UC 577,1; 61 ISSN 0354-3447

Jugoslov Med Biohem 25: 153–159, 2006 *Originalni naučni rad*
Original paper

SPECIFICITY OF CYTOKINE PROFILE AND OXIDANT STRESS IN PATIENTS WITH BRONCHIAL ASTHMA

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Summary: Since nowadays morbidity and mortality of bronchial asthma are increasing more and more, this issue presents a global challenge despite the significant development of knowledge about the pathogenetic mechanism of asthma, role of inflammatory cells, regulation of inflammatory and immune responses, about many mediators and their co-regulation. Cytokines and their modulators are becoming increasingly important because they are crucial intercellular transmitters which contribute to the development of airways remodeling. The aim of this study is to evaluate Th1/Th2 ratio and to estimate the inter-dependence between cytokines and pro/antioxidant parameters in patients with newly diagnosed bronchial asthma, which could be the basis for some new possibilities in the prevention and therapeutic application of this chronic disease. The parameters were determined in the patients' plasma, erythrocytes and cultures of lymphocytes treated with Concanavaline A (Con A) and Phorbol 12-myristate 13-acetate (PMA). Contrary to Th1 cytokines, Th2 profile of cytokines shows a significant increase ($p<0.01$) vs. control group, with higher level in extrinsic (atopic asthma) vs. intrinsic asthma. The results of oxidant stress markers (increased levels of lipid peroxides, xanthine oxidase activity in blood of patients with BA) and antioxidant parameters (decreased superoxide dismutase (SOD) activity, increased glutathion peroxidase (GPx) and catalase activities, and glutathione (GSH) concentration) suggest that asthma is associated with disturbances in pro/antioxidants indicating that oxidant stress occurres in patients with newly diagnosed BA. Investigation of cytokines in lymphocyte cultures of patients with BA showed a significant increase in basal TNF-a concentration ($p<0.05$), IL-13 and IL-4 ($p<0.05$) after stimulation with Con A, and TNF-a after stimulation with PMA ($p<0.01$). The study of subtle cellular mechanisms in the initiation and development of asthma offers the possibility of applying some blockers of inflammatory and immune mediators as well as transducers, or some supplements which may compensate for defects in asthma regulation mechanisms, regulate disbalance and reorganize pathogenesis of the disease in the desired direction.

Key words: bronchial asthma, Th1/Th2 cytokines, oxidant stress, antioxidants

Introduction

Bronchial asthma (BA) is a serious global health problem. About 300 million people are affected by this chronic airway disorder which is followed by more frequent complications (including fatal) than in the past. This disease, together with various pathologic conditions is associated with changes in cytokine and antioxidant profiles (1, 2). Special attention of many studies focused on factors responsible for specific polarization to predominantly Th1 or Th2 immune response. The primary signals which activate Th2 cells are unknown, but they may be related to the presentation of a restricted panel of antigens in the presence of appropriate cytokines (3). Therefore, dendritic cells are ideally suited to being the primary contact between the immune system and external allergens. Co-stimulatory molecules on the surface of antigen presenting cells, in particular B7.2/CD28 interaction, may lead to the proliferation of Th2 cells (4). Some cytokines, as well as chemokines, prosta-

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glandins, nitric oxide and oxidants can directly influence the polarization of Th cells acting via transcriptional factors (5–7). Certainly, the domination of Th2 cytokines leads to secretion and release of many mediators which determine the type of immune-inflammatory response (8).

The disturbances in cytokine profile, oxidant stress and response of antioxidant enzymes to cytokines have a key role in both the initiation and the development of asthma. These events are being increasingly recognized as important in remodeling airways and critical in orchestrating the type of inflammatory response (9). In experimental models, oxidants and specific pattern of Th1/Th2 cytokines can produce many of the features typical for asthma: they induce bronchoconstriction and increase in airway responsiveness to several agonists (10). Also, in these models oxidants and cytokines can lead to increased permeability of the airways. Cytokines induce synthesis and secretion of endothelial adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (11), endothelial leukocyte adhesion molecule-1 (ELAM-1), E selectin, chimiotactic cytokines, such as IL-8 and an additional degranulating factor which can induce neutrophil degranulation (12) and stimulate oxidant stress followed by the formation of free radicals. The inflammatory cells, stimulated by cytokines and recruited to the asthmatic airways, have an exceptional capacity for producing oxidants. In addition, activated eosinophils, neutrophils, monocytes and macrophages, as well as resident cells such as bronchial epithelial cells, can generate oxidants (10, 13–15) as well.

Lung cells experience enhanced oxidant stress because of their direct exposure to environmental irritants and pollutants. Also, specific features of the lung (large surface area, high partial pressure of oxygen) contribute to its distinct model of antioxidants (including SOD and GPx) for protection against exposure to noxious oxidants, compared to other organs (16, 17). When an imbalance exists between oxidants and antioxidants in favor of oxidants, oxidative stress occurs.

The purpose of this study was to evaluate the level of circulating cytokines and antioxidant enzyme production in patients with newly diagnosed bronchial asthma, and the results were compared with those from healthy immunocompetent individuals. The study included the examination of plasma Th1 (TNF-α, INF-γ, IL-1β) and Th2 (IL-4, IL-10, IL-13) cytokines, antioxidant enzyme activities SOD, GPx, catalase and the level of thiobarbituric acid-reactive substances (TBARS) as markers of lipid peroxidation. The measurement of cytokine levels in plasma may not adequately reflect the cytokine-producing potential of immune cells due to the short life of cytokines and the presence of soluble receptors, anticytokine antibodies, and receptor antagonists in human plasma. Therefore, we also observed the effect of Concanavaline A (Con A) and Phorbol 12-myristate 13-acetate (PMA) on the expression of Th1 (TNF-α, INF-γ) and Th2 cytokines (IL-4, IL-13) before and after stimulation.

Patients and Methods

Patient selection

In this study we observed 52 patients, aged 40–58 years, with newly diagnosed bronchial asthma, as defined by the Global Initiative for Asthma (GINA) (18). Patients were divided into two main groups: intrinsic (non-allergic) and extrinsic (atopic, allergic) type of BA, on the basis of GINA recommendations (immunologic parameters, clinical finding, specific cells for asthma and other relevant functional tests). Patients with abnormal renal or hepatic function or with a recent atherosclerotic event were excluded.

The blood was taken just before patients inhaled glucocorticoides and received other adjuvant therapy. Obtained results were compared with control group. The control group included 49 volunteers.

Cytokine and parameters of oxidative stress assays

The sera of all participants as well as cell culture supernatants were collected and frozen for a later analysis of cytokines by the enzyme-linked immunosorbent assay (ELISA) technique with commercially available kits. IL-1β, TNF-α and IL-10 kits were obtained from R&D Systems (Abingdon, Oxon, UK). IFN-γ kit was obtained from Bender Med System Diagnostics (Vienna, Austria) and IL-4, IL-13 were obtained from Beckman Coulter, Imunotech (Marseille, France). In cell culture supernatants cytokines were analyzed by ELISA kits obtained from Bender Med System (Vienna, Austria). All cytokine assays were calibrated in accordance with the World Health Organization international standards by the kit manufacturer. Sensitivities for individual assays are as follows: TNF-α, $<$ 4.4 pg/mL; IL-1β, 1 pg/mL; IFN-γ, 1.5 pg/mL; IL-4, $<$ 2 pg/mL; IL-10, $<$ 0.5 pg/mL; IL-13, 1.5 pg/mL.

The concentration of thiobarbituric acid-reactive substances (TBARS) was determined by thiobarbituric acid method: lipid peroxidation products reacting with thiobarbituric acid in the buffer of 1% orthophosporic acid solution, pH 2.0, and the addition of 1 μmol of iron sulphate. The absorbance was measured at 535 nm (19). Catalase (EC 1.11.1.6) activity was determined with the method of Beutler based on the decomposition of H_2O_2 followed directly by the decrease in absorbance at 230 nm (20). Glutathione was determined with Ellman reagents (5,5-ditiobis-2 nitrobens acid-DNTB) and absorption was

measured at 412 nm (21). Activities of GSH-Px (EC 1.11.1.9) and SOD (EC 1.15.1.1) were performed in erythrocytes with the commercial kit Ransel and Ransod (Randox Lab, UK) on the Beckman Synchron CX 5 analyzer. Activity of MnSOD was obtained by KCN inhibition.

Isolation and culture of human PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from 10 mL freshly drawn heparinized blood in 9 patients with newly diagnosed bronchial asthma, through density gradient centrifugation by Histopaque-1077. The cells were washed twice in RPMI 1640 culture medium containing 25 mmol/L HEPES, 2 mmol/L glutamine, penicillin (100 U/mL), streptomycin (100 mg/mL) and resuspended at the concentration of 2×10^6/mL in the same medium supplemented with 10% fetal calf serum. PBMC were treated for 72h with Concanavaline A (Con A, SERVA, Germany, 10 mg/mL) and Phorbol 12-myristate 13-acetate (PMA, Sigma, Germany, 10 ng/mL) at 37 °C in an atmosphere of 95% air and 5% CO_2. After incubation, the concentration of some Th1 (TNF-α, INF-γ) and Th2 (IL-4, IL-13) cytokines was measured in supernatant and cell homogenates.

Statistical significance of differences was estimated using the Student's t-test and two-way ANOVA. P values lower than 0.05 were considered significant. The results are expressed as mean ± SE.

Results

Crucial parameters for the division of patients into two main groups are presented in *Table I*. Patients with extrinsic asthma are characterized by a higher level of IgE, number of eosinophiles and lower concentration of CRP in comparison with those of the intrinsic type. All of these BA features were increased in comparison with the control group (*Table I*).

Oxidative stress was estimated on the basis of disturbances in pro/antioxidative status. We noted an increased activity of prooxidant enzyme (XO) and increased concentration of TBARS, markers of lipid peroxides. Antioxidant enzymes showed different activities: increased activity of catalasa and GPx in erythrocites as well as catalasa in plasma and significant decrease in erythrocyte SOD activity (*Table II*).

Th1/Th2 ratio was disturbed: contrary to Th1 cytokines, Th2 cytokine profile shows a significant increase ($p<0.01$) vs. control group, being higher in

Table I Main immunological biomarkers in patients with BA

Parameters	Control	BA	Extrinsic BA	Intrinsic BA
CRP (mg/L)	1.1 ± 0.5	3.2 ± 3.9[a]	1.7 ± 5.0	4.7 ± 0.9[b]
Eosinophilis (G/L)	0.20 ± 0.07	0.71 ± 0.18[b]	0.81 ± 0.21[b]	0.68 ± 0.17[b]
IgE (U/ml)	32.5 ± 10.1	91.4 ± 71.2[b]	142.5 ± 60.4[b]	34.5 ± 18.2

Values are means ± S.E.
[a]$p<0.01$ vs. control group
[b]$p<0.001$ vs. control group & vs. intrinsic BA group

Table II Pro/antioxidant parameters in patients with BA

	Control	BA	Extrinsic BA	Intrinsic BA
XO pl (U/L)	8.98 ± 1.74	9.97 ± 1.84[a]	10.08 ± 2.05[a]	9.85 ± 1.88
TBARS pl. (μmol/L)	7.76 ± 1.21	8.51 ± 1.58[a]	8.15 ± 1.13	8.90 ± 2.36[a]
TBARS er. (μmol/L)	5.66 ± 0.89	6.41 ± 11.6	6.67 ± 1.08	6.12 ± 1.16
Catalase er. (U/gHb $\times 10^4$)	5.54 ± 0.81	7.17 ± 1.96	8.35 ± 1.71[b]	5.86 ± 1.32
Catalase pl. (kU/L)	39.6 ± 14.5	81.1 ± 20.1[c]	82.8 ± 19.3[c]	79.2 ± 22.7[c]
SOD er. (U/g Hb)	1272 ± 498	1102 ± 472	1282 ± 524	902 ± 184[a,d]
GPx er. (U/g Hb)	27.1 ± 11.2	42.9 ± 15.2[a]	45.6 ± 14.0[a]	39.9 ± 16.7
GSH (μmol/L)	7.38 ± 1.42	9.83 ± 1.78[a]	8.63 ± 1.88	11.16 ± 1.66[b]

Values are means ± S.E.
[a]$p<0.05$ vs. control group
[b]$p<0.01$ vs. control group
[c]$p<0.001$ vs. control group
[d]$p<0.05$ vs. extrinsic BA group

Table III Concentration of plasma cytokines in patients with BA

		Control	BA	Extrinsic BA	Intrinsic BA
Th1 (pg/mL)	IL-1β	16.69 ± 3.62	17.04 ± 4.73	17.58 ± 5.45	16.44 ± 3.75
	INF-γ	5.89 ± 1.67	8.45 ± 1.95	7.96 ± 1.88	9.00 ± 1.94
	TNF-α	5.89 ± 2.47	6.72 ± 3.82	7.31 ± 2.52	6.07 ± 1.77
Th2 (pg/mL)	IL-4	8.97 ± 1.62	13.03 ± 2.07[a]	13.53 ± 1.78[a]	12.47 ± 1.75[a]
	IL-10	5.13 ± 3.27	19.75 ± 11.74[b]	21.36 ± 14.04[b]	17.96 ± 10.27[a]
	IL-13	2.17 ± 1.18	7.06 ± 5.31[a]	8.33 ± 4.42[a]	5.65 ± 4.24

Values are means ± S.E.
[a]$p<0.05$ vs. control group
[b]$p<0.01$ vs. control group

Table IV IFN-γ and TNF-α production by stimulated cultured PBMC

Group	Mitogen	IFN-γ (pg/mL)	TNF-α (pg/mL)
Control (n = 6)	Con A	95 (8–1180)	1465 (1050–1730)
	PMA	19 (10–80)	3050 (750–5280)
	None (cell control)	6 (5–6)	350 (330–435)
BA (n = 10)	Con A	103 (18–1260)	1381 (680–1730)
	PMA	29 (6–1150)	12345 (860–95780)[b]
	None (cell control)	6 (6–7)	1050 (105–1470)[a]

[a]$p < 0.05$ vs. unstimulated control group
[b]$p < 0.05$ vs. PMA control group
Median values and range are given in parentheses.

Table V IL-4 and IL-13 production by stimulated cultured PBMC

Group	Mitogen	IL-4 (pg/mL)	IL-13 (pg/mL)
Control (n = 6)	Con A	9 (7–70)	11 (6–415)
	PMA	6.5 (6–9)	22.5 (8–150)
	None (cell control)	5 (5–6)	5 (4–5)
BA (n = 10)	Con A	13 (6–57)[a]	137 (7–1180)[b]
	PMA	6 (5–72)	49 (5–2630)
	None (cell control)	5 (5–7)	5 (4–6)

[a]$p<0.05$ vs. Con A stimulated control
[b]$p <0.01$ vs. Con A stimulated control
Median values and range are given in parentheses.

extrinsic vs. intrinsic asthma. The highest level was noted in the concentration of IL-10 in patients with extrinsic type of BA (*Table III*).

Median values and range for the production of each cytokine by cultured PBMC from patients with BA were compared with controls and are given in *Tables IV* and *V*. The results show homogeneous values of basal (unstimulated) cytokine production (except basal TNF-α production). Although the results for IFN-γ production (*Table IV*) show a wide range of values, the median values demonstrate a clear mitogen stimulatory effect, but significant differences were not observed. Also, it was noticed that Con A induces much higher IFN-γ production than PMA in both control and patient PBMC. In contrast, PMA-stimulated PBMC produced a higher concentration of TNF-α in comparison with Con A-treated cells. Unstimulated patient PBMC produced three times larger amounts of TNF-α than PBMC of healthy persons, and this difference was significant ($p<0.05$).

Production of IL-4 was significantly higher in patients than in controls after Con A stimulation. IL-13

production showed a wider range of values than IL-4 production in both groups. Stimulation with Con A leads to a greater production of IL-13 in comparison with healthy subjects ($p<0.01$).

Discussion

The results of this investigation showed disturbance of Th1/Th2 balance in patients with BA: a significant increase in Th2 and unaffected Th1 cytokines. The highest increase was noted in concentrations of IL-10 and IL-13 in patients with extrinsic asthma, and the increase in IL-13 and IL-4 was in significant correlation with the degree of disease. Cytokines are released from consecutive airway cells and contribute to a characteristic inflammation which leads to clinical features of BA. Despite the well known fact that IL-4 is the crucial cytokine of Th2 response (22, 23), recent studies show that some other ligands can bind to IL-4a and activate STAT6, which is an important step in asthma signaling cascade of IL-4 (24, 25). Thus, IL-13 can express effects similar to those of IL-4 (26, 27) by activation of IL-13R/IL-4R receptor complex which activates different transducer-signaling mechanisms, including STAT6 and insulin receptor substrate-2 (IRS-2). A recent study demonstrates the key role of STAT6 in the development of phenotype BA (24).

In this study we used two mitogens, Con A and PMA, to stimulate cultured lymphocytes. Con A is known to stimulate the proliferation of cytotoxic T cells, suppressor inducer T cells, or »virgin« T cells, while PMA stimulates some kind of oxidative stress. Unlike PMA which did not show any significant effect on cytokine production, a significant increase in IL-13 and IL-4 after Con A stimulation showed different response of patient lymphocytes in comparison with lymphocytes of healthy persons. The reason why different mitogens stimulate cells to produce different levels of cytokines is not clear, although the spectrum of target cells for each mitogen is known to be somewhat different. A spontaneous *in vitro* release of cytokines by PBMC may reflect a measure of their activation *in vivo*. *In vitro* TNF-α basal production from patient PBMC showed a significant increase in comparison with the basal production of control cells. The results from our study suggest that disturbance in cytokine concentrations in unstimulated and especially in stimulated cultures of patients with bronchial asthma is a result of a specific pattern in patients with probably chronic oxidative stress and disturbances in cytokine network.

Higher level of IL-13 is important because IL-13 plays a key role in bronchial hyperactivity affected by B cell (production of IgE), VCAM-1, eotaxin and Ca^{+2} activated chloride channel gene-1 (CACL1) (28). However, higher level of serum IL-10 in patients with BA probably has a beneficial effect on clinical and serological findings. It is known that IL-10 downregulates both Th1 and Th2 immune responses. Also, administration of IL-10 in patients with BA leads to improvement through clinical signs of the disease (29) by many anti-inflammatory effects (decrease Th1/Th2 cytokines, chemokines, inflammatory enzymes and increase IL-1RA and TIMPs) (3).

Specificity of oxidant stress in patients with BA is characterized by higher concentrations of TBARS and changed activities of antioxidants' enzymes, particularly in the extrinsic type of asthma: decreased SOD activity, increased glutathion peroxidase GPx and catalase activities. These changes suggest that asthma is associated with disturbances in pro/antioxidants and indicate that oxidant stress occurred.

It is known that main generators of free radicals include NADPH oxidase complex, XO, reactions of eikosanoide biosynthesis and mitochondrial respiratory chain (14–16). In asthma, activated eosinophils, neutrophils, macrophages and bronchial epithelia cells (characteristic finding in BA), stimulated by cytokines, represent crucial cells responsible for the generation of free radicals (29, 30). Eosinophils possess several times greater capacity for generating oxidants than neutrophils, and the EPO content of eosinophils is several times higher than that of MPO in neutrophils. The finding of lower SOD level may be the consequence of permanent consumption and inactivation of this enzyme by superoxide anion and by H_2O_2. Also, other studies indicated that SOD derangement in bronchoalveolar lavage (BAL) and increase in extracellular SOD activity may occur. Increased level of GSH may be a transitory finding in the blood of newly diagnosed patients with BA.

Oxidants have a direct influence on NF-κB and AP-1, crucial transcriptor factors which regulate the production of cytokines. Cytokines play an integral role in the coordination and persistence of inflammatory processes. In chronic inflammation of the airways, oxidants and/or antioxidants directly or indirectly participate in the main pathophisiological mechanisms of asthma: airways remodeling, bronchoconstriction and responsiveness.

A general and complete study of subtle cellular mechanisms in the initiation and development of bronchial asthma provides conditions for the administration of modulators of different inflammatory, immune mediators or transducers, or supplement application which may compensate for defects in asthma regulation mechanisms, balance disbalance and reorganize pathogenesis of disease in the desired direction.

Acknowledgments. This work (Project 1714: Inter-dependence between cytokines, antioxidative enzymes and adhesion molecules in Th1 and Th2 cellular responses) was supported by the Ministry of Science and Environmental Protection of Serbia.

SPECIFIČNOST CITOKINSKOG PROFILA I OKSIDANTNOG STRESA U BOLESNIKA SA BRONHIJALNOM ASTMOM

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Kratak sadržaj: Bronhijalna astma (BA), zbog sve većeg morbiditeta i mortaliteta, danas predstavlja globalni izazov uprkos velikom pomaku koji je napravljen u saznanjima o patogenetskim mehanizmima astme uključujući ulogu inflamatornih ćelija, regulaciju inflamatornih i imunih odgovora, brojne medijatore i njihovu koregulaciju. Sve veći značaj se pridaje citokinima i njihovim modulatorima s obzirom na to da su ključni interćelijski transmiteri koji doprinose razvoju remodeliranja disajnih puteva. Cilj ove studije je evaluacija Th1/Th2 odnosa i procena međuzavisnosti citokina sa pro/antioksidantnim parametrima u bolesnika sa novootkrivenom bronhijalnom astmom, na osnovu čega bi se mogle naći nove mogućnosti u preventivnom i terapijskom pristupu ovoj hroničnoj bolesti. Navedeni parametri određivani su u plazmi, eritrocitima i u kulturama limfocita pacijenata tretiranih Koncanavalinom A (Con A) i forbol 12-miristat 13-acetatom (PMA). Za razliku od Th1 citokina, Th2 citokinski profil (IL-4, IL-10, IL-13) pokazuje značajan porast ($p < 0,01$) u odnosu na kontrolnu grupu, sa većim nivoom u ekstrinzik (atopijskoj) astmi u odnosu na intrinzik astmu. Rezultati markera oksidantnog stresa (povišeni lipidni peroksidi, ksantin oksidaza u krvi bolesnika sa BA) i antioksidantnih činilaca (smanjena aktivnost SOD, povišena aktivnost GPx, katalaze i koncentracije GSH) sugerišu da je došlo do narušavanja ravnoteže pro/antioksidanata te da je prisutan oksidantni stres u bolesnika sa novootkrivenom astmom. Ispitivanje citokina u kulturama limfocita bolesnika sa BA pokazalo je značajan porast koncentracija TNF-a pre ($p<0,05$), IL-13 i IL-4 ($p<0,05$) nakon stimulacije sa Con A i TNF-a po stimulaciji sa PMA ($p<0,01$). Proučavanjem suptilnih ćelijskih mehanizama u inicijaciji i razvoju astme pruža se mogućnost primene određenih blokatora inflamatornih, imunih medijatora i transduktora, ili primene suplemenata koji mogu da kompenzuju nedostatke u regulacionim mehanizmima astme, čime se može uravnotežiti disbalans i preusmeriti patogeneza bolesti u željenom pravcu.

Ključne reči: bronhijalna astma, Th1/Th2 citokini, oksidantni stres, antioksidanti

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Received: January 25, 2006

Accepted: February 25, 2006