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Research paper

Accelerated reactivity of blood granulocytes in patients with atopic bronchial asthma out of exacerbation

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ABSTRACT

Reactive oxygen species (ROS) are important in bronchial asthma (BA) pathogenesis owing to accumulation of activated granulocytes in the lungs. But the ROS-producing activity of the cells is insufficiently understood in the blood of BA patients. This study analyzes the kinetics of phagocyte respiratory burst in the blood to improve the methods of BA patients monitoring. Patients with atopic BA out of exacerbation ($n = 60$) and healthy controls ($n = 43$) were recruited. The time-course of respiratory response to opsonized zymosan (OZ) was recorded in the whole blood using luminol-dependent chemiluminescence (CL), and its activation kinetics (lag-time, rate, amplitude, ROS production) was calculated. The discriminative power of ROS generation kinetics was defined by Receiver Operating Characteristic (ROC) curve analysis. Standard physiological respiratory parameters of patients did not differ from the controls. More rapid response to OZ was recorded in BA patient samples versus the controls. The primed state of phagocytes in the blood of BA patients was corroborated by significant weakening formyl peptide priming effect. The adhesion of granulocytes to cultured human endothelial cells was two-fold higher in BA patients versus controls. ROC curve analysis exhibited good discriminative effectiveness of the CL kinetics to compare BA individuals with the controls. The highest power (86% sensitivity and 90% specificity) was achieved at a linear combination of the parameters. We assume that the assessment of phagocyte reactivity based on the analysis of the response kinetic profile is a good test for monitoring of the state in BA patients.

1. Introduction

Bronchial asthma (BA) is a heterogeneous disease, usually characterized by chronic airway inflammation (Global Initiative for Asthma, 2015; Hinks et al., 2016; Koczulla et al., 2017). The heterogeneity of asthma with different phenotypes and endotypes is one of the obstacles to successful management (Agache and Akdis, 2016). The clinical phenotype of atopic BA is the most recognizable one, since it is associated with the history of allergic diseases and reversible lung obstruction (Macdowell and Peters, 2007; Monteseirin et al., 2002; Virchow, 2016). Allergens contact with the airway epithelium, disrupt its integrity as a physical barrier and trigger the release of alarmins

including cytokines, uric acid, adenosine triphosphate, colony-stimulating factors and other substances (Hoffmann et al., 2016; Lambrecht et al., 2017). These agents activate and recruit the innate immune cells (dendritic cells, granulocytes, monocytes) from the bloodstream, ultimately leading to inflammation in the lungs (Kim et al., 2016; Lambrecht et al., 2017; Thiriu et al., 2017; Virchow, 2016). The severity of asthma affects the functioning of peripheral blood cells: in severe forms the number of granulocytes is significantly increased in the blood and bronchoalveolar lavage, and the expression profile of proinflammatory cytokines is also altered (Agache and Akdis, 2016; Brightling et al., 2008; Ciepiela et al., 2015; Nakagome et al., 2012; Shannon et al., 2008; Thiriu et al., 2017). Peripheral blood

Abbreviations: a.u, Arbitrary units; AUC, area under the curve; BA, bronchial asthma; CL, chemiluminescence; CI, confidence interval; CR3, CR4, complement receptors 3 and 4; EC₅₀, half maximal effective concentration; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; fMLF, peptide N-formyl-Met-Leu-Phe; FPR, formyl peptide receptor; GINA, Global Initiative for Asthma; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IgE, immunoglobulin E; LFA-1, lymphocyte function-associated antigen 1; OZ, opsonized zymosan; PEF, peak expiratory flow; PMA, phorbol 12-myristate 13-acetate; ROC, receiver operating characteristic; ROS, reactive oxygen species; TNF- α , necrosis factor- α ; WKYMVM, peptide Trp-Lys-Tyr-Met-Val-D-Met-NH₂

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granulocytes in BA patients have a higher metabolic response and sensitivity to bacterial or viral agents compared to healthy donor cells (Asman et al., 1997; Tang et al., 2015; Tang et al., 2016; Ramirez-Velazquez et al., 2014; Vargas et al., 1998). Excess granulocyte activity is considered a factor of complications and exacerbation in asthma (Fahy, 2009).

Granulocytes circulating in blood are in a resting state, they can be physiologically primed (preactivated), activated, inactivated, and can die by apoptosis (El-Benna et al., 2016). Priming of granulocytes is particularly associated with an enhanced functionality of adhesion-associated responses that facilitates the recruitment of the cells from the blood to the airways (Kanters et al., 2007). Physiologically primed neutrophils was supposed can be deprimed in lung *in vivo* and return back in the pulmonary circulation as resting neutrophils (Singh et al., 2012; Summers et al., 2014). Primed neutrophil phenotype has been identified in humans with various inflammatory diseases, such as rheumatoid arthritis, chronic kidney disease, traumatic injury, and acute respiratory distress syndrome (Miralda et al., 2017). Not much is known regarding priming of inflammatory cells in an allergic asthma (Kanters et al., 2007; Luijk et al., 2005). Distinct priming profiles are associated with different phases, phenotypes, or both of allergic asthma (Kanters et al., 2007). Literature analysis showed that little attention is paid the state of the blood granulocytes in BA out of exacerbation.

Reactive oxygen species (ROS) play a key role in asthma pathogenesis due to accumulation and activation of granulocytes in the lungs (Al-Harbi et al., 2015; Jiang et al., 2014; Nadeem et al., 2014; Zuo et al., 2013). Excessive ROS production contributes to the development of oxidative stress, which leads to bronchospastic syndrome and the transition of the inflammatory process to a chronic form (Qu et al., 2017; Zanatta et al., 2012). However, a clear relationship between the ROS-producing activity of the blood cells and the disease severity is not currently established. Teramoto et al. (1996) found increased spontaneous and stimulated production of superoxide per an individual neutrophil in the whole blood of BA patients using a lucigenin-dependent chemiluminescence (CL). Hyperactivated phenotype with decreased (Mattheyse et al., 2001) or increased ROS production (Vargas et al., 1998) was detected during the acute BA stage using luminol-dependent CL. Also increased production of superoxide anion was recorded on reduction of cyt c in BA (Kanazawa et al., 1991). During exacerbations, even without stimulation granulocytes of BA patients had an increased ROS-generating response versus controls and patients without exacerbation. Contradictory data were obtained also in the remission period: without alterations in ROS level, as well as either increased or decreased ROS compared with healthy donors using luminol-dependent CL (Mattheyse et al., 2001; Monteseirin et al., 1996; Vargas et al., 1998). The isolated neutrophils of BA patients demonstrated increased ROS production in response to different stimuli estimated by various methods: reduction of cyt c (Meltzer et al., 1989; Styrt et al., 1988), nitroblue tetrazolium test (Mosca et al., 2015), lucigenin-dependent CL (Teramoto et al., 1996). Given that divergent results can be a consequence of the use of different methods for measuring ROS level (Caldefie-Chézet et al., 2002; Freitas et al., 2009; Monteseirin and Vega, 2011). The development of tests for monitoring the state of the blood cells is a difficult task. Serum ROS level was proposed to be a biomarker for predicting severe BA exacerbations. It was tightly associated with the degree of airway obstruction, neutrophil count and severe exacerbation (Nakamoto et al., 2016).

The granulocytes are the main effector cells in inflammation: they receive an alarm signal, leave the vascular bed and migrate to the inflammatory site along the chemotactic gradient of inflammatory factors. Adhesion interactions accompany different granulocytes' functions: diapedesis, migration through intercellular matrix to inflammatory centre, phagocytosis (Langereis, 2013). The adhesion to the endothelium, intracellular matrix and microorganisms is provided mainly by the expression of β_2 -integrins including $\alpha_M\beta_2$ (MAC-1, or CR3, or CD18/CD11b), $\alpha_L\beta_2$ (CD18/CD11a or LFA-1) and $\alpha_X\beta_2$ (CD18/

CD11c, or CR4, or gp150/95). Fc γ R and CR3 receptors expressed on the cell membrane mediate the binding of opsonized particles and participate themselves in activation of ROS production (Nguyen et al., 2017). Opsonins are considered as molecules of antibodies IgM and IgG classes, fragments of complement components C3b, C4b, C-reactive protein and others. Neutrophils from patients with severe BA are known to express much more adhesion molecules than healthy donor cells (Aroca et al., 2014; Mukhopadhyay et al., 2014). Increased adhesion of peripheral blood eosinophils to E-selectin and ICAM-1 and neutrophils to ICAM-1 was observed in BA patients with a high degree of PEF variability. It was most probably caused by a functional upregulation of the receptors CD11b/CD18 and CD49d, because the number of receptors per cell was unchanged (Hakansson et al., 1995). The level of the soluble form of ICAM-1 serves as a marker of BA severity and is considered as a therapeutic target for asthma control (Mukhopadhyay et al., 2014).

Chronic inflammatory process underlies BA and a risk of frequent exacerbations exists, despite the permanent use of medications by patients (Hinks et al., 2016; Koczulla et al., 2017). So, simple and quick methods are necessary for monitoring the state of BA patients to personalize treatment and to prevent exacerbations. In this work we suggest testing the reactivity of the blood phagocytes in BA based of the time-course of respiratory response to opsonized zymosan (OZ) in the whole blood using luminol-dependent CL, as a measure of ROS production, and its activation kinetics. The fluorimetric, chemiluminometric and colorimetric probes were elaborated to identify and quantify ROS formed during the oxidative (respiratory) burst (Freitas et al., 2009). Many authors prefer to use luminol-dependent CL to detect ROS what is explained its advantage as following: the method has a high sensitivity; membrane-permeability of luminol allows to reflect the extra- and intracellular ROS unlike isoluminol detecting only extracellular ROS; luminol detects many oxygen intermediates like superoxide anion radical, hydrogen peroxide, hydroxyl radical, hypochlorite (Allen, 2015; Bedouhène et al., 2017; Dahlgren et al., 2008; Freitas et al., 2009; Monteseirin and Vega, 2011; Vladimirov and Proskurnina, 2009). Also nitric oxide produced by NO-synthase contributes luminol CL (Wang et al., 1993). All these oxygen spices were revealed in phagocytosis (Segal, 2005). The whole blood was more appropriate for our task to study cell reactions in experimental conditions which are maximal closed to native since the serum components, the products of phagocytes themselves and other cells can influence the phagocyte responses in complex. Microbe particles, e.g. zymosan, covered with serum opsonins, among which molecules of antibodies IgM and IgG classes, fragments of complement components C3b, C4b, C-reactive protein and others, are the experimental model of pathophysiological impact. When the microbial targets are opsonized, they are recognized more effectively by phagocytic receptors. Phagocytosis, physiological process critical in inflammation, initiates rather quick ROS production after recognition and internalization of opsonized microbes by the professional phagocytes (Cheson and Morris, 1981; Lim et al., 2017; Segal, 2005). Analysis of CL kinetics (K_s – the apparent Michaelis constant; V_{max} – the maximum velocity; T_n – the nodal time) revealed that luminol detects a relatively early event in OZ phagocytosis unlike lucigenin (Allen, 1986). The kinetic parameters of CL curves, which were recorded in the whole blood during phagocytosis of OZ, have shown the high discriminative possibilities in different diseases. The integral CL response and the time of reach half maximal value were useful indicators of CL defects in recurrent infections, myeloperoxidase deficiency, phagocytic defects and other (Carulli et al., 1995). Magrisso et al. (1995, 2000) presented OZ-initiated CL responses of isolated neutrophils and phagocytes in the blood as a sum of three biological components: (the first) processes that take place near the plasma membrane, connected with phagocytosis, cause extracellular CL; (the second) processes located inside the cell, connected with phagocytosis, and which cause intracellular CL; (the third) processes that lead to intracellular CL, not connected with phagocytosis. On the basis of the conception of three components of the CL curves the discriminative

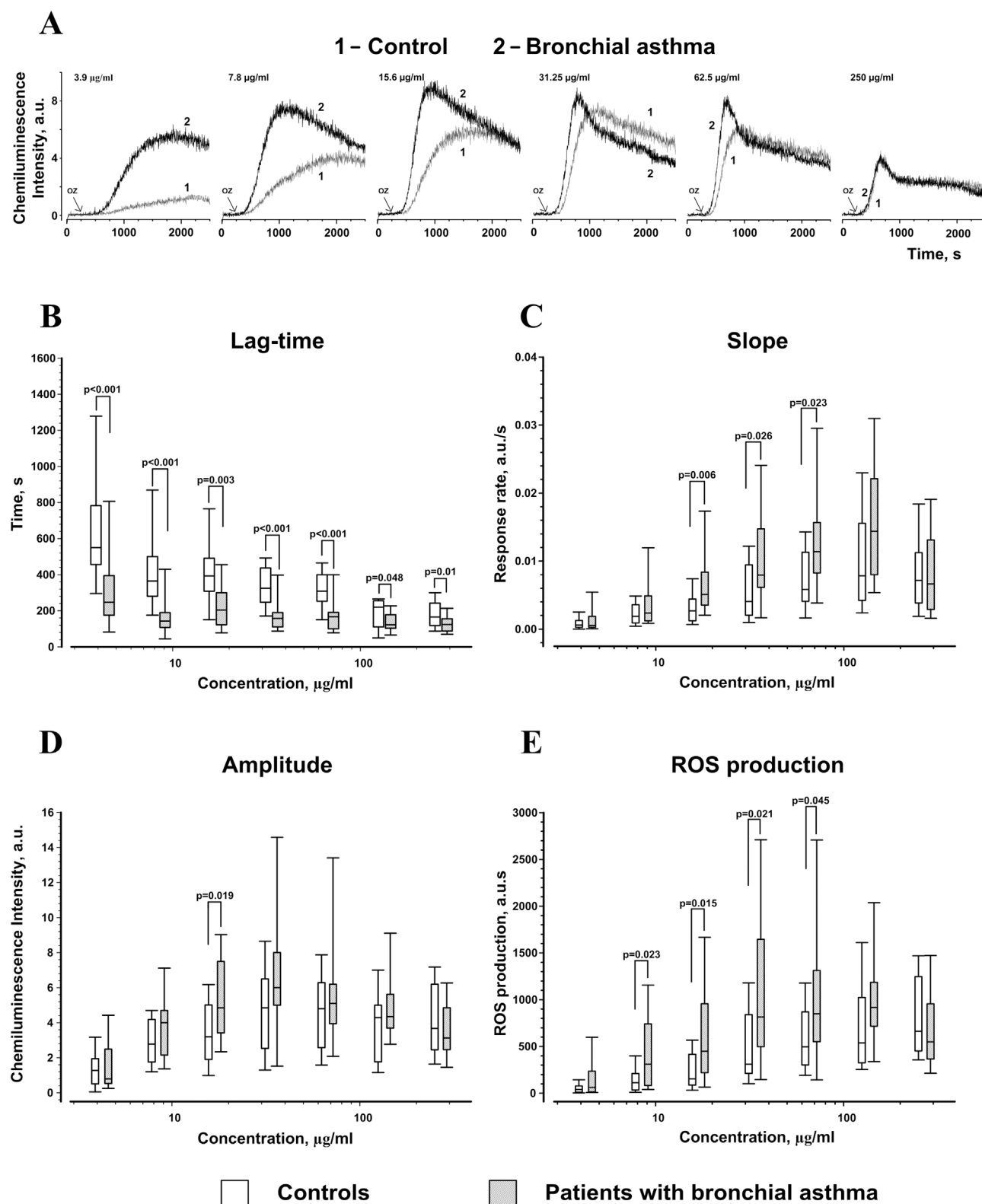


Fig. 1. Dependencies of the kinetic parameters of the respiratory response on the concentration of opsonized zymosan (OZ) in the whole blood. (A) – experimental records of cell responses initiated with OZ (3.9–250.0 µg/ml) in the samples from a healthy donor (1) and BA patient (2). B – E – a comparison of the cell response kinetics: B – lag-time; C – rate of response (slope); D – amplitude of the response (maximal intensity of ROS generation); E – total ROS production (integral). Median and 5th-95th percentiles are shown. White boxes in each graph – controls, grey boxes – BA patients. Statistical significance of differences between the parameters of the controls and BA patients estimated in Mann-Whitney rank sum test is shown.

criteria were elaborated for classification of infections and differentiation of bacterial and virus infections (Prilutsky et al., 2011a; Prilutsky et al., 2011b). There are not available data concerning the kinetic analysis of the CL curves in BA out exacerbation. This work aimed to identify the kinetic characteristics of the respiratory response on microbial provocation in the peripheral blood, estimate granulocytes priming state and adhesion properties to improve the methods for monitoring the state of BA patients out of exacerbation.

2. Subjects and methods

2.1. Reagents

Hanks' solution, phosphate buffered saline (PBS), peptide N-formyl-MLF (fMLF), luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione), Hoechst 33342, trypan blue, zymosan, heparin, gentamycin, accutase, gelatin, medium 199, collagenase type I from *Clostridium histolyticum*, Na₂CO₃, ethylenediaminetetraacetic acid (EDTA), xanthine oxidase, xanthine, medium 199 with Earl's salts, ficoll were purchased from Sigma-Aldrich (St-Louis, MO, USA). Penicillin and streptomycin were obtained from Biotol (Saint-Petersburg, Russia). Bovine fetal serum was from Invitrogen (Carlsbad, CA, USA). Peptide WKYMVM was from Alomone Labs (Jerusalem, Israel). Romanovsky-Gimza's dye was purchased from Minimed-R (Bryansk, Russia), ethyl alcohol was from Donskoi' (Tula region, Epifan', Russia), isopropyl alcohol (2-propanol) was from Component-Reactive (Moscow, Russia). Urografin was from Bayer AG (Leverkusen, Germany).

2.2. Patients

Patients with atopic BA out of exacerbation (60 individuals) were recruited at the Hospital of Pushchino Scientific Center, Russian Academy of Sciences. The atopic BA diagnosis was verified by the criteria of the Global Initiative for Asthma (GINA – 2012/2116). Patients received basic therapy with a combination of inhaled glucocorticosteroids (salmeterol/fluticasone propionate, 100–1000 µg/day in a mode of constant dispensing). The inclusion criteria were: lack of BA symptoms for more than 4 weeks before research; no restrictions on physical activity; requirement in short-range β₂-agonists more than once a week; no severe accompanying diseases. The exclusion criteria were: exacerbations for the last 3 months before the study; intake of systemic glucocorticoids; severe and chronic diseases. The control group included 43 individuals without asthma, other bronchopulmonary, chronic and acute diseases. All examinees gave a written consent for participation in the study after being informed on the procedures. In the formation of observation groups, BA patients and the controls were comparable by sex, age, socioeconomic and social conditions. The research protocol corresponds to the [World Medical Association Declaration of Helsinki \(2013\)](#) and was approved by the Local Ethic Committee of the Hospital of Pushchino Scientific Center (№2 of 10.04 2014).

2.3. Estimation of respiratory function

The respiratory function of BA patients was assessed by standard parameters: forced expiratory volume in 1 s (FEV₁), forced vital capacity (FVC) and peak expiratory flow (PEF). These parameters are expressed as percent predicted of normal reference values. Lung-tester MIR Spirodoc (Medical International Research, Italy) was used.

2.4. Leukocyte count

The leukocyte differential count was performed for each patient by blood processing on hematology analyzer AB-X Micros 60 (HORIBA ABX SAS, France).

2.5. Kinetics of ROS generation in the whole blood

The intensity of ROS generation was measured by chemiluminescent technique as described in our previous work (Safronova et al., 2013). Chemiluminometer device CHEMLUM-12 (Institute of Cell Biophysics, Russian Academy of Sciences) was used. Luminol-dependent CL served as a measure of different oxygen intermediates in the extra- and intracellular localization (Dahlgren et al., 2008; Monteseirín and Vega, 2011; Vladimirov and Proskurnina, 2009). The whole blood was studied to save closed to a physiological environment of phagocytes. The blood sampled using Sarstedt tubes with heparin (Sarstedt, Germany) was diluted with Ca²⁺-free Hanks' solution (1:1) and kept for 1 h at 4 °C. Then samples were prepared in polypropylene minidishes as follows: 173 µl Hanks' solution with 1 mM Ca²⁺, 20 µl diluted blood and 7 µl luminol (final concentration of 0.35 mM). After registration of the base level the respiratory burst was induced with serum-opsonized zymosan (OZ), what reproduced *in vivo* situation in considerable degree (Lim et al., 2017). Since phagocytes function in gradient of chemotactic factors producing by microbes and own disrupted cells, we used OZ in concentration range 1.95–250.0 µg/ml (Fig. 1). Measurements were carried out simultaneously in 12 minidishes for nearly 50 min at 37 °C, the signal from each individual minidishes was registered every 2.5 s.

To study the primed state of phagocytes a bacterial peptide N-formyl-MLF (fMLF) in subthreshold concentration of 0.01 µM was added into the blood samples. After 30 min incubation the respiratory response was initiated with 15.6 µg/ml OZ, close to EC₅₀. Primed state of ROS-producing system of phagocytes is verified by enhanced responsibility to activation by different stimuli (El-Benna et al., 2016). CL-curve parameters as lag-time, rate of OZ-response (slope) and ROS production are more informative for estimation of the ability of the blood phagocytes to priming.

2.6. Isolation of granulocytes from the blood

Granulocytes were isolated in ficoll/urografin gradient (1.077/1.119 g/ml). Granulocytes count was estimated as 85–95% based on Hoechst 33342 staining using fluorescent microscope Leica DM2500 (Leica, Germany). The survival of the cells was nearly 98% under trypan blue staining. Before measurements the cell suspension (10⁷ cells/ml) was kept in Ca²⁺-free media for 1 h at 4 °C.

2.7. Culture of endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated and cultivated as described earlier (Nadeev et al., 2016). Briefly, an umbilical vein washed out with Hanks's solution containing 50 U/ml penicillin and 50 µg/ml streptomycin was filled with medium 199 with the addition of 0.1% collagenase type I from *Clostridium histolyticum* and incubated for 40 min at 20–22 °C. After washing the cells grew in a flask covered with 0.2% gelatin in medium 199 with Earl's salts supplemented with 20% bovine fetal serum, 300 µg/ml endothelial growth component from rabbit brain (Maciag et al., 1979), 100 µg/ml heparin and 100 µg/ml gentamycin. The cells were used for experiment after 2–3 passages when the monolayer density was about 10⁵ cells/cm². Accutase solution was used to remove the cells from the well bottom.

2.8. Adhesion test

HUVEC were cultivated in a 96-well plate for three days, and then granulocytes (3 × 10⁵ cells) were added. Three different tests were carried out: intact cells; cells with addition of 1 µM fMLF or 1 µM WKYMVM, a synthetic agonist of low affinity fMLF receptors (FPR2) (Bufe et al., 2015). The wells with HUVEC only served as a control. All samples were incubated in a CO₂-incubator (Sanyo, Japan) for 30 min at 37 °C. Subsequent protocol of treatment was as described in our previous work (Safronova et al., 2016). In brief, saline solution was

removed after cell incubation, then 30 μ l of 70% ethanol was added to each well to fix the cells within 3 h at 20–23 °C. After alcohol removal the plate was dried out within 14 h at 37 °C and 100 μ l Romanovsky-Gimza's dye (azur-eosin solution) diluted in PBS (1:9) was added to stain the attached cells within 40 min. Then the dye was removed, the plate was washed out thrice. After that 250 μ l isopropanol was added to each well and absorption was measured with Multiscan Plus spectrophotometer (Lab System, USA) at 492 nm.

2.9. Data analysis

Data were processed with software developed for CHEMLUM-12 in ICB RAS. The parameters of CL-curve were calculated: base level of ROS, lag-time, rate of response development (slope), maximum of ROS generation intensity (amplitude). Also integral under CL curve for 1000 s (ROS production) was calculated in according with conception about the participation of extra- and intracellular stages of phagocytosis in early events of ROS generation (Magrisso et al., 2000). Authors presented the OZ-initiated CL response in blood is a sum of three kinetic components: (1) phagocytosis-dependent extracellular CL; 2) phagocytosis-dependent intracellular CL; 3) phagocytosis-independent intracellular CL. Analysis was based on CL records for 20–25 min, at that the peaks of the first two components are in the first half of the total recording time (not more 10 min), and their kinetics is associated with extra- and intracellular phagocytosis. We choose 1000 s as fixed time so we studied the phagocytosis-dependent extra- and intracellular ROS production.

The results were analyzed with the Matlab software (MathWorks, Natick, USA) and presented as a median and 5th-95th percentiles. The baseline physiological characteristics of examinees are presented as mean $\pm$ SEM and compared using Student's *t*-test. Mann-Whitney Rank Sum Test was applied to compare the parameters of the responses for the BA patients and controls. Distinctions were considered as statistically significant at $p < 0.05$.

Receiver operating characteristic (ROC) curves were used to define the discriminative power of the respiratory burst kinetics. The generalized linear regression algorithm was applied to: 1) kinetic parameters (lag-time, response rate and ROS production) separately; 2) their linear combination. A logistic model with a binomial distribution was chosen. A 95% confidence interval (CI) was estimated using a bootstrap method with a number of replicas of 1000 and using the vertical averaging algorithm. To assess the significance level (p -value) of the difference between the regression model ($AUC \neq 0.5$) and the constant model ($AUC = 0.5$), the Chi-square criterion was used. The odds ratio was calculated using the sensitivity/(1-specificity) formula.

Table 1

Baseline characteristics of patients with bronchial asthma and healthy donors (mean $\pm$ SEM).

Physiological characteristics	Patients, n = 60	Controls, n = 43
Age (years)	58.6 $\pm$ 1.7	50.6 $\pm$ 2.9
Male/Female	13/47	20/23
Weight (kg)	75.2 $\pm$ 2.2	67.3 $\pm$ 3.2
Height (cm)	165.8 $\pm$ 7.1	171.1 $\pm$ 2.5
Body mass index (kg/m ²)	28.6 $\pm$ 4.1	23.3 $\pm$ 1.1
Arterial blood pressure (mmHg)		
Systolic	138.5 $\pm$ 12.6	116.4 $\pm$ 14.8
Diastolic	83.1 $\pm$ 8.7	76.3 $\pm$ 10.1
Heart rate (bpm)	68.6 $\pm$ 9.1	69.1 $\pm$ 7.1
The clinical blood analysis		
Leukocytes, $\times 10^9$ cells/L	6.8 $\pm$ 0.5	6.2 $\pm$ 0.3
Monocytes, %	6.6 $\pm$ 0.4	7.5 $\pm$ 0.3
Segmented neutrophils, %	48.1 $\pm$ 2.6	48.3 $\pm$ 1.2
Eosinophils, %	2.0 $\pm$ 0.6	1.1 $\pm$ 0.1

3. Results

3.1. Physiological characteristics of participants

Patients with bronchial asthma (BA) out of exacerbation (n = 60) and healthy individuals (n = 43) were characterized by age, height, weight, body mass index, blood pressure and heart rate (Table 1). In BA patients the values of FEV₁, FVC and PEF were 87.6 $\pm$ 4.3; 89.5 $\pm$ 4.9 and 78.0 $\pm$ 5.7% predicted, respectively. All values were more than 80% in the controls. There was no difference between the parameters in the BA patients out of exacerbation and the controls.

According to the clinical blood analysis the leukocyte count and the blood cell proportions in the BA patients did not differ from the control values (Table 1). This fact allowed comparing data from BA patients and the controls without normalization to amount of phagocytes in blood.

3.2. Resting ROS level in the whole blood

The intensity of spontaneous ROS generation did not differ in the whole blood of the controls and BA patients: 0.15 (0.08–0.30) versus 0.14 (0.02–0.26), respectively. This indicated that phagocytes were not activated in the blood of BA patients out of exacerbation.

3.3. The respiratory burst kinetics in the whole blood

Opsonized zymosan (OZ) initiated concentration-dependent responses (Fig. 1A). Their kinetic parameters were also concentration-dependent (Fig. 1B–E). As the concentration of OZ increased from 1.95 to 250.0 μ g/ml, the lag-time decreased in both the controls and BA patients, but the lag-time was significantly shorter in the samples from BA patients versus controls (Fig. 1B). The response rate was significantly higher in the BA patient samples versus the controls at the concentration range of 15.6–62.5 μ g/ml. But differences between the groups were not observed at low (3.9 and 7.8 μ g/ml) and high (125 and 250 μ g/ml) concentrations (Fig. 1C). There were no differences between the response amplitudes in the controls and BA patients except the dose of 15.6 μ g/ml (Fig. 1D). The ROS production over 1000 s (the early stage of CL responses) was significantly higher in the BA patients versus the controls at the concentration range of 7.8–62.5 μ g/ml (Fig. 1E).

Thus, three kinetic parameters of respiratory response were significantly different in BA patients and healthy individuals: lag-time, response rate (slope) and ROS production (integral) in the representative concentration range of 15.6–62.5 μ g/ml OZ. It testifies that reactivity of blood granulocytes in the BA patients out of exacerbation is accelerated compared with the controls.

ROC curves analysis showed that the kinetic parameters of the respiratory response such as lag-time, response rate (slope) and ROS production (integral) have a fair or good effectiveness in discriminating asthmatic individuals out of exacerbation from the controls in concentration range 15.6–62.5 μ g/ml OZ with the median AUC values were than 0.72 (Table 2). It was observed a bad effectiveness of the test for other concentrations with the median AUC values in range 0.48–0.63 for slope, amplitude and ROS production, so further we took into account the middle concentrations. A combination of three parameters (Combination) improved the effectiveness to good for three OZ doses taken into account (Table 2) and provided a high sensitivity and specificity of the test (Fig. 2). Cutoff thresholds for the combination of parameters (lag-time, response rate, ROS production) were: for a dose of 15.6 μ g/ml–280.25 s, 0.007 a.u./s, 514.9 a.u.s, respectively; for a dose of 31.25 μ g/ml–327.7 s, 0.018 a.u./s, 1704.1 a.u.s; for a dose of 62.5 μ g/ml–293.0 s, 0.019 a.u./s, 1485.7 a.u.s. These cutoff thresholds indicated a high power in discriminating BA individuals out of exacerbation from controls taking into account the odds ratio: for a dose of 15.6 μ g/ml–3.095 (95% confidence interval [CI] 1.85–4.29); for a

Table 2

Receiver operating characteristic (ROC) curves analysis for the kinetic parameters of the OZ response in the whole blood.

Kinetic parameter	Dose, µg/ml								
	15.6			31.25			62.5		
	AUC ^a	95% CI ^b	<i>p</i> ($\times 10^{-4}$)	AUC	95% CI	<i>p</i> ($\times 10^{-4}$)	AUC	95% CI	<i>p</i> ($\times 10^{-4}$)
Lag-time	0.826	0.652 0.930	2.2	0.854	0.607 0.956	2.9	0.841	0.599 0.949	4.0
Response rate (slope)	0.801	0.631 0.916	7.7	0.780	0.574 0.900	13.6	0.750	0.555 0.890	40.7
ROS production	0.783	0.571 0.904	6.4	0.729	0.524 0.875	53.6	0.744	0.549 0.883	87
Combination ^c	0.860	0.695 0.947	6.6	0.875	0.643 0.968	10.1	0.878	0.620 0.976	14.7

Notes: AUC^a – Area Under Curve; CI^b – confidence interval; Combination^c – linear combination of the kinetic parameters was taken for analysis. The generalized linear regression algorithm was used to approximate the experimental data. A logistic model with a binomial distribution was chosen. A 95% CI was estimated using a bootstrap method with a number of replicas of 1000 and the vertical averaging algorithm. To assess the significance level (*p*-value) of the difference between the regression model (AUC $\neq$ 0.5) and the constant model (AUC = 0.5), the Chi-square criterion was used.

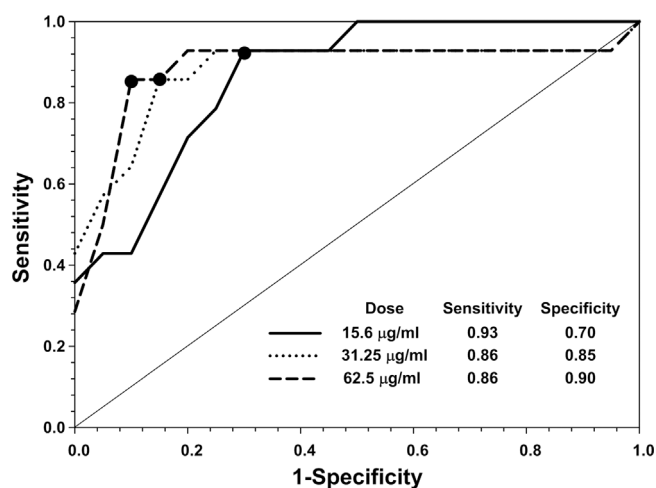


Fig. 2. Receiver Operating Characteristic (ROC) curve for a linear combination of the kinetic parameters of the respiratory response. The kinetic characteristics were taken into account: lag-time, response rate (slope), ROS production. ROC curves for three doses of opsonised zymosan are shown. Values of the area under the curve (AUC), 95% confidence intervals and *p*-values are listed in Table 2. Cutoff points (black), the optimal relationship between the sensitivity and specificity of the test, are indicated.

dose of 31.25 µg/ml–5.71 (CI 3.71–12.0); for a dose 62.5 µg/ml–8.57 (CI 2.79–10.29). It should be noted that this test has a high discriminative power over the entire concentration range with the highest power at a dose of 62.5 µg/ml OZ.

3.4. Cell priming in the blood samples

We assumed the response kinetics reflected the altered granulocyte phenotype retained in BA patients out of exacerbation. An unchanged baseline level of ROS together with an increased response rate indicated that granulocytes were rather in the primed (preactivated) than in the activated state in BA patients. The treatment of blood samples with bacterial peptide fMLF in a subthreshold concentration of 0.01 µM modified the OZ-induced respiratory burst (Fig. 3). The kinetic parameters of the respiratory response were changed: the lag-time was shortened, the rate and ROS production were increased in the control samples (Fig. 3A). In the samples from BA patients, the changes were less pronounced (Fig. 3B). fMLF significantly shortened the lag-time in the controls: 320.0 (234.4–510.0) s in the intact samples versus 205.0 (147.50–313.75) s in the fMLF-treated samples (*p* = 0.006), which was about 35% (Fig. 3C). At the same time, there was no significant change of the lag-time in BA patients: 242.5 (201.25–318.85) s versus 210.0

(166.25–251.25) s, respectively. The effect of 0.01 µM fMLF was almost negligible in the blood samples of BA patients versus the controls (Fig. 3C).

Priming with fMLF increased the response rate (slope) by almost 2.5 times in the controls and by less than 50% in the BA patients, showing a significant difference (Fig. 3D). But there were no significant differences in the fMLF effect on the amplitude of the response between the healthy and BA individuals (Fig. 3E). The production of ROS was increased by 2.4 times in the fMLF-pretreated samples from the controls and almost by 50% in the BA patients (Fig. 3F), showing a significant difference.

Thus, the priming effect was significantly weaker in the blood samples of BA patients compared with the controls. Consequently, the systems involved in the priming of phagocytosis and ROS generation (receptors, their signal systems or targets) had been preliminarily primed or activated in the BA patients.

The differences between the kinetics of the respiratory response to OZ in BA patients and controls were observed at the earliest stages of phagocytosis after adhesion interaction between the cell membrane and particles. NADPH oxidase, an enzyme catalyzing superoxide anion production, became activated and the intensity of ROS generation increased. We assumed that the adhesion properties of phagocytes in the BA patients were changed.

3.5. Adhesion of isolated peripheral blood granulocytes to HUVEC

According to the adhesion test the absorbance was two-fold higher in the wells with granulocytes from BA patients compared with those from healthy individuals (Table 3). The addition of 1 µM fMLF activating the high and low affinity fMLF receptors (FPR1 and FPR2, respectively) significantly increased the granulocyte adhesion on HUVEC in the control samples, but was not altered in the samples from BA patients (Table 3). Also, 1 µM WKYMVM, a synthetic FPR2 agonist, enhanced the cell adhesion in the controls, but was ineffective in the BA patients (Table 3).

4. Discussion

Atopic BA patients out of exacerbation were examined in our study. Their physiological parameters and external respiration functions did not vary from the controls. The absolute number of leukocytes was also comparable with the control values as well as the proportion of monocytes and neutrophils in the blood (Table 1). A higher proportion of eosinophils was observed in the BA patients versus the controls, but the difference was not significant. The elevated level of eosinophils can persist in the blood, bronchoalveolar lavage and bronchial biopsy in

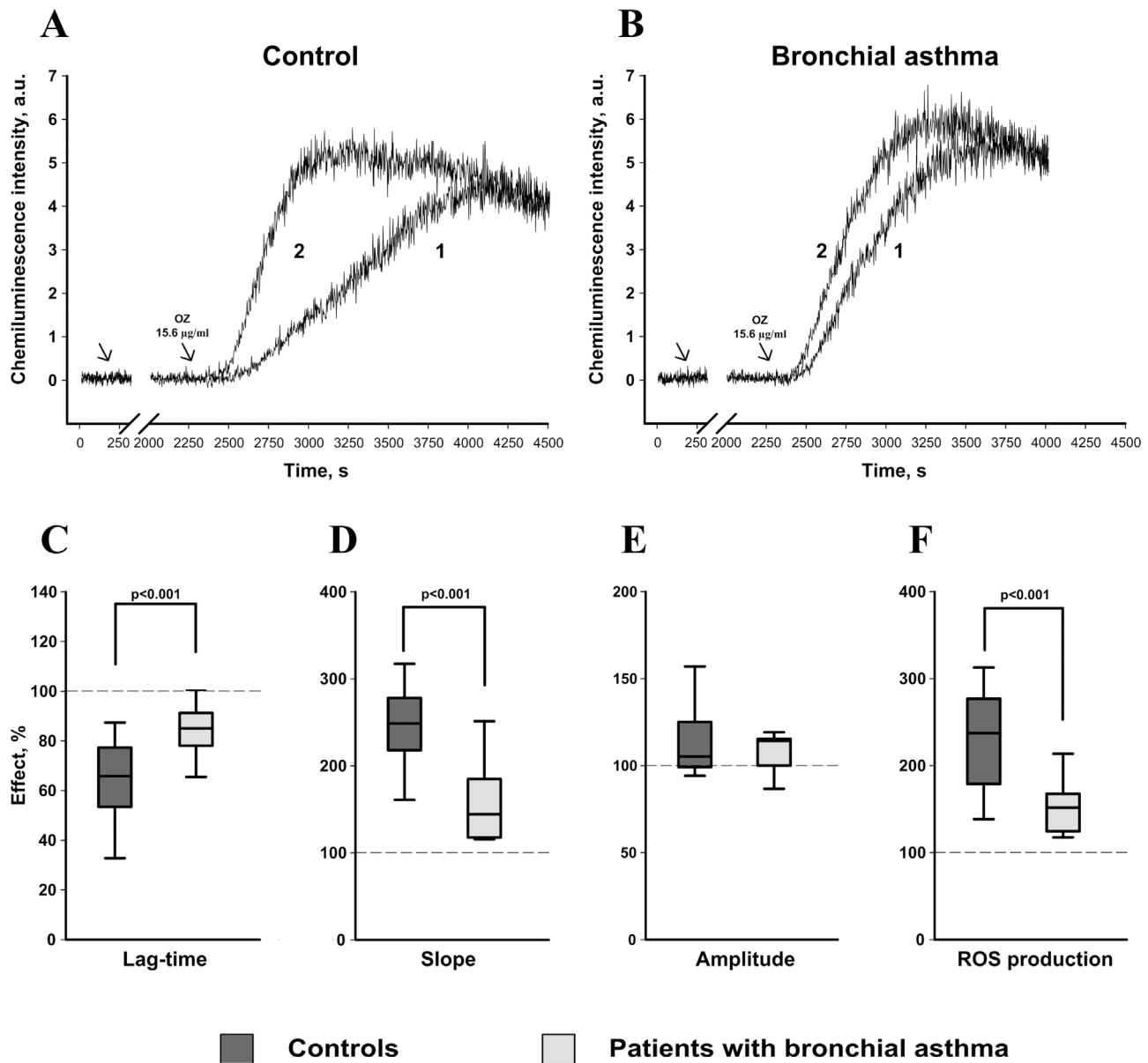


Fig. 3. The kinetic characteristics of the response to OZ under fMLF priming.

A, B – experimental records of the phagocyte responses to 15.6 µg/ml OZ in the blood samples of a healthy person and a BA patient, respectively. In each plot the responses to OZ after addition of 0.001% DMSO as solvent (1) or 0.01 µM fMLF (2) are shown. Arrows indicate the moment of agent addition. Pre-incubation was continued for 30 min at 37 °C. C – F – priming effect of 0.01 fMLF on the kinetic parameters of the response to 15.6 µg/ml OZ: lag-time (C); slope (D, response rate); amplitude (E); ROS production (F, integral). The effect was estimated as the ratio of the value obtained from a blood sample with fMLF, to the parameter of an intact sample taken as 100%.

Table 3

Absorbance values upon adhesion of granulocytes to HUVEC.

Conditions	Absorbance	
	Patients, n = 6	Controls, n = 8
Intact cells	0.1815 (0.087–0.248)*	0.083 (0.051–0.128)
1 µM fMLF	0.164 (0.080–0.308)	0.108 (0.071–0.354)#
1 µM WKYMYM	0.153 (0.127–0.271)	0.1235 (0.085–0.205)#

Notes: * comparison of the BA patients with the controls, $p < 0.02$; # comparison of the intact and peptide-treated cells, $p < 0.04$.

patients with the abatement of asthma symptoms (Boulet et al., 2012). It means that residual inflammation is observed under BA out of exacerbation, which is also manifested by the attenuation of the suppressive function of T-regulatory lymphocytes (Boulet et al., 2012). Thus, the mechanisms of immunity regulation in these patients remain

incorrect, that is fraught with the recurrence of the disease.

Studying the ROS generating activity of phagocytes in the peripheral blood of BA patients is important since (i) ROS play a key role in the asthma pathogenesis (Al-Harbi et al., 2015; Jiang et al., 2014; Nadeem et al., 2014; Zuo et al., 2013); (ii) professional phagocytes, neutrophils predominantly, are main source of ROS in the peripheral blood; (iii) the granulocyte phenotype is altered in the peripheral blood and respiratory tract of BA patients (Ramirez-Velazquez et al., 2014); (iv) circulating granulocytes differ on the sensitivity to bacterial or viral agents in healthy individuals and BA patients (Tang et al., 2015; Tang et al., 2016). This issue has been given some attention, but no clear answer has been received until now. During exacerbation of BA, ROS production was reported to be decreased (Mattheyse et al., 2001) or increased (Kanazawa et al., 1991). Contradictory data were also obtained in BA patients out of exacerbation: no difference in ROS level (Mattheyse et al., 2001) as well as either increased (Vargas et al., 1998)

or decreased (Monteseirín et al., 1996) ROS levels in contrast to healthy individuals. After the capture of microbes or their fragments by phagocytes phagolysosomes are formed and generation of superoxide-anion radicals occurs (Babior, 1999; Jaillon et al., 2013). The kinetics of the phagocytic reaction is determined by the adhesive interaction between an opsonized foreign object and a phagocyte; activation and inactivation of the opsonin receptors and components of signaling pathways; the kinetics of activation and inactivation of NADPH oxidase (El-Benna et al., 2016; Makni-Maalej et al., 2013; Nordenfelt and Tapper, 2011).

Comparing the phagocyte state and ROS generation in the blood of BA patients and controls we revealed: 1) similar ROS spontaneous levels; 2) changed kinetics of respiratory burst activation as shortened lag-time, accelerated response to OZ and augmented ROS production; 3) attenuated reaction on respiratory burst priming; 4) increased adhesion of granulocytes to the endothelium. These evidences indicate that a pool of primed phagocytes is present in the peripheral blood of BA patients out of exacerbation.

Kanters et al. (2007) have shown multiple priming phenotypes of peripheral blood granulocytes associated with different phases, phenotypes, or both of allergic asthma. Long-time priming by endogenous substances results in modified gene expression in BA (Baines et al., 2010; Croteau-Chonka et al., 2017; Sordillo and Raby, 2014). ROS production can be short-time potentiated also with proinflammatory cytokines and chemotactic agents, e.g. fMLF. Main priming mechanisms of ROS-generating activity fulfils Ca^{2+} -dependent mechanisms and partial phosphorylation of NADPH oxidase components, p47phox dominantly (El-Benna et al., 2016; Kelher et al., 2003). The leading role in this case is given to tyrosine phosphorylation with participation of mitogen-activated protein kinases (Dang et al., 2006; Miralda et al., 2017; Safronova and Gabdoulkhakova, 2009). The short-time priming effect of fMLF was significantly weaker in the blood samples of BA patients compared with the controls (Fig. 3). Consequently, the systems involved in the priming of phagocytosis and ROS generation (receptors, their signal systems or targets) had been preliminarily primed or activated in the BA patients out exacerbation.

The primed state of granulocytes is reflected in the phenomenon of elevated expression of adhesion molecules in peripheral blood under allergic BA (Aroca et al., 2014; Hakansson et al., 1995; Mukhopadhyay et al., 2014; Verdegaal et al., 1998). We revealed increased adhesion to HUVEC and lack of effect of FPR agonists (fMLF and WKYMVM) on adhesion of granulocytes in BA patients out of exacerbation compared with the controls (Table 3). It also indicates the altered granulocyte state. OZ particles were coated with various serum proteins, so our results could be explained by an analysis of the functional expression of adhesion proteins on the phagocyte membrane, including the complement C3bi receptor, CD11b/CD18 (CR3) and CD11c/CD18 (CR4) molecules. They are important for adhesion, chemotaxis, phagocytic activity (Vargas et al., 1998). The bacterial peptide fMLF and synthetic peptide WKYMVM increased adhesion of the cells to HUVEC in the health individual samples, but they did not influence adhesion of BA patient cells (Table 3), i.e. maximal activation of adhesion properties of granulocytes in blood of BA patients in the state out of exacerbation. Probably, during the BA period out of exacerbation, a high expression of adhesion molecules in granulocytes is maintained, which leads to enhanced adhesion properties. This problem requires further investigations in patients with BA out of exacerbation.

The pathogenesis of asthma is associated with an elevated level of certain endogenous factors (e.g. GM-CSF, TNF- α , and IL-8) which can alter a state of phagocytes (Charrad et al., 2016; Mahajan and Mehta, 2006; Seys et al., 2013; Tamai et al., 2017). The cytokines and inflammatory mediators, which concentrations are elevated in the blood under BA (Ciepiela et al., 2015), can influence the state of phagocytes and change the kinetic characteristics of ROS production in phagocytosis. Their effect led to granulocyte transformation from resting to primed state, but did not activate ROS generation, since the level of

spontaneous ROS production in the blood did not increase. The lag-time was decreased and the response rate increased at rather low OZ doses (Fig. 1). Circulating cytokines change the cell's absorbing capacity, the level of expression of membrane receptors interacting with the opsonized particles, the regulation of NADPH oxidase activity and intracellular signaling systems (Makni-Maalej et al., 2013; Mosca et al., 2015). According to the data obtained, phagocytosis-induced ROS generation is activated much faster in the blood of BA patients compared to the controls. The amplitude of response to OZ, i.e. the maximum intensity of ROS generation, in BA patients out of exacerbation did not change significantly compared to the controls, but the ROS production during the early stage of CL responses was significantly increased (Fig. 1). It is clear, that some parameters of the respiratory burst (e.g. amplitude) do not allow revealing the differences in the functional state of the cells in the whole blood. The faster development of the response to the pathogen is a result of the primed (not activated) phenotype of phagocytes in BA patients out of exacerbation. It makes difficult to compare data obtained in different laboratories. In addition, discrepancies can take place owing to the use of different methods for measuring the ROS level (Caldefie-Chézet et al., 2002; Monteseirín and Vega, 2011). The luminol-chemiluminescent assay is considered the most applicable on the basis of its high sensitivity, ability of luminol to penetrate through the membrane and detect both intra- and extracellular sets of different reactive oxygen forms (Monteseirín and Vega, 2011).

ROC curve analysis of kinetic parameters of chemiluminescence records demonstrated high power to discriminate patients with asthma out of exacerbation and healthy individuals (Table 2, Fig. 2). Previously the assessment of phagocyte reactivity based on the analysis of the response kinetic profile was not suggested for monitoring. Some tests for diagnosis of BA and monitoring of BA patients have been elaborated based on ROC curve analysis. For example, the serum ROS level was proposed to be a biomarker for predicting severe exacerbations (Nakamoto et al., 2016). In another study, serum baseline tryptase levels have been offered as potentially useful in supporting the diagnosis of asthma in children and as a predictor of disease severity (Gao et al., 2016). Our results show significant differences between the controls and atopic BA patients out of exacerbation for 3 parameters: lag-time, response rate and ROS production at OZ concentrations from 15.6 $\mu\text{g}/\text{ml}$ (close to EC_{50}) to 62.5 $\mu\text{g}/\text{ml}$ (close to saturation). The lag-time magnitude has higher AUC values than other kinetic parameters (the response rate and ROS production), thus it is more effective in this test. At the same time, when all the three parameters were taken into account (Combination), the effectiveness of the test increased (Table 2, Fig. 2), being the highest at the combination of the three kinetic parameters of the response to the dose of 62.5 $\mu\text{g}/\text{ml}$. We suggest applying the kinetic parameters of chemiluminescence records to discriminate patients with asthma out of exacerbation from healthy individuals. It would be extremely interesting to analyze the kinetics of the respiratory response during BA exacerbation or under allergens.

5. Conclusions

The work revealed that atopic BA patients out of exacerbation had normal physiological respiratory parameters, their blood was characterized by normal spontaneous ROS level, but activated phagocytosis and ROS generation were more rapid versus the same parameters in healthy donors. Adhesion of granulocytes to the endothelium in BA patients was significantly increased and was not regulated through the receptors of bacterial peptides. These facts indicate a pool of primed phagocytes. According to significant differences of kinetic parameters (lag-time, response rate) in all used stimulus concentrations, the expression of receptors involved in phagocytosis is proposed to be changed in BA patients out of exacerbation. A combination of the kinetic parameters of the response on microbial provocation provides a highly sensitive and specific test for monitoring atopic BA patients out

of exacerbation and other pathologies.

Conflicts of interest

None.

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Ethics approval and consent to participate

The study was approved by the Local Ethic Committee of the Hospital of Pushchino Scientific Center (№2 of 10.04 2014). Informed consent was taken from each of the examinees after being informed on the procedures.

Consent for publication

Not applicable. The present paper does not contain any individual person's data.

Availability of data and material

The datasets used during the current study are available from the corresponding author on reasonable request.

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