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Role of Genetically Determined Production of Immunoregulatory Cytokines in Immunopathogenesis of Chronic Viral Hepatitides

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Immunopathogenesis of chronic viral hepatitides was studied by modern immunological, molecular, genetic methods. We revealed an imbalance in the production of immunoregulatory cytokines by mononuclear leukocytes (primarily of the Th2 type). The risk of progression and chronic course of viral hepatitides in Caucasian population was associated with alleles of promoter regions -330G and -592A in the *IL-2* and *IL-10* genes, respectively, as well as with the T/T genotype of the polymorphic region C590T in the *IL-4* gene. The C/C genotype of the *IL-10* gene promoter region C592A was shown to be a factor determining resistance to long-term persistence of hepatitis B and C viruses.

Key Words: *hepatitis B and C viruses; cytokines; cytokine gene polymorphism*

The biological stage of virus activity and immune response of the macroorganism are major factors that determine the pathogenesis of chronic viral hepatitides (CVH). Biological properties of viruses were established. Much is known about clinical forms of infections caused by hepatitis B (HBV) and C viruses (HCV) [2,3,8]. The severity of viral infection is not exclusively determined by genetic properties of the virus, but also depends on complex interaction between the infectious agent and host organism. Immune activity of the macroorganism is as important as its taxonomic group. Published data show that the human genotype determines function of the immune system and direction of the immune response during infection [5,6].

The effectiveness of cell-cell interactions during the antiviral immune response depends on genotypic characteristics of the macroorganism. Allelic polymorphism in the promoter regions of cytokine genes that modulate spontaneous and induced production of cytokines is one of the factors, which determines individual differences in immune function in people of the same ethnic group living under similar social and ecological conditions. Normal function of the immune system depends on genetically determined balance between Th1 and Th2 lymphocytes, which is mainly based on the well-balanced production of regulatory cytokines [1,9,10,14,15].

The search for markers of human predisposition to chronic viral infections (*e.g.*, viral hepa-

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titides) is an urgent problem of molecular medicine. These studies are performed to evaluate risk groups and asymptomatic virus carriers, to determine the prognosis and outcome of the infectious process, and to provide individual therapy. The relationship exists between allelic variants of cytokine genes and severity and duration of disease. Previous studies revealed that structural peculiarities of protein products of polymorphic cytokine genes determine differences in the immune response and, therefore, variations in the course and outcome of disease [4,10,15].

Clinical determinants of chronic or self-resolving viral infection remain unknown [6,9,10]. Study of various outcomes of long-term viral persistence is focused on genotypic characteristics of the infectious agent. However, the course of the disease also depends on activity of the immune system [11]. According to the general features of immunogenesis of viral infections, activation of T cells results in the induction of expression of genes of growth factors and their receptors. Interleukin-2 (IL-2) is the major autocrine growth factor for T lymphocytes, which provides clonal expansion in response to the antigen [7]. The effects, production, and biological activity of this cytokine depend on various factors, including the ratio between receptors of agonists and antagonists and relationship with components of the extracellular matrix and plasma proteins. One of these compounds, α_2-macroglobulin, contributes to inactivation of circulating cytokine [5,7,17].

Here we studied the ability of mononuclear cells to produce proinflammatory and antiinflammatory cytokines (IL-2, IL-4, and IL-10) during CVH-B and CVH-C. Immunogenetic criteria for predisposition to the chronic course or resistance to progression of viral hepatitides were evaluated by studying the distribution of allelic variants in promoter regions of IL-2, IL-4, and IL-10 cytokine genes in the Caucasian population. The association with cytokine production was estimated.

MATERIALS AND METHODS

We examined patients with viral hepatitides B, C, and B+C (89 men and 69 women, 18-50 years, average age 31±3 years). The diagnosis of CVH was made from several syndromes, including hepatomegaly, splenomegaly, cholestasis, cytolysis, and mesenchymal inflammation. The diagnosis was verified by the presence of HBV DNA, HCV RNA (polymerase chain reaction, PCR), and serological markers of HBV and HCV in blood plasma. Activity of viral hepatitis was estimated by the severity of necrosis in the hepatic parenchyma and degree of inflammatory cell infiltration. HCV genotyping revealed genotype 1b of HCV in 57% patients. The remaining patients had genotypes 2a and 3a. These results correspond to the average distribution of HCV genotypes in the Siberian region. The duration of CVH was 1-18 years (7±3 years). The control group included 80 healthy donors of similar age and sex. The venous blood was taken from fasting subjects in the morning and stabilized with 25 U/ml heparin.

The concentrations of IL-2, IL-4, and IL-10 were measured in culture fluids. Mononuclear cells were isolated on a Ficoll-Paque density gradient (Pharmacia) and cultured (2×10^6 cells/ml) in RPMI-1640 medium containing 10% inactivated fetal bovine serum, 0.3 mg/ml L-glutamine, 10 mM HEPES (Flow), 100 μg/ml gentamicin, and 5% CO_2 for 24 h. Cytokine production was stimulated by addition of phytohemagglutinin (PHA, Difco) to the medium. Cytokine content in supernatants was measured by solid-phase enzyme immunoassay using Cytimmune test systems (Procon). Optical density of solutions was measured on a Multiskan EX microplate photometer (ThermoLabSystems). Cytokine concentration was calculated from the calibration curve.

DNA was isolated by the method of phenol extraction using a VektoDNAekstraktsiya commercial kit (Vektor-Best). The concentration and purity of isolated DNA were estimated spectrophotometrically. DNA was frozen and stored at -20°C. Allelic variants were genotyped by restriction analysis of amplification products from specific genomic regions. Amplification was performed by PCR on a Tercik MC2 amplifier (DNA-technology). We examined 3 polymorphic variants of 3 cytokines (IL-2 T330G, IL-4 C590T, and IL-10 C592A). All mutations were localized in promoter regions of the corresponding genes.

The reaction medium (total volume 20 μl) consisted of PCR buffer (Sibenzim), which included 60 mM Tris-HCl (pH 8.5), 25 mM KCl, 1.5 mM $MgCl_2$, 10 mM 0.1% mercaptoethanol, Triton X-100, 30 pmol of each oligonucleotide, 125 μM of each deoxyribonucleoside-5'-triphosphate (Sibenzim), 50-200 ng genomic DNA, and 1-2 U Taq polymerase (Sibenzim). The program of amplification suggested denaturation (94°C, 5 min), 30-35 cycles of annealing at temperatures specific for each pair of primers (1 min), chain elongation (72°C, 1 min), and denaturation (94°C, 1 min); final elongation was performed at 72°C for 5 min.

After PCR, 3-5 μl amplificate was separated in 2% agarose gel with 0.5 mg/ml ethidium bromide at 120-130 V for several minutes. Further visualization in UV light confirmed the presence of the

amplification product. Amplification products were restricted with the corresponding endonucleases. The restriction medium to study *IL-2* gene polymorphism contained 5 μl amplificate, 5 μl 2× restriction buffer, and 1-2 U *MaeI*. The restriction medium to study *IL-4* and *IL-10* gene polymorphism contained 7-9 μl amplificate, 1.0-1.2 μl 10× restriction buffer (New England Biolabs), and 1-2 U enzyme. We performed restriction of amplification products from the *IL-2* gene (45°C, 4 h), *IL-4* gene (65°C, 6 h), and *IL-10* gene (37°C, 10-12 h). Restriction products were separated by electrophoresis in 2% agarose gel containing 0.5 mg/ml ethidium bromide at 120-130 V for 30-45 min and visualized in UV light. *MspI*-cleaved *pUC19* plasmid (Sibenzim) served as DNA length marker.

The normality of data distribution was estimated by Kolmogorov—Smirnov test. The equality of sample means was evaluated by Student's *t* test and Mann—Whitney test. The distribution of genotypes by the studied polymorphic loci was tested for correspondence to the Hardy—Weinberg equilibrium by means of Fisher exact test. Intergroup differences in the frequency of alleles were estimated by Pearson χ^2 test and Fischer exact test. For the analysis of genetic data odds ratios (OR) and 95% confidence intervals were calculated. The following conclusions were made: OR=1, no relationship between compared factors; OR<1, negative relationship between compared factors; and OR>1, positive relationship between compared factors.

RESULTS

Induced production of IL-2 in patients with CVH-B, CVH-C, and CVH-B+C was much lower than in healthy donors (Fig. 1). The inhibition of induced IL-2 production in patients with moderate CVH was more pronounced than in patients with mild hepatitis. Little is known about the mechanisms of inhibition of induced IL-2 production during viral infections. Probably, there are several types of inhibition [9]. Previous studies showed that protein products of HCV can block intracellular signal transduction from receptors and decrease IL-2 secretion by activated T lymphocytes. These changes also shift the balance towards secretion of Th2 cytokines, which is a part of virus survival strategy [13].

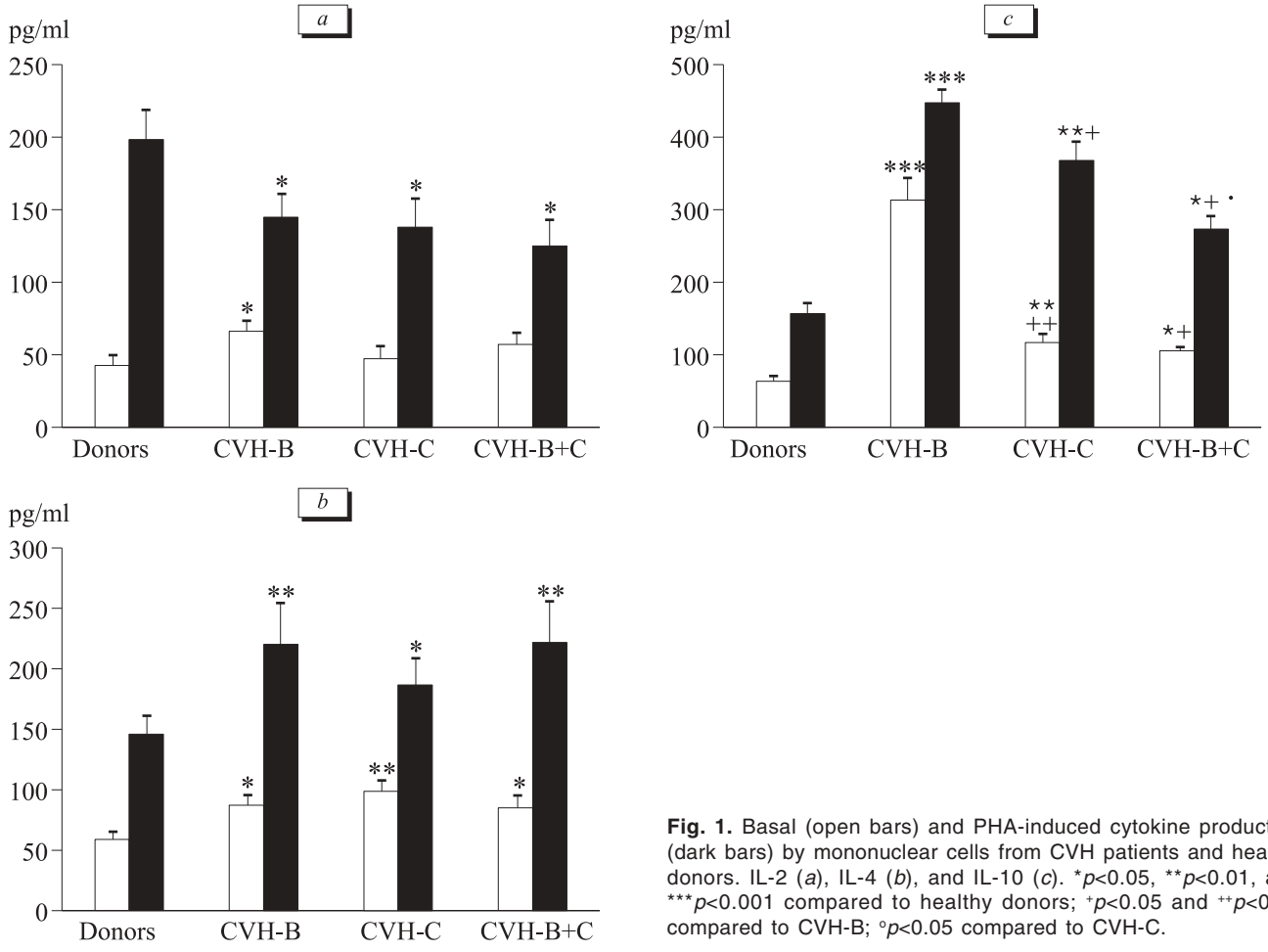

Fig. 1. Basal (open bars) and PHA-induced cytokine production (dark bars) by mononuclear cells from CVH patients and healthy donors. IL-2 (*a*), IL-4 (*b*), and IL-10 (*c*). **p*<0.05, ***p*<0.01, and ****p*<0.001 compared to healthy donors; ^{+}p<0.05 and ^{++}p<0.01 compared to CVH-B; $^{\circ}p$<0.05 compared to CVH-C.

Since the nucleotide exchange is localized in the promoter region of the *IL-2* gene, we assumed that its phenotypic effect consists in modulation of gene expression and production of the corresponding protein. Study of polymorphism in *IL-2* gene promoter region T330G in CVH patients revealed a positive association of the T/G heterozygous variant with CVH-B (OR=2.24) and CVH-C (OR=3.48). There were no hepatitis B patients with the homozygous genotype of the G allele. It should be emphasized that the frequency of genotypes of the polymorphic region T330G in the *IL-2* gene were redistributed in patients with CVH-C. In these patients the frequency of the T/T genotype was much lower (χ^2=8.71, p<0.01), while the ratio of the T/G variant exceeded that in healthy donors (χ^2=8.08, p<0.01). The homozygous G/G variant of the *IL-2* gene promoter region T330G was positively associated with the risk of chronic hepatitis B+C (OR=3.07, Table 1). A relationship was found between the G/G genotype of the *IL-2* gene promoter region T330G and risk of chronic viral hepatitis (grade II and III fibrosis). However, spontaneous and induced IL-2 production did not differ in CVH patients carrying various genotypes of the polymorphic region T330G in the *IL-2* gene.

Published data show that the imbalance in cytokine production and prevalence of mediators of the Th1 or Th2 immune response play a key role in immunopathogenesis of long-term viral persistence [7,12]. Th2 lymphocytes stimulate the humoral response and produce a variety of cytokines, including IL-4, IL-5, IL-6, and IL-10. Their antiinflammatory activity is mainly related to the inhibition of IL-2 and interferon-γ (IFN-γ). An antagonistic relationship exists between Th1 and Th2. One type of helpers prevails over another type. These features determine the predominant type of the immune response [7].

TABLE 1. Distribution of Genotypes and C590T Alleles of *IL-4* Gene in Healthy Donors and Patients with CVH

Parameter	Healthy donors	CVH patients		
		B	C	B+C
T/T	66.67 (32)	52 (13)	35.71 (20)	64.71 (11)
χ^2		0.94	8.71*	0.02
			1.28	0.25
				3.38
OR		0.54	0.28	0.92
		(0.18-1.63)	(0.11-0.67)	(0.25-3.42)
T/G	29.17 (14)	48 (12)	58.93 (33)	23.53 (4)
χ^2		1.79	8.08*	0.02
			0.45	1.64
				3.20
OR		2.24	3.48	0.75
		(0.74-6.90)	(1.43-8.62)	(0.17-3.10)
G/G	4.16 (2)	0	5.36 (3)	11.76 (2)
χ^2		0.08	0.03	0.28
			0.29	1.04
				0.14
OR		0	1.3	3.07
		(0.0-8.1)	(0.17-11.73)	(0.28-34.39)
T	0.81 (78)	0.76 (38)	0.65 (73)	0.76 (26)
G	0.19 (18)	0.24 (12)	0.35 (39)	0.24 (8)
χ^2		0.28	3.93	0.12
			1.41	0.04
				1.05
OR		0.73	0.43	0.75
		(0.30-1.81)	(0.22-0.86)	(0.27-2.14)

Note. *p<0.01 compared to healthy donors. Here and in Tables 2 and 3: figures in parentheses show percent or data range (for OR).

Some authors showed that production of IL-4 and IL-10 increases, while synthesis of IFN-γ decreases in patients with CVH and liver cirrhosis associated with HCV. It was hypothesized that activated Th2 cells play a role in the development of chronic infection [9,10]. However, other investigators revealed that cells of the hepatic infiltrate are mainly presented by Th1 during viral hepatitis. They increase the concentrations of IL-2 and IFN-γ. These changes are accompanied by quantitative and functional deficiency of Th2 [7].

CVH is accompanied by significant increase in not only constitutive production of IL-4, but also reserve capacity to induced secretion of one of the major antiinflammatory cytokines. Spontaneous and PHA-induced production of IL-10 significantly increased in patients with CVH-B, CVH-C, and CVH-B+C (Fig. 1). One of the properties of hepatotropic viruses (*e.g.*, HCV) appeared after long-term evolution is modulation of the immune response toward the prevalence of Th2 reactions at the early stage of disease. Moreover, they determine the development of chronic infection. These features probably contribute to long-term persistence of viruses in the macroorganism [13].

Predisposition to chronic viral hepatitis is probably associated with the allelic variant C590T of the *IL-4* gene. Previous studies revealed that the T/T genotype of the *IL-4* gene promoter region C590T is associated with IL overproduction, particularly at the early stage of HIV infection. It contributes to the rapid progression of the infection [4]. The distribution of genotypes of the *IL-4* gene polymorphic region C590T in patients with CVH-B, CVH-C, and CVH-B+C differed insignificantly from that in healthy donors. However, the frequency of the homozygous C/C genotype of the *IL-4* gene promoter region C590T in patients with CVH-B+C was

TABLE 2. Distribution of Genotypes and C590T Alleles of *IL-4* Gene in Healthy Donors and Patients with CVH

Parameter	Healthy donors	CVH patients		
		B	C	B+C
C/C	29.17 (14)	33.33 (8)	14.04 (8)	52.63 (10)
χ^2		0.01	2.75	2.32
			2.84	0.93
				9.71^{o}
OR		1.21	0.4	2.7
		(0.37-3.93)	(0.13-1.15)	(0.79-9.33)
C/T	58.33 (28)	37.5 (9)	75.44 (43)	31.58 (6)
χ^2		2.01	2.74	2.90
			8.99^{+}	0.01
				10.13^{o}
OR		2.88	2.19	0.33
		(0.73-11.65)	(0.88-5.50)	(0.09-1.15)
T/T	12.5 (6)	29.17 (7)	10.52 (6)	15.79 (3)
χ^2		1.98	0.01	0.01
			3.08	0.84
				0.04
OR		0.43	0.82	1.31
		(0.14-1.31)	(0.21-3.16)	(0.23-7.03)
C	0.58 (56)	0.52 (25)	0.52 (59)	0.68 (26)
T	0.42 (40)	0.48 (23)	0.48 (55)	0.32 (12)
χ^2		0.29	0.66	0.78
			0.02	1.72
				2.57
OR		0.78	0.77	1.55
		(0.36-1.65)	(0.43-1.37)	(0.65-3.71)

Note. $^{+}p<0.01$ compared to CVH-B; $^{o}p<0.01$ compared to CVH-C.

TABLE 3. Distribution of Genotypes and C592A Alleles of *IL-10* Gene in Healthy Donors and Patients with CVH

Parameter	Healthy donors	CVH patients		
		B	C	B+C
C/C	18.75 (9)	0	0	0
χ^2		3.75*	9.77***	2.96*
OR		0	0	0
		(0-1.02)	(0-0.42)	(0-1.37)
C/A	77.08 (37)	96 (24)	96.61 (57)	100 (19)
χ^2		3.02*	7.71**	3.67*
			0.26	0.02
				0.01
OR		7.14	8.47	—
	(0.85-157.31)	(1.62-58.92)		
A/A	4.16 (2)	4 (1)	3.39 (2)	0
χ^2		0.34	0.04	0.01
			0.26	0.02
				0.01
OR		0.96	0.81	0
		(0-14.61)	(0.08-8.42)	(0-10.85)
C	0.43 (41)	0.48 (24)	0.48 (57)	0.5 (19)
A	0.57 (55)	0.52 (26)	0.52 (61)	0.5 (19)
χ^2		0.37	0.46	0.33
			0.02	0.01
				0.01
OR		1.24	1.25	1.34
		(0.59-2.60)	(0.70-2.24)	(0.59-3.05)

Note. *p<0.05, **p<0.01, and ***p<0.001 compared to healthy donors.

much higher than in patients with CVH-C (χ^2=9.71, p<0.001). The frequency of C/T in patients with CVH-C was 2-fold higher than in patients with CVH-B and CVH-B+C. The C/T genotype was positively associated with long-term persistence of HBV (OR=2.88) and HCV (OR=2.19). The C/C genotype was positively associated with HBV+ HCV infection (OR=2.70, Table 2). We studied whether the genotype of IL-4-producing cells is associated with spontaneous and induced production of this cytokine. The C/T genotype was mainly found in subjects with high level of cytokine production. These data are consistent with the hypothesis of competitive relations between Th1 and Th2 immune reactions.

We evaluated the dependence of polymorphism in the *IL-10* gene promoter region C592A in CVH patients on taxonomic characteristics of the infectious agent. The heterozygous C/A variant was positively associated with the chronic course and risk of progression and unfavorable outcome of CVH-B, CVH-C, and CVH-B+C. There were no subjects with the C/C genotype. The homozygous genotype by the A allele was not found in patients with hepatitis B+C (Table 3). The C/C genotype of the *IL-10* gene in the site of C592 allelic polymorphism is an immunogenetic factor determining the resistance to CVH [4,13]. The 592A allelic variant of *IL-10* can serve as an immunogenetic factor determining predisposition to viral hepatitis (by *IL-10* gene polymorphism). Exactly this variant is associated with the increased spontaneous production of IL-10 in cell cultures.

It should be emphasized that overproduction of IL-4 and IL-10 during long-term persistence of viruses is not related to one allelic variant of the gene, but depends on a variety of genes regulating production of Th2 cytokines [14,15].

Interindividual differences in the cytokine profile are partially determined by allelic polymorphism in cytokine gene promoters. The polymorphic cytokine network is also formed by alternative

splicing. Its biological role is associated with several functions. Isoforms are usually involved in the processes regulated by proteins of the cytokine network. The regulatory influence of alternative cytokines occurs in various types of cells. The character of regulation by splice variants (even mutual exclusion of effector pathways) suggests that proteins of this group play an important role in cellular regulation under conditions of viral persistence. Moreover, the regulatory effect of cytokines is associated with the rate of metabolism in these regulatory biomolecules during cell-cell interactions. The important events are expression of the corresponding receptors on cell membranes and loss of some receptor molecules accepting soluble cytokines.

These data suggest that polymorphism in cytokine genes serves as a factor of predisposition to persistence of hepatitis viruses in the macroorganism. The character of mutations and type of mutant genes that play a key role in this process remain unknown. An important role is attributed to combinations, but not to individual gene alleles. Evaluation of the role of allelic combinations in predisposition to infection and long-term persistence of viruses in the organism is an urgent problem of immunogenetic studies.

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