GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

Allelic Polymorphism of Cytokine Genes during Pulmonary Tuberculosis

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 148, No. 8, pp. 137-142, August, 2009
Original article submitted February 3, 2009

Modern immunological and molecular genetic studies showed that tuberculosis is accompanied by an imbalance in the production of immunoregulatory cytokines by mononuclear leukocytes. *T* allele and homozygous *TT* genotype of *T-330G* polymorphism in the *IL2* gene, *T* allele and *TT* genotype of *C-590T* polymorphism in the *IL4* gene, and *CC* genotype of *A-1188C* polymorphism in the *IL12B* gene are immunogenetic factors that have protective activity against susceptibility to pulmonary tuberculosis. Susceptibility to tuberculous infection is associated with *A1A2* genotype of the polymorphic region +3953 A1/A2 in the *IL1B* gene; *G* allele and *TG* and *GG* genotypes of *T-330G* polymorphism in the *IL2* gene; *C* allele and *CC* and *CT* genotypes of *C-590T* polymorphism in the *IL4* gene; and *AC* genotype of the polymorphic region *A-1188C* in the *IL12* gene.

Key Words: *cytokines; cytokine gene polymorphism; tuberculosis*

From the genetic point of view, the majority of infections (*e.g.*, tuberculosis) are multifactorial diseases that result from the interaction of numerous genes with a variety of environmental factors [1,4]. Susceptibility to disease is determined by a combination of allelic variants of genes in the individual genotype forming unfavorable hereditary background, which is realized during the interaction with *Mycobacterium tuberculosis*. Recent advances in molecular genetics open new perspectives in studying the pathogenesis of tuberculosis: genes whose expression products are involved in the infectious process were identified [1,6].

Functional activity of the cytokine network during tuberculosis depends on a variety of factors, including individual differences in the production of immunoregulatory molecules determined by some genetic peculiarities [3,6,10,12]. The search for predictors of susceptibility to infection among cytokine gene alleles is a new perspective trend of research [5]. Structural characteristics of protein products of polymorphic regions in cytokine genes affect differentiation of organism's immune response to bacterial aggression determining the course and outcome of tuberculosis.

Here we studied the association between polymorphic variants of genes modulating the immune

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0007-4888/09/1482-0175

response and secretion of the corresponding protein products.

MATERIALS AND METHODS

The study included 78 Caucasian individuals living in Tomsk and Tomsk region, patients the Tomsk Regional Clinical Tuberculosis Hospital (46 men and 32 women, 19-35 years, average age 32.7±2.1 years). The patients had a primary diagnosis of infiltrative pulmonary tuberculosis. The diagnosis was made by clinical symptoms of the disease, X-ray examination of the lungs, and microscopic or bacteriological study of sputum. Clinical signs of infiltrative pulmonary tuberculosis included the syndrome of inflammatory intoxication (abnormally high body temperature, nocturnal sweating, sensation of chill, easy fatigability, weakness, reduction or loss of appetite, body weight loss, and tachycardia) and bronchopulmonary symptoms (cough with duration of more than 2-3 weeks, sputum expectoration, hemoptysis, dyspnea, and chest pain during breathing). Excretion of bacteria was documented in 100% patients. The patients were examined before specific antituberculous chemotherapy. The control group consisted of 82 healthy age- and sex-matched donors without history of pulmonary diseases, pathological changes in the lungs (plain radiography), chronic infectious diseases, allergic reactions, and acute respiratory infections (over 3 months before the trial).

The concentration of immune cytokines was measured in culture fluids. Mononuclear cells (2×10^6 cells/ml) were isolated in a Ficoll-Paque density gradient (Pharmacia) and cultured in RPMI 1640 medium with 10% inactivated fetal bovine serum, 0.3 mg/ml L-glutamine, 10 mM HEPES (Flow), and 100 μg/ml gentamicin at 5% CO_2 for 24 h. The content of immune cytokines in supernatants was measured by a solid-phase enzyme immunoassay according to manufacturer's instructions (Proteinovyi Kontur). Optical density was measured on a Multiscan EX microplate photometer (ThermoLabSystems). Cytokine concentration was calculated using a calibration curve.

Peripheral blood DNA was isolated by the sorbent method using DNA-sorb-B kit according to manufacturer's instructions (InterLabService). Polymorphic regions of cytokine genes were studied by the restriction analysis of amplification products of specific genomic regions (restriction fragment length polymorphism analysis, RFLP analysis). The following four polymorphic variants of cytokine genes were studied: +3953A1/A2 of the *IL1* gene; *T-330G* of the *IL2* gene; *C-590T* of the *IL4* gene; and *A-1188C* of the *IL12B* gene. Amplification was performed according to the instructions for an AmpliSens-200-1 commercial kit (InterLabService). During amplification, PCR was conducted in Eppendorf tubes. The structure of primers and parameters of temperature cycles were described previously (Tertsik MS2 amplifier, DNA-technology). The PCR mixture contained 0.5-2.0 μl specific pair of primers (1 opt. unit/ml), 1.2-1.8 μl 10× buffer for amplification with 0.5-2.0 mM $MgCl_2$, 0.5-1.0 U Taq DNA polymerase (Sibenzim, Medigen), and 100-200 ng genomic DNA. The mixture was put in 0.5-ml Eppendorf tubes. Mineral oil was layered onto the mixture to prevent evaporation. Amplification was performed as follows: predenaturation at 94°C for 5 min; 30-35 cycles of annealing, chain elongation, and denaturation; and final elongation. The amplificate was hydrolyzed with restrictase at the enzyme temperature optimum for 12-24 h. The restriction mixture consisted of 5-7 μl amplificate, 1.0-1.2 μl 10× buffer for restriction (Sibenzim), and 1-5 U enzyme. The restriction products were fractionated in 3% agarose gel at 120 V for 30 min. DNA fragments were stained with ethidium bromide and visualized in UV light.

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 in polymorphic loci was tested for conformity 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. The results of genetic studies were analyzed by odds ratio (OR). Confidence intervals (95%) were calculated. The following conclusions were made: OR=1, no relationship between factors; OR<1, negative relationship between factors; and OR>1, positive relationship between factors.

RESULTS

The reaction of CD4 T cells to the antigen is the major event of the specific immune response [1,3,4]. The type of the immune response (prevalence of antibodies or cellular reactions) is determined at this stage. Differentiation of native T helper lymphocytes (Th0 cells) to Th1 or Th2 depends on a variety of factors, including the quality and dose of antigen, cytokine microenvironment, and presence or absence of cytokine-binding receptors. Macrophages and Th1 play the major role in the development of antituberculous immunity (mechanism of delayed-type hypersensitivity response) [2,4,11]. Secretion of IFN-γ and IL-2 by Th1 lymphocytes contributes to high activity of accessory cells and effector cytotoxic T lymphocytes, thus maintaining the cellular immune response [7,8,13]. Activation of the main cells producing immune factors (monocytes/macrophages and lymphocytes) is followed by succes-

sive interrelated stages of cytokine gene transcription, processing and translation of mRNA, and secretion of bioactive proteins into the environment [7,10].

Spontaneous production of IL-1β, TNF-α, and IL-2 was reduced in patients with pulmonary tuberculosis before the start of antituberculous chemotherapy (Table 1). The impairment of immune cytokine production in the acute stage of pathological process is probably related to toxic effect of *Mycobacterium tuberculosis* on the biosynthesis of DNA and protein in immunocompetent cells [1,3,13].

It cannot be excluded that metabolic disturbances in lymphocytes from pulmonary tuberculosis patients contribute to a decrease in the synthesis of not only cytokines, but also of their receptors. Inhibition of spontaneous production of IL-2 during pulmonary tuberculosis is associated not only with reduced synthesis or secretion of its inductor (IL-1β), but also with low expression of type I IL-1 receptors (IL-1RI) on Th1 lymphocytes. These receptors are essential for the biological effect of IL-1β, which depends on functional activity of nuclear transcription factors NF-κB and AP-1. Activation of these factors is followed by an increase in the synthesis of various molecules playing a role in the regulation of inflammatory reactions [7,10]. Moreover, the decrease in cytokine concentration can be related to elevated content of soluble receptors (IL-1RII, IL-2R, IL-12R, and TNFR) competing for binding with the corresponding interleukins and reducing the effectiveness of interleukin-mediated activation of immunocompetent cells [7]. The cytokine network is characterized by receptor-mediated autocrine and paracrine regulation of the production of immunoregulatory molecules. These processes can determine the impaired production of competitive transmitters (*e.g.*, proinflammatory and antiinflammatory cytokines) [8].

Published data show that the inflammatory response and directionality of anti-infectious immunity depend on genetically determined characteristics of intercellular cooperation between immunocompetent cells [5,12]. The detected association between some genotypes/allelic variants of cytokine genes required the improvement of methods of identification of new polymorphic loci. A special field of modern immunogenetics is directed toward the search for pathogenetically important variants of immunomodulatory genes playing a role in the development and outcome of various diseases. We studied genetic factors determining the type of defense reactions (*e.g.*, interrelation between genetic heterogeneity of blood cytokines and secretion/reception of Th1 and Th2 cytokines by blood mononuclear leukocytes *in vitro*) [5].

Immunogenetic study showed that homozygous *A1A1* genotype of the polymorphic region +3953 A1/A2 of the *IL1B* gene is especially typical of tuberculosis patients (57.69%). However, the risk of tuberculous infection was associated with the genotype *A1A2* in 37.18% patients (OR=1.27; Table 2). The proinflammatory cytokine IL-1β play a key role in the immunopathogenesis of tuberculous infection. We believe that allelic variants of IL-1β can produce various phenotypic effects, which leads to a change in gene expression and content of the protein product. However, spontaneous and induced production of IL-1β did not differ in tuberculosis patients with various genotypes of the polymorphic region +3953 A1/A2 of the *IL1B* gene.

T-330G polymorphism in the promoter region of the *IL2* gene was studied. The heterozygous *TG* variant of the polymorphic region *T-330G* in the *IL2* gene was found in 44.4% patients with pulmonary tuberculosis (much more frequently than in healthy donors, 34%). By contrast, homozygous *TT* genotype of *T-330G* polymorphism in the *IL2* gene was more typical of healthy donors (60.5 *vs*. 46.9% patients). statistical analysis revealed a positive correlation of the *G* allele, *TG* genotype, and *GG* genotype with pulmonary tuberculosis. Therefore, the presence of these allelic variants reflects high risk of tuberculous infection (Table 2).

TABLE 1. Production of Immune Cytokines by Mononuclear Cells (pg/ml) in Healthy Donors and Pulmonary Tuberculosis Patients (Me ($Q_{25\%}$:$Q_{75\%}$))

Parameter	Healthy donors	Pulmonary tuberculosis patients
IL-1β	282.33(154.55:355.52)	207.33(141.32:226.16) $p<0.05$
TNF-α	274.89(194.36:325.07)	96.57(74.92:121.63) $p<0.01$
IFN-γ	491.96(294.23:527.37)	689.41(487.35:725.11) $p<0.01$
IL-2	25.00(19.91:32.50)	19.38 (14.55:25.52) $p<0.05$
IL-4	55.04 (48.31:65.50)	37.62 (40.54:55.21) $p<0.05$
IL-12	108.79(58.29:127.74)	264.67(181.42:301.75) $p<0.01$

Note. Here and in Table 2: *p*: significant differences from healthy donors.

TABLE 2. Distribution of Genotypes and Polymorphic Alleles in Healthy Donors and Pulmonary Tuberculosis Patients (%, abs.)

Parameter		Healthy donors	Pulmonary tuberculosis patients	χ^2	OR (95% CI)
+3953A1/A2 of the *IL1B* gene	A1A1	63.41 (52)	57.69 (45)	0.55 p=0.45	0.79 (0.40-1.56)
	A1A2	31.71 (26)	37.18 (29)	0.53 p=0.46	1.27 (0.63-2.58)
	A2A2	4.88 (4)	5.13 (4)	0.01 p=0.9	1.05 (0.21-5.25)
	A1	0.79 (130)	0.76 (119)	0.41 p=0.5	0.84 (0.48-1.47)
	A2	0.21 (34)	0.24 (37)		1.19 (0.68-2.08)
T-330G of the *IL2* gene	TT	60.5 (49)	46.9 (38)	4.041 p=0.044	0.577 (0.34-0.99)
	TG	34.0 (28)	44.4 (36)	4.355 p=0.037	1.792 (1.03-3.11)
	GG	5.6 (5)	8.6 (7)	0.836 p=0.36	1.306 (0.58-4.49)
	T	0.77 (126)	0.69 (112)	2.872 p=0.09	0.653 (0.40-1.07)
	G	0.23 (38)	0.31 (50)		1.532 (0.93-2.51)
C-590T of the *IL4* gene	CC	25.3 (21)	42.0 (34)	7.030 p=0.008	2.135 (1.21-3.76)
	CT	70.4 (57)	50.6 (41)	9.121 p=0.003	1.771 (0.58-5.46)
	TT	4.3 (4)	7.4 (6)	1.016 p=0.313	0.432 (0.25-0.75)
	C	0.60 (99)	0.67 (109)	1.41 p=0.24	1.316 (0.84-2.07)
	T	0.40 (65)	0.33 (53)		0.760 (0.48-1.19)
A-1188C of the *IL12* gene	AA	50.00 (41)	43.59 (34)	0.66 p=0.41	0.77 (0.39-1.51)
	AC	37.80 (31)	50.00 (39)	2.42 p=0.012	1.65 (0.84-3.25)
	CC	12.20 (10)	6.41 (5)	1.51 p=0.21	0.5 (0.14-1.70)
	A	0.69 (113)	0.69 (107)	0.001 p=0.9	0.99 (0.86-1.15)
	C	0.31 (51)	0.31 (49)		1.01 (0.73-1.67)

The regulation of *IL2* gene transcription is closely related to the promoter region, which includes binding sites for transcription enhancers NFAT1, NF-κB, AP-1, and erg-1. All sites in the gene promoter region are required for activation in response to the TCR-mediated stimulation of lymphocytes. Blockade of NFAT expression (major element in the signal pathway for regulation of *IL2* gene expression) in T lymphocytes is accompanied by inactivation of the *IL2* promoter and inhibition of cytokine synthesis. The interaction between transcription enhancers is realized via a key-lock mechanism. Therefore, the substitution of even one nucleotide in the gene promoter region is followed by structural changes in binding sites. Transcription factors gain a higher or lower affinity for regulatory regions, which is directly related to transcription of the encoded cytokine [4,13]. *T-330G* polymorphism in the *IL2* gene is revealed in the promoter region and, probably, determines production of the encoded cytokine. Our study showed that the homozygous *T* allele genotype is associated with more intensive secretion of IL-2 (21.76±3.08 pg/ml) compared to heterozygotes (20.95±4.36 pg/ml) and *G* allele homozygotes (16.92±1.56 pg/ml). We conclude that the *G* allele of *T-330G* polymorphism in the *IL2* gene (human genotype) reflects a deficiency of the cellular immune response, which serves as the risk factor of tuberculous infection.

Published data show that *C-590T* allelic polymorphism in the *IL4* gene can be considered as an immunogenetic marker of tuberculous infection. This polymorphism is associated with increased production of IgE, which results in atopic reactions [1,7,12,14]. Studying the distribution of allelic polymorphism in the *IL4* gene promoter showed that 50% pulmonary tuberculosis patients carry the heterozygous *CT* variant of *C-590T* polymorphism in the *IL4* gene (Table 2). The number of *C* allele homozygotes among these

patients was higher compared to healthy donors. A positive association was found between pulmonary tuberculosis and *C* allele, *CC* genotype, and *CT* genotype. The *T* allele and homozygous *TT* genotype had a protective effect and prevented the development of tuberculous infection (Table 2).

Previous studies (*e.g.*, experiments on transgenic animals) showed that transcription factors STAT4, STAT6, c-Maf, NIP45, GATA-3, NFAT1, and NFAT2 play a role in the regulation of *IL4* gene transcription. The binding site for a specific octamer and NFAT proteins is localized at the 5'-end of the *IL4* gene. IL-4 production is an inducible process. The increase in *IL4* gene transcription requires activation of protein kinase C and Ca^{2+}-calmodulin system, which leads to phosphorylation of NFAT proteins and binding of these proteins to the gene regulatory region. The *-590T* allelic variant of the promoter region was shown to be associated with high-intensity production of IL-4 during pulmonary tuberculosis (61.40±5.07 pg/ml).

Recent studies showed that IL-12 play a key role in activation of the cellular immune response and induction of anti-infectious protection [9,15]. The protective effect of this immune cytokine is mediated by a IFN-γ-dependent mechanisms, which includes increased secretion of NO, T cell infiltration, strong expression of adhesion molecules, production of chemokines, and increase in cytotoxic activity of NK cells and cytotoxic lymphocytes [12,15]. IL-2 not only affects the cellular immune response, but also stimulates humoral immunity. This cytokine contributes to differentiation of B lymphocytes into plasma cells and shifts the synthesis of immunoglobulins [9]. IL-12 production was studied in patients with infiltrative pulmonary tuberculosis before the start of specific therapy [14]. We showed that IL-12 production in patients increases during the acute stage of pathological process (Table 1).

Studying the genotype distribution of *A-1188C* polymorphism in the *IL12B* gene showed that this polymorphic region is associated with tuberculosis. The number of heterozygotes with the *AC* genotype was much higher among tuberculosis patients (50%). This genotype was associated with high risk of the tuberculous process (OR=1.65, p=0.012). The risk of tuberculosis development in *CC* homozygotes was lower than in subjects with the heterozygous and homozygous *AA* genotype of *A-1188C* polymorphism in the *IL12B* gene. The allelic distribution of *A-1188C* polymorphism in the *IL12B* gene did not differ in tuberculosis patients and healthy donors (Table 2). We studied the association of genotype in IL-12-producing cells with spontaneous IL-12 production. The *CC* genotype of the *IL12B* gene was more typical of individuals with intensive production of this cytokine. Our findings are consistent with the hypothesis on competitive interactions between Th1/Th2-dependent immune components. However, the dependence of cytokine production on the genotype of promoter region *A-1188C* in the *IL12B* gene was statistically insignificant.

Our results indicate that the course of infiltrative tuberculosis in residents of Tomsk and Tomsk region is accompanied by a decrease in the production of IL-1β, TNF-α, IL-2, and IL-4 by peripheral blood mononuclear cells. Secretion of IFN-γ and IL-12 was shown to increase in these patients. Low secretion of IL-4 during tuberculous infection is associated with the homozygous *C* allele of *C-590T* polymorphism in the *IL4* gene. The *T* allele and homozygous *TT* genotype of *T-330G* polymorphism in the *IL2* gene, *T* allele and *TT* genotype of *C-590T* polymorphism in the *IL4* gene, and *CC* genotype of *A-1188C* polymorphism in the *IL12B* gene are immunogenetic factors that have a protective activity against the susceptibility to pulmonary tuberculosis. The susceptibility to tuberculous infection is associated with the *A1A2* genotype of the polymorphic region +3953 A1/A2 in the *IL1B* gene; *G* allele and *TG* and *GG* genotypes of *T-330G* polymorphism in the *IL2* gene; *C* allele and *CC* and *CT* genotypes of *C-590T* polymorphism in the *IL4* gene; and *AC* genotype of the polymorphic region *A-1188C* in the *IL12* gene. The highest risk of infiltrative tuberculosis is associated with a combination of the *GG* genotype of *T-330G* polymorphism in the *IL2* gene and *CT* genotype of *C-590T* polymorphism in the *IL4* gene (*GG/CT*).

This work was performed within the framework of the Federal Target Program "Research and Development in Priority Fields of S&T Complex of Russia for 2007-2012" (State Contract No. 02.512.12.0013). Our study was supported by the Council on Grants of the President of Russian Federation for State Support of Leading Scientific Schools of the Russian Federation (NSh-2334.2008.7) and Young Scientists of Russia (MD-3659.2007.7).

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