



Original article

Delayed kinetics of phagocytosis related respiratory burst in blood is a distinctive feature of moderate exacerbation of bronchial asthma

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ABSTRACT

Atopic bronchial asthma based on allergy history and chronic inflammation is hazardous to patients due to the risk of exacerbation. The sign of severe exacerbation is considered an abundant number and high activity of granulocytes in respiratory system and blood. Relationships between the ability of cells in blood to produce reactive radicals and their metabolites and the severity of asthma remain largely unclear. Kinetics of respiratory burst evoked by microbe particles in blood samples of patients was studied to reveal the most significant predictors distinguishing states of moderate exacerbation and out of exacerbation. Asthmatic patients with exacerbation ($n = 18$) or out of exacerbation ($n = 62$) and healthy individuals ($n = 43$) were characterized on respiratory function, cell count in blood and kinetics of generation of reactive radicals and their metabolites during phagocytosis. Mean values of respiratory parameters forced expiratory volume in 1 s and peak expiratory flow rate in patients with exacerbation were significantly differed compared with same of patients out of exacerbation and healthy individuals. Mean values of cell count in blood did not significantly differed in patients with exacerbation and out of exacerbation. Receiver operating characteristic analysis showed that both cell count and respiratory indexes did not discriminate patients with exacerbation from out of exacerbation. A delayed response to opsonized zymosan was revealed in patients with exacerbation compared to other examinees: lengthened lag-time and T_{max} , reduced production of reactive species. T_{max} was the most statistically significant predictor to discriminate bronchial asthma exacerbation from bronchial asthma out of exacerbation (area under curve $> 90\%$, $p < 10^{-5}$) and controls (area under curve $> 80\%$, $p < 10^{-5}$).

Thus kinetic parameters of the phagocyte response to opsonized zymosan in the whole blood are the best predictors of bronchial asthma exacerbation in comparison with respiratory parameters and blood cell count. This test can be used for immunological monitoring of bronchial asthma status to prevent exacerbation.

1. Introduction

Allergic (atopic) bronchial asthma (BA) is based on allergy history and inflammatory causation [1]. The sign of severe BA phenotypes and exacerbations is an abundant number and high activity of granulocytes (neutrophils and eosinophils) in the blood, airways and lung [2–7]. In BA allergens or infections activate granulocytes for the migration from blood to lungs and release of many substances [8]. It leads to the generation of an excessive amount of reactive oxygen species (ROS) and/or their metabolites that aggravate BA patient state. The role of

ROS as a main player of oxidative stress is underlying maintenance of chronic inflammation and provocation of exacerbation [9,10]. Acute exacerbation is associated with a great increase of ROS level in serum [9]. It is considered as a biomarker of exacerbation closely related to the polymorphonuclear neutrophils (PMN) count, the degree of airway obstruction and severity of exacerbation [11]. However inflammatory markers strong correlated with increased level of oxidative stress markers even in patients without deterioration of asthma symptoms [12]. Thus, high level of ROS in serum presents in BA, that is considered a predictor of exacerbation in some cases, but its effect on asthma

Abbreviations: BA, bronchial asthma; CL, chemiluminescence; COPD, chronic obstructive pulmonary disease; DUOX1, dual oxidase 1; ESR, erythrocyte sedimentation rate; FEV₁, forced expiratory volume in 1 s; fMLF, N-formyl-Met-Leu-Phe; FVC, forced vital capacity; GCS, glucocorticosteroid; GINA, the Global Initiative for Asthma; IgE, immunoglobulin E; NOX, nicotinamide adenine dinucleotide phosphate oxidase; PEF, peak expiratory flow; PEFR, peak expiratory flow rate; PMA, phorbol 12-myristate -13- acetate; ROC, Receiver Operating Characteristic

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severity should be clarified.

NADPH oxidase is the main enzyme catalyzing the generation of superoxide anion radical O_2^- during phagocytosis. O_2^- is a short-living radical that further forms other reactive metabolites in the reaction named as “respiratory burst”. In asthma NADPH oxidase isoforms (NOX2, NOX4, DUOX1) presented in different lung tissues are sources of O_2^- [13]. ROS produced by myeloid cell with participation of NOX2 are associated with worsened asthma phenotypes. NADPH oxidase-derived ROS regulate lung inflammation and injury through the redox regulation of nuclear factor NF- κ B [14]. A consequence is a change of gene expression profile of proinflammatory cytokines. An altered activity of isolated granulocytes to produce ROS was shown in BA patients with exacerbation [15,16]. The production of O_2^- was higher in BA patients versus healthy individuals and correlated with forced expiratory volume in 1 s (FEV_1). It increased with the disease progression and was greater in exacerbation versus stable disease [15]. Controversially, low activity of neutrophils in respiratory burst induced with bacterial peptide was demonstrated during BA exacerbation [16]. Both opposite facts suggested the presence of an altered neutrophil population in the circulation during exacerbation. In the whole blood an increased spontaneous and stimulated production of superoxide radicals per an individual neutrophil was shown in BA patients without specifying the disease stage [17]. Thus, relationships between the ability of blood cells to produce reactive radicals and their metabolites and asthma severity remain largely unclear.

In our previous work an accelerated reactivity of granulocytes to produce ROS was shown in blood of BA patients out of exacerbation [18]. According receiver operating characteristic (ROC) curve analysis kinetic parameters of response to opsonized zymosan (OZ) are informative for definition of BA patient state. Earlier the kinetics of ROS generation was taken into account very fragmentarily in the case of BA. Whereas high discriminative possibilities of the kinetics of chemiluminescent (CL) response recorded in whole blood samples during phagocytosis of OZ have been shown in different diseases [19]. The integral CL response and the time of reach half maximal value were suggested as indicators in recurrent infections, myeloperoxidase deficiency, phagocyte defects.

It is important to monitor the state of BA patient out of exacerbation for the recognition and treatment of moderate exacerbation on time to prevent severe exacerbation. We suggest the kinetics of ROS generation as a perspective discriminative test for the monitoring of BA exacerbation. In current work we revealed that kinetics of respiratory burst during phagocytosis of microbe particles was different in whole blood samples of BA patients with moderate exacerbation and BA patients out of exacerbation. The most statistically significant predictor for asthma monitoring was time to response maximum (T_{max}).

2. Materials and methods

2.1. Study design and examinees

The study was carried out at the Hospital of Pushchino Scientific Centre of the Russian Academy of Sciences. Three groups of examinees were surveyed: Group 1 - atopic BA patients with moderate exacerbation ($n = 18$), Group 2 - BA patients out of exacerbation ($n = 62$), Group 3 - healthy individuals ($n = 43$), all Caucasian (Table 1). Atopic BA was diagnosed and verified by criteria of GINA [1]. The severity of exacerbation was defined as “moderate” according to the criteria: deterioration in symptoms and lung function (PEF (peak expiratory flow) or FEV_1 from 50 to 80% predicted), increased rescue bronchodilator use. These features lasted more than 2 days without of the need of hospitalization and systemic corticosteroids use. Status of such patients corresponded with modern concepts of moderate exacerbation of asthma [1,20].

All participants gave a written consent for the personal data processing. The study protocol was adjusted to the World Medical

Table 1

Baseline characteristics of examinees.

	Group 1 ^a , $n = 18$	Group 2, $n = 62$	Group 3, $n = 43$
Physiological characteristics			
Age (years)	58.7 ± 3.3	58.8 ± 1.7	50.6 ± 2.9
Sex (male/female)	2/16	13/49	20/23
Body mass index (kg/ m^2)	$29.1 \pm 1.0^*$	$27.8 \pm 0.7^{\#}$	22.9 ± 0.7
Disease duration (years)	13.1 ± 2.4	15.0 ± 3.7	0
Arterial blood pressure (mmHg)			
Systolic	$139.1 \pm 4.2^*$	$138.6 \pm 2.3^{\#}$	116.1 ± 1.6
Diastolic	$84.7 \pm 2.6^*$	$84.1 \pm 1.3^{\#}$	76.1 ± 1.5
Heart rate (bpm)	71.3 ± 2.1	68.1 ± 1.1	68.9 ± 1.2
Respiratory functions			
FVC, %	$76.05 \pm 5.00^*$	85.10 ± 3.05	100.4 ± 4.6
FEV_1 , %	$72.6 \pm 6.3^{*^*}$	$87.3 \pm 3.5^{\#}$	113.9 ± 1.8
PEF, %	67.1 ± 7.0	75.5 ± 3.2	88.1 ± 7.3
PEFR, l/min	$286.7 \pm 35.2^{*^*}$	350.35 ± 13.44	456.0 ± 35.4

Notes. ^a) Here and further: Group 1 – BA patients with moderate exacerbation; Group 2 – BA patients out of exacerbation; Group 3 – Controls. FVC - forced vital capacity, FEV_1 - forced expiratory volume in 1 s, PEF - peak expiratory flow, PEFR - peak expiratory flow rate. Data are presented as mean value $\pm$ SEM. Kruskal-Wallis One Way Analysis of Variance on Ranks was applied to the comparison of three groups: * - differences between Groups 1 and 2, ^ - differences between Groups 1 and 3, # - differences between Groups 2 and 3, $p < 0.05$.

Association Declaration of Helsinki [21] and confirmed by the Local Ethic Committee of the Hospital of Pushchino Scientific Centre, the Russian Academy of Sciences (№2 of 10.04.2014).

Inclusion criteria for Group 1 were: burdened allergic anamnesis, paroxysmal unproductive cough after contact with triggers, dyspnea, wheezing, positive test with bronchodilator (salbutamol 400 μg , 20% increase FEV_1), eosinophils in sputum and nose mucus, positive skin prick-tests with allergens, increased serum level of IgE. Exclusive criteria were: severe exacerbations of BA requiring systemic glucocorticosteroids and hospitalization; infectious or oncological diseases; severe concomitant diseases of endocrine or cardiovascular systems; acute myocardial infarction or cerebrovascular accidents, surgery 2 years before the study. Basic therapy was: inhalation with glucocorticoids as monotherapy (fluticasone propionate, beclomethasone), combined inhaled corticosteroids (formoterol/budesonide, salmeterol/fluticasone propionate, vilanterol/fluticasone furoate); antagonists of leukotriene receptors (montelukast) in a mode of constant dispensing.

Inclusion criteria for Group 2 and Group 3 were listed in our previous work [18]. They were for Group 2: a good control over BA symptoms, lack of BA symptoms for more than 4 weeks before research; physical activity without restrictions; requirement in short-range β_2 -agonists more than once a week; no severe accompanying diseases. Exclusive criteria were: exacerbations 3 months before the study; severe and chronic diseases; intake of systemic glucocorticosteroids. Basic therapy included: inhaled corticosteroids in combination: salmeterol/fluticasone propionate, 100–1000 $\mu\text{g}/\text{day}$ in a mode of constant dispensing.

Inclusion criteria for Group 3 were: absence of asthma, bronchopulmonary, cardiovascular and other chronic pathologies, lack of acute diseases one month before the study. Exclusive criteria were: bronchopulmonary, cardiovascular pathologies, diabetes, allergy, smoking and intake of medications, alcohol or caffeine 12 h before the study.

2.2. Measurement of respiratory parameters

The respiratory function was estimated by standard method on forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC) and peak expiratory flow (PEF). The parameters were expressed as percent predicted of normal reference values. Lung-tester MIR Spirodok

(Medical International Research, Roma, Italy) was used. A peak expiratory flow rate (PEFR) was measured by peak flow meter Vitalograph Model 4.300 (Ennis, Ireland).

2.3. Blood cells count

The blood cell differential count was performed for each individual using a hematology analyzer AB-X Micros 60 (HORIBA ABX SAS, Montpellier, France).

Registration of phagocyte respiratory burst in the whole blood and estimation of kinetics.

Luminol-dependent chemiluminescence taken as measure of reactive oxygen species (O_2^- , hydrogen peroxide, hydroxyl radical) and some attendant metabolites (hypochlorite, nitric oxide) in respiratory burst was recorded by chemiluminometer CHEMLUM-12 (Russia) as described early [18]. Experiment design in short was as follow: peripheral blood collected to Sarstedt tubes with heparin (Germany) was diluted with Ca^{2+} -free Hanks' solution (1:1) and kept for 1 h at 4°C. Specimens for measurements was prepared in polypropylene minidishes which contained 173 μ l Hanks' solution with 1 mM Ca^{2+} ; 7 μ l luminol (0.35 mM) and 20 μ l diluted blood. After record of spontaneous level of ROS, serum- OZ was added to initiate the respiratory burst. As phagocytes operate in chemotactic gradient we analyzed their responses in concentration range of 3.9–250.0 μ g/ml OZ (Fig. 1). Experimental model reproduces *in vivo* situation in considerable degree [22]. CL-responses were recorded consecutively from 12 minidishes with 2.5 s intervals, for 2700 s in cases of the controls and BA out of exacerbation, or 6000 s in case of exacerbation. The functional state of phagocytes in the blood was estimated in samples with 0.01 μ M N-formyl-Met-Leu-Phe (fMLF) for priming and 31.3 μ g/ml OZ for activation. All reagents were purchased from Sigma-Aldrich (USA).

2.4. Measurement of IgE level in the serum blood

Total IgE in the serum blood was measured by quantitative immunoturbidimetric method with latex enhancement of fixed-time kinetics using Reagents «DiaSys» (Germany) and Analyzer BS-400 Mindray (China).

2.5. Data processing

Data were handled with CHEMLUM-12 software developed in the ICB RAS (Russia). Following CL-curve parameters were estimated: base level of reactive species, lag-time, rate of response (slope), maximum of reactive species (amplitude), time to maximum (T_{max}) and production of reactive species, calculated as integral under CL-curve for 1000 s interval. This interval was chosen because phagocytosis-dependent chemiluminescence reaches its peak value in no more than 600 s [23].

Results were processed with the Matlab software (USA). Kruskal-Wallis One Way Analysis of Variance on Ranks was applied for comparison of three groups. Distinctions between groups were considered as statistically significant at $p < 0.05$. Correlation between study parameters was estimated using Spearman rank test.

The method of ROC curve analyses was applied to clinical functional parameters and kinetic parameters of respiratory burst named as “predictors” for estimation of their discriminative power regarding to BA patient state. The generalized linear regression algorithm and logistic model with a binomial distribution were applied to the linear combination of predictors under different OZ concentrations [18]. A 95% confidence interval (CI) was estimated by a bootstrap method with a number of replicas of 1000 and the vertical averaging algorithm. The Chi-square criterion was used to assess the significance level (p -value) of the difference between the regression model ($AUC \neq 0.5$) and the constant model ($AUC = 0.5$). Function *stepwiseglm* in Matlab was

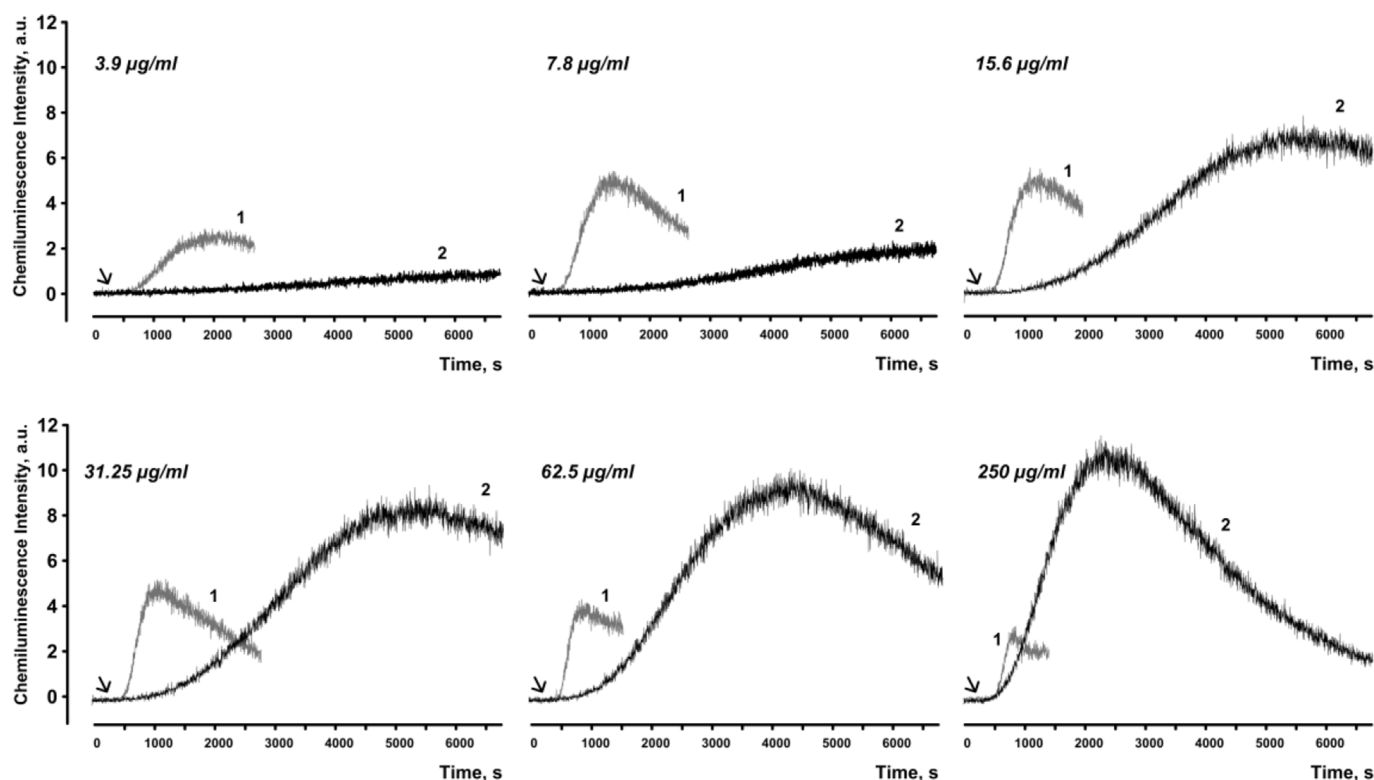


Fig. 1. Original recordings of kinetic curves of respiratory response to opsonized zymosan (OZ) in the whole blood samples. The blood samples were obtained from one of patients with bronchial asthma (BA) in the state out of exacerbation (1 - gray lines) and with moderate exacerbation (2 - black lines). OZ in concentration range 3.9–250 μ g/ml was used to initiate the respiratory burst which was recorded on intensity of luminol-dependent chemiluminescence. Arrows indicate time of OZ addition to blood samples.

applied to identify a linear combination of statistically significant predictors.

3. Results

3.1. Patient characteristics

Body mass indexes and arterial blood pressure were higher in Groups 1 and 2 compared to Group 3 (Table 1). However these parameters were not significant different between Groups 1 and 2.

3.2. Respiratory function

Mean values of considered respiratory parameters in Group 1 were lower than reference values (Table 1). FEV₁ and PEF mean values in Group 1 were significantly lower compared with values in Groups 2 and 3. FVC mean value in Group 1 was significantly lower then in Group 3. PEF did not significantly differ between groups. ROC curve analysis for linear combination of respiratory parameters showed insignificant specificity and sensitivity of the respiratory test to classify Groups 1 and 2 (0.95 and 0.22, respectively, $p = 0.1$). The discriminative power of the test is shown in Table 2. High discriminative power of respiratory parameters was revealed for Groups 1 and 3. Using the *stepwiseglm* function in Matlab it was shown that FEV₁ only was the most statistically significant predictor for Groups 1 and 3, but it had low discriminative power for Groups 1 and 2 (Table 2). Thus, despite that FEV₁ mean values significantly differed in Groups 1 and 2, it had low discriminative power to classify these groups by asthma status.

3.3. Clinic blood analysis

The significant enlargement of proportion of eosinophils, lymphocytes and PMN-lymphocyte ratio was revealed in Group 1 versus Group 3 (Table 3). None of cell count parameters gave a significant difference between Groups 1 and 2. A higher level of IgE was observed in Group 1 (507.9 ± 205.0 ME/ml) than in Group 2 (272.6 ± 77.5 ME/ml) without significant differences.

To estimate discrimination power of clinical blood parameters following parameters were chosen: total leukocyte count, PMN ratio, eosinophil ratio, PMN-lymphocyte ratio, ESR, which considered as significant markers in asthma and chronic obstructive pulmonary disease (COPD) [24,25]. ROC curve analysis of linear combination of these parameters showed a significantly high power to discriminate Group 1 from Group 3, but there was not a significant discriminative power to distinguish Group 1 from Group 2 (Table 2). The most statistically

Table 2
ROC analysis for the respiratory parameters and blood cell count.

Group 1 and Group 2			Group 1 and Group 3		
AUC ^{a)}	95% CI ^{b)}	p	AUC	95% CI	p
A linear combination of FEV₁, FVC, PEV and PEFR^{c)}					
0.72	0.54–0.84	1×10^{-1}	0.96	0.79–1.00	1×10^{-4}
FEV₁ as the most statistically significant predictor					
0.65	0.49–0.79	5×10^{-2}	0.93	0.76–1.00	9×10^{-6}
A linear combination of the blood analysis parameters^{d)}					
0.77	0.56–0.91	8×10^{-2}	0.98	0.90–1.00	2×10^{-5}
A linear combination of PMN ratio and eosinophil ratio as the most statistically significant predictors					
0.73	0.54–0.88	7×10^{-2}	0.90	0.69–0.99	2×10^{-4}

Notes: AUC^{a)} – Area Under Curve; CI^{b)} – Confidence Interval; ^{c)}FVC -forced vital capacity, FEV₁ - forced expiratory volume in 1 s, PEF - peak expiratory flow, PEFR - peak expiratory flow rate; ^{d)}a linear combination of parameters of clinic blood analysis: total leukocyte count, polymorphonuclear neutrophils (PMN) ratio, eosinophil ratio, PMNs/lymphocytes ratio, erythrocyte sedimentation rate, was taken for calculation.

significant predictors were PMN ratio and eosinophil ratio, but even their linear combination did not allow distinguishing Groups 1 and 2. Thus blood parameters did not show significant discriminative power to classify Group 1 and Group 2.

3.4. Kinetics of respiratory burst

We described the kinetics of generation of reactive radicals and their metabolites in respiratory burst as a perspective way to discriminate BA out of exacerbation and the controls [18]. Here we analyzed kinetics of respiratory burst in the whole blood to distinguish the state of moderate exacerbation from out of exacerbation. There were no significant differences between all groups on spontaneous intensity of generation of reactive species. It indicates that phagocytes in blood were not activated in all groups.

Curves of CL-responses initiated with 3.9–250 µg/ml OZ in blood samples of BA patient out of exacerbation and then under exacerbation demonstrated different kinetics: during exacerbation slope decelerated, lag-time became longer and amplitude increased (Fig. 1). Kinetics of responses was concentration dependent: slope, amplitude, production of reactive species increased, whereas lag-time and T_{max} decreased with increasing OZ concentration (Fig. 2). As a whole, kinetics parameters (lag-time, T_{max} and production of reactive metabolites) differed in Group 1 compared to Groups 2 and 3 (Fig. 2).

Lag-time of responses was significant higher in Group 1 compared to Group 2 (in all concentrations) and Group 3 (in concentrations of 7.8–31.25 µg/ml) (Fig. 2A). Slope did not differ between Groups 1 and 3, but values in Group 2 significant exceeded both in concentrations of 15.6 and 62.5 µg/ml (Fig. 2B). Although amplitude values in samples of some patients under exacerbation changed versus out of exacerbation (Fig. 1), average amplitude values did not vary significantly between groups (data are not shown). T_{max} was significant higher in Group 1 than in Groups 2 in all concentration range (Fig. 2C). Group 1 was differed from Group 3 in concentrations of 7.8–62.5 µg/ml. Production of reactive species was lower in Group 1 compared to Group 2 in concentration range of 7.8–62.5 µg/ml, the boundary concentrations of 3.9 and 250 µg/ml did not reveal differences between groups (Fig. 2D). Judging from original records, it was obvious that CL-responses in exacerbation prolonged more than 1000 s and exceeded the response duration in the state out of exacerbation (Fig. 1). We did not calculate total production of reactive species for the time over 1000 s in all groups, because this evaluation would require long-term recording exceeded the response time in Groups 2 and 3.

ROC curve analysis of OZ response kinetic parameters (lag-time, slope, T_{max} and production of reactive species) was carried out for concentration range 7.8–250.0 µg/ml. The calculation revealed that T_{max} was the most statistically significant predictor to discriminate Group 1 from Groups 2 and 3 in all concentrations (Table 4). However, slope, lag-time and production of reactive species were statistically significant predictors only in some OZ concentrations. The values of specificity and sensitivity for T_{max} at optimal points varied in ranges: 0.9–1.0 and 0.56–0.89, respectively, comparing Groups 1 and 2 (Fig. 3A); 0.8–1.0 and 0.5–0.89, respectively, comparing Groups 1 and 3 (Fig. 3B).

There was no significant correlation between T_{max} (in all OZ concentration range) and PMN or eosinophil ratio in blood ($p > 0.05$). It means that the cell count was not a reason for the change of kinetics of OZ-responses in BA exacerbation, possibly the state of cells themselves modified.

3.5. Priming test

Different phases, phenotypes of allergic asthma, or both, were associated with distinct priming profiles of granulocytes [26]. We observed primed state of granulocytes in blood of BA patients out of exacerbation [18]. Priming with 0.01 µM fMLF accelerated response to OZ

Table 3
Cell count in peripheral blood.

	Group 1, n = 18	Group 2, n = 62	Group 3, n = 43
Leukocytes, $\times 10^9$ cells/L	7.47 ± 0.45	6.6 ± 0.3	6.0 ± 0.3
Monocytes, %	6.6 ± 0.4	7.46 ± 0.71	7.3 ± 0.4
Segmented neutrophils (PMN), %	55.5 ± 2.0	52.0 ± 1.4	49.0 ± 1.3
Stab neutrophils, %	1.7 ± 0.3	1.5 ± 0.2	1.1 ± 0.1
Eosinophils, %	$2.5 \pm 0.6^{\wedge}$	2.0 ± 0.3	1.2 ± 0.1
Lymphocyte, %	$33.0 \pm 1.8^{\wedge}$	37.0 ± 1.3	41.2 ± 1.3
PMN lymphocyte ratio	$1.86 \pm 0.25^{\wedge}$	$1.82 \pm 0.14^{\#}$	1.17 ± 0.07
Erythrocytes, $\times 10^{12}$ cells/L	4.6 ± 0.1	4.6 ± 0.1	4.5 ± 0.1
Platelets, $\times 10^9$ cells/L	259.4 ± 12.1	271.3 ± 14.4	226.3 ± 10.7
Haemoglobin, g/l	135.9 ± 3.1	137.0 ± 3.8	139.4 ± 4.5
Erythrocyte sedimentation rate (ESR), mm/h	13.3 ± 2.0	9.7 ± 1.4	6.9 ± 1.3

Notes: Data are presented as mean value $\pm$ SEM. Kruskal-Wallis One Way Analysis of Variance on Ranks was applied to the comparison of three groups: $\wedge$ - differences between Groups 1 and 3 (Controls), $\#$ - between Groups 2 and 3, $p < 0.05$.

in Groups 1 and 3, but not in Group 2. Kinetic parameters as lag-time and production of reactive species were significant different between Groups 1 and 2 (Fig. 4). But they were not differed between Groups 1 and 3, indicating that phagocytes in blood of BA patients with exacerbation and the controls reacted to subthreshold concentration of fMLF contrast to patients out of exacerbation.

4. Discussion

This work was focused on search of criteria to differ the state with moderate exacerbation from out of exacerbation. Main data were obtained: 1) the respiratory parameter FEV_1 and blood PMN ratio and eosinophil ratio did not allow classifying BA patients with moderate exacerbation and out of exacerbation; 2) a delayed CL-response to OZ

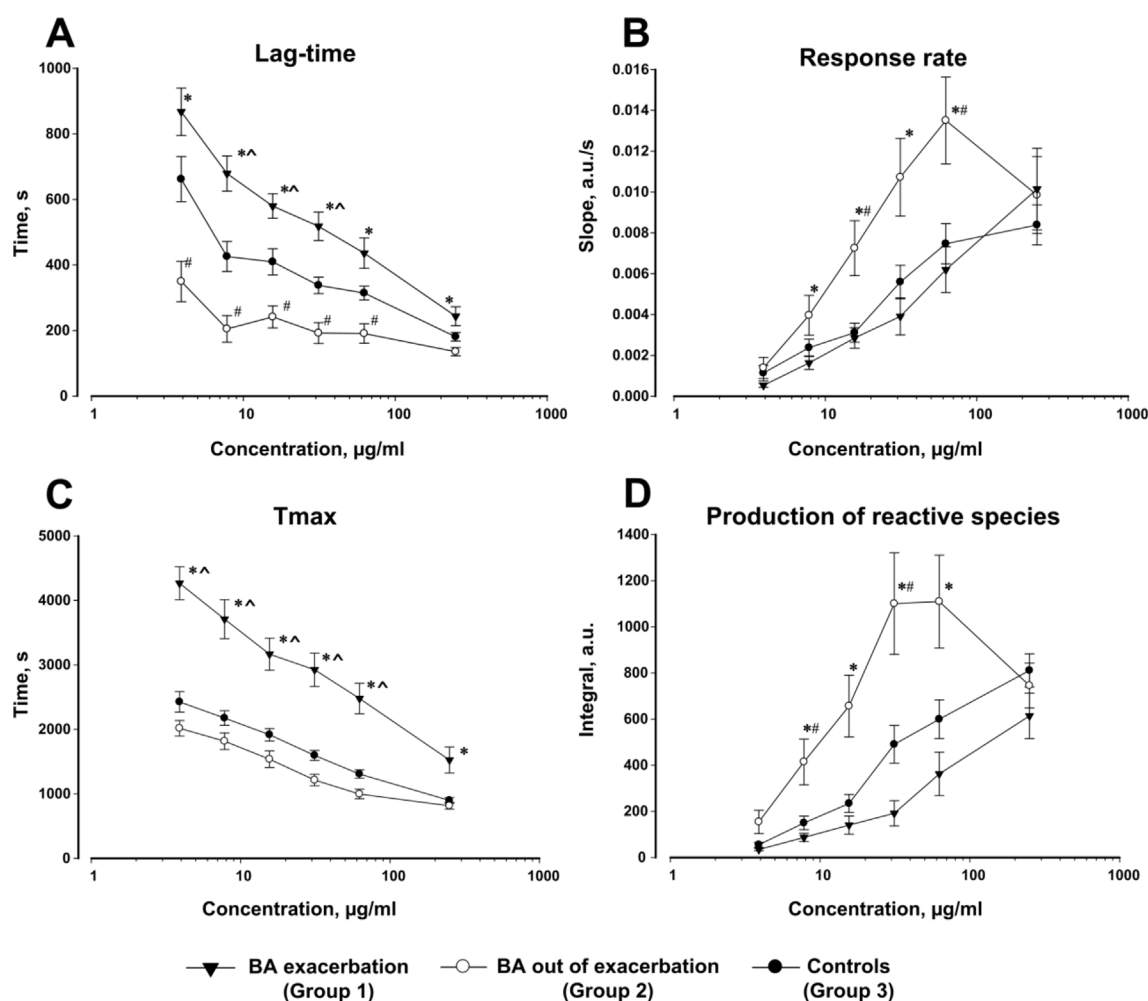


Fig. 2. Concentration dependences of kinetics parameters of phagocyte responses to opsonized zymosan in whole blood samples in bronchial asthma (BA). Kinetic parameters as lag-time (A), response rate (B), time to maximum of response (T_{max} , C) and production of reactive species (D) were calculated for individuals from Group 1 (BA patients with moderate exacerbation), Group 2 (BA patients out of exacerbation) and Group 3 (healthy individuals, Controls). Data are presented as mean and SEM. Kruskal-Wallis One Way Analysis of Variance on Ranks was applied to the comparison of three groups: $\ast$ - differences between Groups 1 and 3; $\#$ - differences between Groups 2 and 3, $p < 0.05$.

Table 4

Receiver operating characteristic (ROC) curves analysis for $T_{\max}$ as a statistically significant predictor.

Dose of OZ ^{a)} , $\mu\text{g}/\text{ml}$	Group 1 and Group 2			Group 1 and Group 3		
	AUC ^{b)}	95% CI ^{c)}	<i>p</i>	AUC	95% CI	<i>p</i>
7.80	0.96	0.79–1.00	4×10^{-7}	0.89	0.68–0.96	2×10^{-6}
15.60	0.92	0.77–0.98	8×10^{-6}	0.84	0.65–0.96	7×10^{-6}
31.25	0.94	0.78–0.99	2×10^{-6}	0.86	0.66–0.95	1×10^{-6}
62.50	0.94	0.78–0.99	1×10^{-6}	0.84	0.62–0.95	3×10^{-6}
250.00	0.69	0.48–0.85	3×10^{-4}	0.66	0.43–0.84	3×10^{-4}

Notes: OZ^{a)} – opsonized zymosan; AUC^{b)} – Area Under Curve; CI^{c)} – Confidence Interval.

was revealed in blood of BA patients with exacerbation; 3) among kinetic parameters of the response $T_{\max}$ was the most statistically significant predictor to discriminate BA exacerbation from BA out of exacerbation and the controls. Therefore kinetic parameters of the phagocyte response to OZ in the whole blood were the best predictors of BA exacerbation in comparison with respiratory parameters and blood cell count.

The importance of respiratory parameters FEV_1 and PEFR as independent predictors of severe exacerbations were confirmed [27–29]. A strong association between FEV_1 and risk of asthma attacks was shown [30]. However, these parameters are not always informative for predicting an exacerbation, especially in case of mild or moderate exacerbation [31]. Persistence of marked inflammation of airways was observed in asthmatic patients even if the disease appears to be controlled [32]. The airway obstruction and disease severity are not always precursors for exacerbation owing different pathophysiologic mechanisms [33]. We also showed that FEV_1 significantly differentiated BA patients with moderate exacerbation from the controls, but it had low discriminative power to classify BA groups (Table 2).

The ratio of blood cell count and their functioning can change depending on BA severity [4–6]. Elevated PMN-lymphocytes ratio and eosinophil count indicate the transition of the disease to a more severe form or exacerbation. Significant differences of mean values of PMN lymphocyte ratio, lymphocyte and eosinophil ratio were revealed between Group 1 and Group 3 (Table 3). However, blood eosinophils may

be associated with airway obstruction and decreased lung function regardless of asthma [34]. Our work demonstrated that PMN ratio and eosinophil ratio distinguished significantly BA patients with exacerbation and the controls. However they did not allow distinguishing BA patients with exacerbation from out of exacerbation (Table 2). Significant correlation between $T_{\max}$ and PMN or eosinophil ratio in blood was not found; therefore the cell count was not a reason for the change of the OZ-response kinetics at BA exacerbation.

Increased spontaneous and stimulated production of superoxide radicals per an individual cell was revealed in the whole blood during BA exacerbation [17]. There are data concerning changed ROS level in serum and isolated granulocytes during exacerbation, but these data are controversial [15,16]. We revealed an unchanged spontaneous ROS level and a delayed response to OZ in BA with exacerbation versus BA out of exacerbation and the controls (Figs. 1 and 2). Observed discrepancies can be explained by the use of various methods, devices and protocols for measuring of ROS level. Moreover, it is incorrect to compare data obtained on isolated cells with results obtained on whole blood samples, since in the latter case various serum components can influence ROS production and detection.

The integral CL response and the time of reach half maximal value are useful indicators in recurrent infections, myeloperoxidase deficiency, phagocytic defects and other [19]. However the kinetics of the response has not been analyzed in BA exacerbation early. We showed that such kinetic parameters as slope, lag-time, production of reactive metabolites and $T_{\max}$ varied significantly in BA patients in different states (Fig. 2). In addition, ROC curves analysis revealed that $T_{\max}$ had a high power and the greatest statistical significance for discrimination of BA exacerbation from BA out of exacerbation and the controls (Table 4, Fig. 3). It should be noted that the lowest values of ROC curve characteristics were obtained for OZ concentration of 250 $\mu\text{g}/\text{ml}$ closed to saturation for isolated human neutrophils [35], so it would be more correct to use lower OZ concentration for testing of BA state.

A state of blood granulocytes in BA exacerbation changed in comparison with BA out of exacerbation according to priming test with subthreshold fMLF concentration (Fig. 4). In general, fMLF effects in individual measurements were more scattered in Group 1 compared to other groups. The reasons could be: the heterogeneity of granulocyte population in blood [36,37]; recruitment of activated cells into lungs in BA exacerbation [16]; the release of incompetent granulocytes from

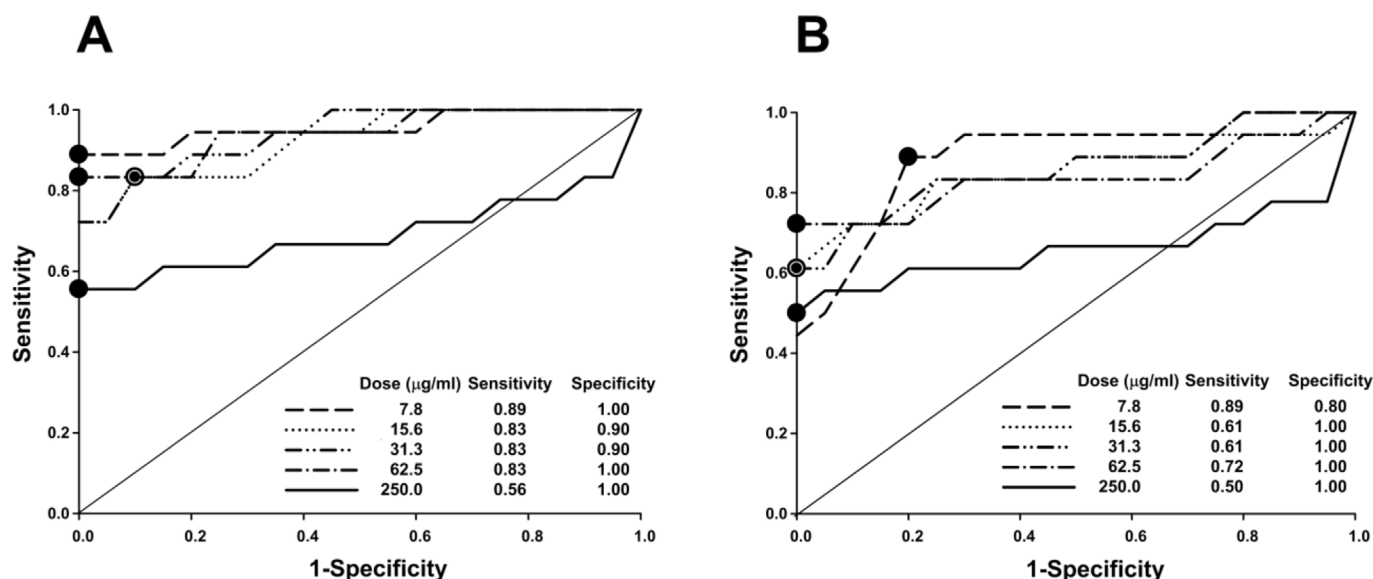


Fig. 3. Receiver Operating Characteristic (ROC) curves of responses to opsonized zymosan (OZ) for $T_{\max}$. Data were analyzed: A – Group 1 (patients with bronchial asthma (BA) with moderate exacerbation) and Group 2, (BA patients out of exacerbation); B – Group 1 and Group 3 (Controls). Concentrations of OZ ranged from 7.8 to 250 $\mu\text{g}/\text{ml}$. Positions of the optimal Cutoff Points are specified in insets for different OZ doses. Values of the area under the curve (AUC), 95% Confidence Intervals (CI) and *p*-values are listed in Table 4.

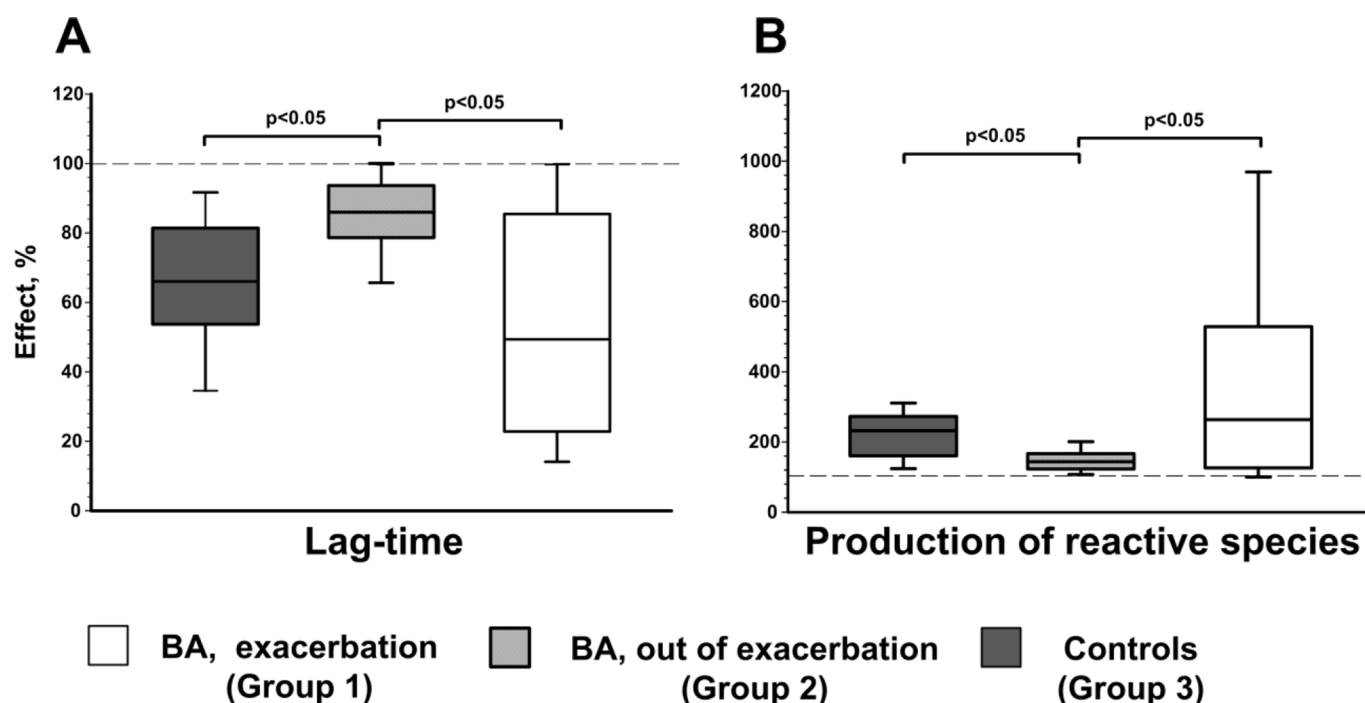


Fig. 4. The effect of bacterial peptide N-formyl-MLF (fMLF) on the respiratory response initiated with opsonized zymosan (OZ). Blood samples from patients with bronchial asthma (BA) and healthy persons were treated with 0.01 μ M fMLF for 10 min, then 31.25 μ g/ml OZ was added to induce respiratory burst. Effect was calculated as a ratio of the parameter from samples treated with fMLF to the same of intact samples, accepted as 100%. Kruskal-Wallis One Way Analysis of Variance on Ranks was applied to the comparison of kinetics parameters as lag-time (A) and production of reactive species (B) in three groups of examinees. Median and 5th–95th percentiles are shown.

bone marrow [38]; de-priming or re-entry of cells into the circulation from a primary inflammatory site [39,40].

5. Conclusions

Our work demonstrated that kinetic characteristics of respiratory burst allowed distinguishing significantly not only BA patients with moderate exacerbation from the controls but BA with moderate exacerbation from BA patients out of exacerbation. Such a parameter as T_{max} has the most discriminative power for this purpose. Therefore, we suggest that kinetic parameters of granulocyte response to microbe provocation in the whole blood samples could be a convenient test for monitoring bronchial asthma status to prevent exacerbation.

Data availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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