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Analysis of Toll-Like Receptor-Dependent Production of Proinflammatory Cytokines *In Vitro* by Human Peripheral Blood Mononuclears of Donors and Patients with Primary Immunodeficiency

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The production of TNF-α and IFN-α cytokines by peripheral blood mononuclears in response to stimulation by TLR2/6, TLR4, TLR5, TLR9 ligands (zymosan, LPS, flagellin, and CpG-oligodeoxynucleotide, respectively) was studied in donors and patients with common variable immunodeficiency. Individual characteristics of TNF-α production by mononuclears were revealed in donors. Reduced stimulated production of TNF-α in response to stimulation with TLR4 and TLR5 ligands *in vitro* was detected in patients with common variable immunodeficiency.

Key Words: *Toll-like receptors; tumor necrosis factor-α; interferon-α; mononuclear cells; common variable immunodeficiency*

Toll-like receptors (TLR) are now regarded as the key receptors mediating innate immunity.

Molecular mechanisms of signal transduction through TLR are characterized at length. Two principally different signal routes are known. Stimulation of one group of TLR leads to production of proinflammatory cytokines (IL-1, TNF-α, IL-6, IL-12), chemokines, and expression of co-stimulatory molecules. Expression of type I IFN genes (IFN-α/β) is activated via the other signal route. Products of these genes regulate the reactions of the innate immunity and direct the development of adaptive immune response [9,12]. The role of TLR in the development of infections, sepsis, and allergic diseases was reported [3,9,13]. The development of approaches to evaluation of the TLR function is therefore an important problem.

Common variable immunodeficiency (CVID) is primary immunodeficiency characterized by low content of serum immunoglobulins; it is a heterogeneous group of diseases with a variety of clinical manifestations and poorly studied immunopathogenesis. Disorders detected in CVID are caused by numerous defects of the immune system. Defects of antigen-presenting cells [4-6] and abnormal expression and functioning of TLR in CVID patients [7] were recently reported.

We studied the effects of TLR ligands on *in vitro* production of TNF-α and IFN-α by mononuclear cells in donors and patients with CVID.

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MATERIALS AND METHODS

The group of donors consisted of 11 healthy subjects (9 men and 2 women) aged 20-40 years.

The group of CVID patients (n=9) included 7 boys aged 11-17 years and 2 girls aged 7 and 8 years. The patients were hospitalized at Department of Clinical Immunology, Russian Pediatric Clinical Hospital. The disease was diagnosed in accordance with the diagnostic criteria developed by the European Society of Immunodeficiency.

Serum levels of IgA, IgM, IgG, counts of $CD4^+$ T lymphocytes, B lymphocytes, and natural killers were sharply reduced in all patients. The patients developed severe relapsing chronic bacterial infections throughout the life.

After diagnosis was made, all patients received adequate intravenous immunoglobulin replacement therapy, in addition to constant preventive antibacterial therapy. The blood was collected in all patients on days 2-6 after replacement therapy.

Mononuclear cells were isolated from heparin-treated (25 U/ml) donor blood in Ficoll-urografin density gradient (ρ=1.077 g/cm^3; Pharmacia). Mononuclear cells were cultured in complete RPMI-1640 with 5% FCS (HyClone, Perbio), 2 mM L-glutamine (M. P. Chumakov Institute of Poliomyelitis and Viral Encephalitides), and 100 μg/ml gentamicin (Dalkhimfarm). The working concentration of mononuclears was 1×10^6 cell/ml.

The production of TNF-α and IFN-α was induced by TLR ligands: polyinosine-polycytidine (P(I:C), a synthetic analog of double-stranded RNA; 10 μg/ml; Amersham Biosciences); LPS (*Escherichia coli* 0127:B8; 0.1 μg/ml; Sigma); zymosan A (*Saccharomyces cerevisae*; 10 μg/ml; Sigma); flagellin (*Salmonella thyphimurium*; 0.5 μg/ml; InvivoGen); CpG-oligodeoxynucleotide (CpG-ODN; 1 μg/ml; InvivoGen), reacting with TLR3, TLR4, TLR2/6, TLR5, and TLR9, respectively [2,9]. Mononuclears were incubated for 24 h at 37°C and 5% CO_2. Mononuclears cultured only in complete RPMI-1640 or with ODN containing no CpG sequence served as the control. After culturing mononuclears were precipitated by centrifugation at 400g for 15 min. The supernatants were stored at -70°C for 2-3 months.

The production of cytokines was evaluated in cell supernatants by EIA (Vector-Best commercial kits for measurements of TNF-α and IFN-α).

Spontaneous cytokine production was taken for one unit and the stimulation coefficient (SC) was estimated as the ratio of cytokine concentration in supernatants of ligand-stimulated mononuclears to the cytokine concentration in not stimulated mononuclear supernatants.

The data were processed statistically using standard methods. The significance of differences was evaluated by nonparametric Wilcoxon and Mann—Whitney tests. The differences in the means were considered significant at $p<0.05$.

RESULTS

Activation of TLR triggers the signal cascade leading to activation of NF-κB, which, in turn, leads to the synthesis of TNF-α and other proinflammatory cytokines, such as IL-1, IL-6, *etc.* [3,9].

The production of type I IFN is stimulated through TLR3, TLR4, TLR7, TLR8, and TLR9 [12]. We studied the effects of TLR ligands on the production of IFN-α, because the synthesis of IFN-α and IFN-β is simultaneous and is triggered by the signal cascade activating the adapter TRIF molecule and IRF3 transcription factor [3,9].

Two groups were distinguished by the results of evaluation of spontaneous production of TNF-α by donor peripheral blood mononuclears: with normal and initially high levels of cytokine secretion. High spontaneous production of TNF-α can be caused by prestimulation of the producer cells *in vivo* and be due to physiological heterogeneity of the donor group [2,8]. The mean spontaneous production of TNF-α in donor group 1 was 38.67±2.54 pg/ml, while in group 2 it was 233.05±20.83 pg/ml. Spontaneous production of IFN-α in donors was 29.94±3.37 pg/ml.

All studied ligands stimulated production of TNF-α by donor mononuclears in both groups without appreciable differences between these groups.

The maximum stimulation of TNF-α synthesis by donor mononuclears was attained with LPS, the minimum with CpG-ODN. Flagellin and zymosan stimulated the production of TNF-α less intensely than LPS (Table 1).

Individual profiles of TLR ligand-stimulated production of TNF-α were detected in donors. In some donors, the maximum production of TNF-α was observed after mononuclear stimulation with zymosan, while the production stimulated with LPS, flagellin, and CpG-ODN was comparable to the control. In other donors, the production of TNF-α was maximum under the effect of LPS or in response to flagellin and CpG-ODN. Individual differences in stimulated TNF-α production in donors were presumably explained by different expression of TLR on mononuclears, initial functional status of cells participating in the production of TNF-α, and were determined by TNF-α and TLR gene polymorphism.

Lipopolysaccharide, CpG-ODN, and P(I:C) stimulated the production of IFN-α by mononuclears.

TABLE 1. Production of TNF-α and IFN-α by Peripheral Blood Mononuclears in Response to TLR Ligands (*M*±*m*)

Ligand	TNF-α		IFN-α	
	donors	CVID patients	donors	CVID patients
Zymosan	2.69±0.23*	2.14±0.17*	1.05±0.08	—
LPS	3.08±0.20*	1.28±0.11	1.69±0.24*	12.3±1.46
Flagellin	2.3±0.2*	1.03±0.05	1.02±0.05	—
CpG-ODN	1.60±0.11*	2.07±0.35*	1.25±0.11*	—
P(I:C)	—	—	2.00±0.28	3.90±0.82

Note. *$p<0.05$ compared to the control (SC=1).

Zymosan and flagellin exhibited virtually no effect of this kind. No individual characteristics of IFN-α production by donor mononuclears were detected.

Spontaneous production of TNF-α by mononuclears in CVID patients was 68.13±5.13 pg/ml, IFN-α production 6.45±5.54 pg/ml.

The TLR ligands stimulated the production of TNF-α by mononuclears of CVID patients. The most intensive production was observed in response to zymosan and CpG-ODN (Table 1). No appreciable increase in the production of TNF-α in response to LPS and flagellin stimulation was detected. Reduced production of TNF-α by peripheral blood mononuclears of CVID patients in response to TLR4 and TLR5 ligands was presumably caused by modulation of the expression of these receptors and high functional activity of cells from these patients *in vivo*.

The TLR ligands stimulated the production of IFN-α by peripheral blood mononuclears from CVID patients (Table 1). Spontaneous production of IFN-α was low, but the absolute values in CVID patients were comparable to those in donors. SC for TLR ligands in the group of CVID patients was higher than in donors.

Interferon-α is the main cytokine with antiviral activity preventing intracellular replication of a virus and activating nucleases destroying viral mRNA. Presumably, high stimulated production of IFN-α by cells of CVID patients detected in our study provides effective antiviral defense, as these patients are more sensitive to bacterial than to viral infections.

The production of TNF-α and IFN-α is mediated by triggering various signal routes and transcription factors. The detected imbalance in the cytokine production by peripheral blood mononuclears of CVID patients presumably indicates a defect at the level of the signal routes, resulting in abnormal production of TNF-α.

Hence, Toll-like receptor ligands to a different degree stimulate the production of TNF-α and IFN-α in donors. The production of IFN-α was stimulated by TLR3, TLR4, and TLR9 ligands. Presumably, cytokine production in response to donor peripheral blood mononuclear stimulation by TLR ligands can be used for evaluation of the TLR function. Since some of TLR ligands are now regarded as adjuvant components of vaccines, analysis of the cytokine production profile can be used, among other approaches, for choosing the individual components of a vaccine for improving the efficiency of vaccination [8,9].

Mononuclears of CVID patients are characterized by low activation of TNF-α production in response to TLR4 and TLR5 ligands *in vitro*. This can lead to deterioration of the defense functions in these patients in case of repeated *in vivo* infection. Changes detected in this study can be involved in the pathogenesis of clinical and immunological manifestations of CVID.

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