chronic patients' group had histological alterations characterized by hepatic biopsy. The number of patients in this group was small, and a definite association with genetic polymorphism of cytokines could not be established. However, all HBV chronic patients with histological alterations in the present study showed the allele T at position +869 of *TGFB1*. According to Perrey et al. (1998) and Letterio et al. (1998), the allele T at position +869 and G at position +915 are associated with higher production of TGF-β1.

One consequence of chronic hepatic injury is the recurrence of tissue repair process, leading to fibrosis

of the liver. TGF-β1 is a cytokine with immunosupressant properties that include regulation of T-cell proliferation and differentiation (Perrey et al. 1998). Moreover, its activity is related to tissue repair and to hepatic fibrosis' formation, since it affects the transcription of genes linked to fibrosis and can retard the hepatocites proliferation (Perrey et al. 1998). As observed by Bissel et al. (2001), there is an inter-individual variation in the response to hepatic injury; and the genetic polymorphism of *TGFB1* seems to affect the progression timing to fibrosis. Awad et al. (1998) observed that patients with both cystic and pulmonary fibrosis showed a greater frequency of the allele +869 T of this cytokine. It was also observed an association of patients with pre-transplant pulmonary fibrosis and the *TGFB1* +915 GG genotype.

The participants of this study were also compared as for the genetic polymorphism of *TNF*, *TGFB1*, *IL-10*, *IL-6*, and *IFNG* with a healthy white Brazilian population of bone marrow donors from the South and Southeast regions of Brazil (Visentainer et al. 2005). It was verified that both the allelic and genotypic frequencies of this population were intermediary as compared with the self-limited infection group and the chronic patient group. Nonetheless, we did not find statistically significant differences between the populations of the two studies.

In this study, we could not characterize definitive evidences pointing to the involvement of the cytokine polymorphisms in the evolution of HBV infection. A greater number of chronic patients with and without histological alterations could allow a more accurate definition of the influence of the genetic polymorphism on infection evolution.

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