

# Measurement of effector properties of neutrophilic granulocytes in patients with allergic hypersensitivity to food

Magdalena Żbikowska-Gotz, Krzysztof Pałgan, Ewa Socha, Michał Przybyszewski, Andrzej Kuźmiński, Zbigniew Bartuzi

Department of Allergology, Clinical Immunology and Internal Diseases, Ludwik Rydygier *Collegium Medicum* in Bydgoszcz, Nicolaus Copernicus University in Torun, Poland  
Head: Prof. Zbigniew Bartuzi MD, PhD

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## Abstract

**Introduction:** Neutrophilic granulocytes (neutrophils) are the most important cells of the non-specific immune response. These cells have the capability of chemotaxis and phagocytosis and also participate in inflammatory processes. Stimulated neutrophils or reactive oxygen species (ROS) and elastase, important mediators of the inflammatory process responsible for tissue injury.

**Aim:** To assess oxygen metabolism as one of the representatives regarding metabolic activity of neutrophilic granulocytes measured with the chemiluminescence test (CL) and analysis of concentrations of leukocyte elastase released from neutrophils and measured in the form of complexes with inhibitor (EL- $\alpha$ 1-IP) in the serum of patients with allergic type of hypersensitivity to food.

**Material and methods:** The study included 30 patients with diagnosed food allergy on the basis of medical history, clinical symptoms, positive prick tests and the presence of allergen-specific IgE against selected food allergens in the serum. The control group contained 10 healthy volunteers. Chemiluminescence of basal neutrophils and neutrophils stimulated for 40 min (fMLP, PMA, OZ) was assessed with the kinetic luminol-dependent method using a luminometer (Luminoscan – Labsystem) and elastase concentration was estimated in the serum with the ELISA method, using Bendermed Systems kits.

**Results:** Mean values of obtained chemiluminescence from basal and stimulated neutrophils and elastase concentrations assessed in a complex with its inhibitor  $\alpha$ 1-IP were statistically significantly higher in patients with allergic hypersensitivity to food than values in the group of healthy persons.

**Conclusions:** The results of performed analyses indicate that neutrophils participate and have increased activity in the process of allergic inflammation in patients with food allergy.

**Key words:** food allergy, chemiluminescence, neutrophils, elastase.

## Introduction

Incidence of allergic reactions has significantly increased during the last several years. This problem also concerns allergic hypersensitivity to food in children, young people and adults [1-3].

The ECAP Studies (Epidemiology of Allergic Diseases in Poland) revealed that about 9% of children aged 6-7 years and about 4% of adults aged 22-44 years present symptoms after consumption of sensitizing food [4].

Diverse clinical symptoms triggered by consumption of sensitizing food can be a result of various, already well-known immune pathogenic mechanisms and can concern various organs and systems. Examinations regarding immune system function concentrate first of all on evaluation of adaptive response indicators in patients with allergic type of food hypersensitivity. It is also worth paying attention to participation of the innate immunity system, which not only initiates, but also influences and

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**Address for correspondence:** Magdalena Żbikowska-Gotz MD, PhD, Department of Allergology, Clinical Immunology and Internal Diseases, Ludwik Rydygier *Collegium Medicum*, Nicolaus Copernicus University, 7 Zbożowy Rynek, 85-116 Bydgoszcz, Poland, e-mail: magda.zb@wp.pl

forms a further specific response. It is known that complicated interactions among various cells constitute the basis of the allergic inflammatory process. Besides already confirmed participation of eosinophilic cells (Eo), also neutrophils (Ne) can substantially participate in this process, as is emphasized more and more often. Proinflammatory properties of Ne depend on their ability to produce and release many important mediators of inflammatory processes. These cells are the most important source of or reactive oxygen species (ROS) in the human organism [5, 6]. Membranous and intracellular chemical reactions that take place in the cell under the influence of various stimulators constitute the source of emitted light. The range of oxygen metabolism, which constitutes one of the components of neutrophil metabolic activity, can be assessed with the chemiluminescence test (CL). Activity of these cells is also associated with release of many proteases from lysosomal granules, among them elastase, cathepsin G, proteinase 3 and many more. Elastase (serine protease) is an enzyme of high activity and a wide activity spectrum. Increased ROS generation and release of proteolytic enzymes can happen in the case of increased neutrophil activation. This fact results in a destructive effect of these mediators on tissues when tissue defensive mechanisms are unsatisfactorily efficient [7-10].

## Aim

The aim of the study was to assess oxygen metabolism as one of the representatives regarding metabolic activity of neutrophilic granulocytes measured with the CL and analysis of concentrations of leukocyte elastase released from neutrophils and measured in the form of complexes with inhibitor (EL- $\alpha$ 1-IP) in the serum of patients with allergic type of hypersensitivity to food.

## Material and methods

The analysed group included 30 adult patients, 18 women and 12 men (mean age 41  $\pm$  8.7 years), in whom detailed diagnostics was performed to exclude diseases other than allergic diseases.

Food allergy was diagnosed on the basis of medical history, physical examination and performed laboratory diagnostics and also double-blind placebo controlled oral provocation test. Most often bloating, abdominal pain, nausea and diarrhoea occurred in the analysed patients. All patients had incidents of acute urticaria in their past medical history. Patients with exacerbated complaints associated with food allergy were qualified for analyses. The following food most often caused allergy: peanuts, celery, apple, eggs and fish. Allergy concerned more than one allergen in 8 patients. Patients with increased concentration of allergen-specific IgE (asIgE) – class  $\geq$  2 (0.70 KU/l) were qualified for the analysed group.

The reference group consisted of 10 healthy volunteers, 5 women and 5 men (mean age 37  $\pm$  6.3 years), with negative atopic past history, without symptoms of infection and who did not take any medications.

The blood for the analyses was taken from the ulnar vein using a closed Vacutainer system into a test-tube with lithium heparin with final concentration of 10 U/ml and also as clot into a test-tube that did not contain anticoagulants. Additionally basic parameters of the blood cell count were measured in all analysed patients.

Allergen-specific IgE measurement was performed with the fluoro-enzyme-immunoassay (FEIA) method on the UNICAP100 system using kits of Phadia company. Concentrations of asIgE antibodies in class  $\geq$  2 were regarded as a positive result.

Evaluation of neutrophil oxygen metabolism was performed with the chemiluminescence method (CL) intensified with luminol (5-amino-2,3 dihydrophthalazine-1,4-dione), Sigma, dissolved in 0.4% NaOH solution up to the concentration 28  $\mu$ mol/ml. Luminol is a compound that evolves into the arousal state during the process of oxidation and this fact allows significant increase of light effects. The analyses were performed using the Luminoscan Ascent system (Thermo LabSystems, Helsinki, Finland). Measurements were performed with the kinetic method for 40 min at a temperature of 37  $\pm$  1°C with CL measurement at 2-min intervals. Results were presented as integration CL values, i.e. surface area under emission curve in time function measured for 40 min and presented in RLU (relative light units).

We evaluated non-stimulated without stimulation cells and cells stimulated with formyl-methionyl-leucyl-phenyl alanine (fMLP)  $2 \times 10^{-6}$  M, phorbol myristate acetate (PMA) 200 ng/ml and opsonized zymosan (OZ) 0.33 mg/ml.

Every analysed sample contained the whole blood, a stimulator (but in the case of measurement of spontaneous chemiluminescence without a stimulator) and luminol, and was also filled up with PBS for a constant volume. The blood was added directly before reading. The readings were performed within 2 h from the moment of material collection. Every measurement was repeated twice and the mean value was calculated. Chemiluminescence values were corrected in accordance with values of haemoglobin concentration and absolute number of neutrophils and were expressed as RLU according to the formula:

$$\text{CL calculated} = \text{CL measured} \times \{ \text{Hb}[\%] / (\text{WBC} [\text{thousands}/\mu\text{l}] \times \text{PMN} [\%]) \}$$

The obtained result (RLU) was related to 1000 cells. This fact allowed us to eliminate the influence of a diverse number of neutrophilic granulocytes in the sample, thereby achieving greater optimization of obtained results.

Elastase bound with long-lasting complex with the proteinase inhibitor  $\alpha$ 1-IP (EL- $\alpha$ 1-IP) was measured in the serum with the ELISA enzymatic method using commer-

cial kits of Bender MEDSYSTEMS company. Analysis was conducted according to instructions provided by the producer. Calibration curve and concentration calculations were performed using the system and software of BIO-TECH INSTRUMENTS INC ELX 800 company.

The following statistical methods were applied: arithmetic mean estimations ( $\bar{x}$ ); estimations of standard deviation for mean ( $s$ ). Analysis of distribution form concerning analysed characteristics was performed using the Shapiro-Wilk test. Mann-Whitney  $U$  test was used to analyse significance of differences among groups whose distribution differed significantly from normal distribution (Shapiro-Wilk test  $p < 0.05$ ). Spearman correlation was used to prove interdependence among analysed variables. Statistica v. 6.0 software of StatSoft company was used in the statistical analysis.

Studies were performed with the consent of the University Bioethical Committee of *Collegium Medicum* in Bydgoszcz, Nicolaus Copernicus University [consent number KB 683/2009].

## Results

Results of studies assessing the basal and stimulated state of neutrophil activation on the basis of ROS and elastase concentration in the serum are presented in Table 1 and graphically in the Figures 1-4 (together with probability values).

Analysis of the results showed in patients with allergic hypersensitivity to food higher mean values of the CL test both for non-stimulated cells and those activated with stimulators fMLP, PMA and OZ in relation to persons from the reference group. There were statistically significantly higher CL values of basal neutrophils and chemiluminescence after PMA and OZ stimulator application in analysed patients in comparison with the group of healthy persons. However, significant differences were not found in CL quantity among analysed groups in the case of use of chemotactic peptide (fMLP).

Similarly, in the group of patients statistically significantly higher elastase concentrations were obtained in measurements with complex (EL- $\alpha$ -IP) in comparison with the group of healthy persons.

No statistically significant correlations were found in the study between complex (EL- $\alpha$ -IP) and CL-BS in the

group of patients ( $p = 0.7874$ ) and in the control group ( $p = 0.5533$ ).

## Discussion

Despite intensive studies, the pathogenesis of food allergy is still not completely explained. More and more often analyses undertake the subject regarding the possibility that neutrophils participate especially in allergic reactions to food. Neutrophilic granulocytes are cells of basic significance in the fight against pathogens. The condition of neutrophils' efficiency is the normal course of their metabolic transformations. The process of intracellular damage is associated with activation of a series of important enzymes and its consequence consists among others in production and release of active oxygen derivatives. This phenomenon is called the "respiratory burst" (or "oxidative burst") [11, 12]. This reaction is accompanied by light emission – chemiluminescence. The number of formed photons can be measured using a luminometer. Neutrophils circulating in the blood are not very active metabolically until the moment of contact with stimulating factors. Only signals transduced by many stimulators regardless of the way of their transmission can cause intensification of oxygen metabolism [13-15].

Produced oxygen compounds can disturb the metabolism of main cellular elements, can influence nuclear transcription factors and stimulate synthesis of proinflammatory cytokines. They can also cause inactivation of important proteinase inhibitors and result in a significant increase of proteolytic enzymes' effects on tissues.

Chemiluminescence in neutrophilic cells can be induced via many ways: via a chemotactic receptor (fMLP), via a receptor for Fc fragment of antibody and complement (OZ), but also via direct activation of PKC (protein kinase C) via a specific activator (PMA) [12, 16].

Assessment of cells' capacity for chemiluminescence was performed by evaluation regarding spontaneous basal chemiluminescence as well as after addition of stimulating factors. We observed in the present study increased ROS production both by basal and stimulated neutrophils of peripheral blood in patients with food allergy and clinical symptoms from various organs. Obtained CL values were significantly higher than values in the group of healthy persons.

**Tab. 1.** Results of measurements and chemiluminescence ranges of blood granulocytes depending on used stimulators

Analysed patients	Chemiluminescence CL (RLU total [40 min])				Elastase EL- $\alpha$ -IP [ng/ml]
	BS	fMLP	PMA	OZ	
Analysed group (n = 30)	$\bar{x} = 1.24$ SD = 0.76	$\bar{x} = 1.69$ SD = 0.79	$\bar{x} = 2.44$ SD = 0.86	$\bar{x} = 15.94$ SD = 8.65	$\bar{x} = 339.46$ SD = 208.06
Control group (n = 10)	$\bar{x} = 0.34$ SD = 0.14	$\bar{x} = 1.14$ SD = 0.64	$\bar{x} = 1.47$ SD = 0.61	$\bar{x} = 8.61$ SD = 2.21	$\bar{x} = 79.84$ SD = 39.01

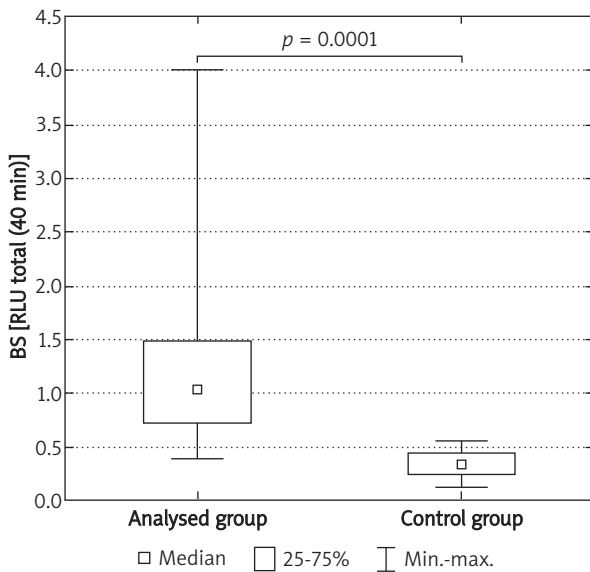


Fig. 1. Neutrophils not stimulated with BS chemiluminescence in analysed groups

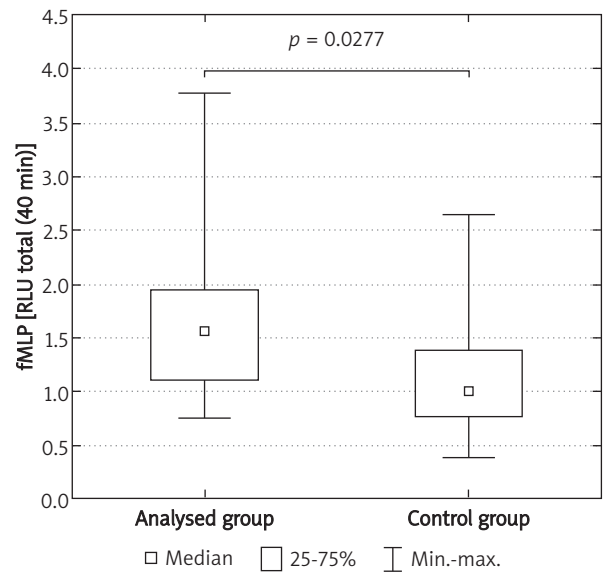


Fig. 2. Neutrophils stimulated with fMLP chemiluminescence in analysed groups

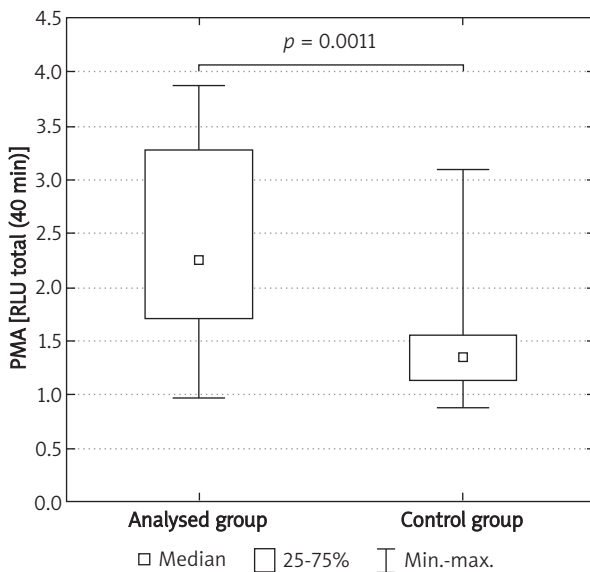


Fig. 3. Neutrophils stimulated with PMA chemiluminescence in analysed groups

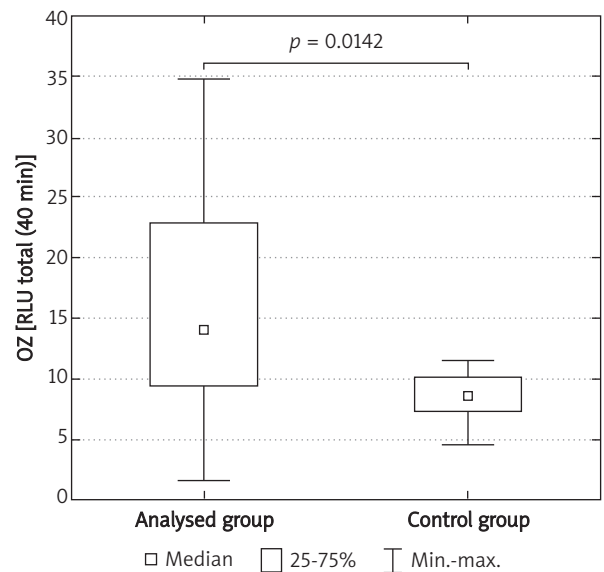


Fig. 4. Neutrophils stimulated with OZ chemiluminescence in analysed groups

Our previous studies in asthmatic patients allergic to allergens of house dust mite also showed significantly higher ROS production by granulocytes in basal circumstances and when activated by stimulants [17, 18]. Participation and importance of these mediators in inflammatory processes are also shown by studies of other authors, performed in groups of adults and children [19-24].

It was noted that neutrophils of asthmatic patients are characterized by increased ability to generate reac-

tive oxygen metabolites that can be associated with the phenomenon of pre-activation of these cells in circumstances *in vivo*. Triggering neutrophils priming can be caused by many inflammatory mediators released during allergic reactions. The result of such influence can be an excessive functional response to stimulating factors in comparison with cells that did not undergo earlier reactivation [25-27]. It seems that this situation can also occur in our own described studies.

Interesting studies were performed by Monteseirin *et al.*, who proved that anti-IgE class antibodies and specific inhaled antigens conditioning clinical symptoms in selected patients with asthma can be responsible for increased oxygen metabolism of granulocytes and its range can be modulated by specific immunotherapy [28].

Similarly to our studies, excessive ROS production by basal Ne and Ne induced by stimulators was noted in a large group of children with well-documented food allergy [13]. The same authors in subsequent reports also emphasize participation of TLR4 receptors present on neutrophilic cells, suggesting involvement of the system of innate immunity in mechanisms of allergy development. TLR receptor activation constitutes signal activating mechanisms of non-specific immunity. It causes increased synthesis of antibacterial factors and proinflammatory cytokines, and maturation of dendritic cells (increased expression of co-stimulating molecules and major histocompatibility complex (MHC) that obtain higher ability to present antigens and proper activation of acquired (specific) immunity as a result.

Wiktorowicz *et al.* direct attention to the previously unknown potential of proteins of lupine seeds for excessive induction of oxidative transformations in human neutrophil cells. Studies performed with flow cytometry confirm this feature, but the fact that the studies were performed in healthy persons is significant and worth emphasizing, because it is well known that lupine seeds are increasingly used in human nutrition [29].

The role of elastase is closely connected with the function of neutrophilic cells. Its enzymatic activity allows granulocytes to have the proper course of many physiological functions.

Elastase participates in the degradation process of membrane and protein cell components, elastin, several collagen types, fibronectin, laminin, proteoglycans, soluble serum proteins and transport proteins. Serum endogenous protein inhibitors proteases  $\alpha$ 1-inhibitor ( $\alpha$ 1-IP),  $\alpha$ 2-macroglobulin ( $\alpha$ 2-MG), elafin and secretory leukocyte proteases inhibitor (SLPI) control and are responsible for extracellular elastase activity, forming inactive complexes with it. Increased Ne activity is also associated with release of increased amounts of elastase that in the form of a complex with  $\alpha$ 1-IP is transferred to the blood. Increased concentration of these complexes in the serum or in the plasma is universally regarded as a measure of inflammatory activity and a significant indicator of neutrophilic granulocyte stimulation in the inflammatory focus [30].

Our studies revealed differences regarding elastase values assessed in a complex with  $\alpha$ 1-IP in persons showing hypersensitivity to food of allergic type in relation to analysed persons from the reference group, indicating and confirming participation of elastase in the inflammatory process triggered by allergy.

Increased elastase concentrations were observed in patients with asthma during the periods of disease exacerbation (values about 15 times higher than the upper limit of the reference value) as well as increased concentrations of this protease in the serum in the group of patients with mild, moderate and severe asthma with a tendency to increase together with degree of asthma severity [31].

Increased activity of neutrophil elastase in the serum of patients with asthma, atopic dermatitis and allergic rhinitis is found in studies in which the significance of this parameter as an important indicator occurring during the course of atopic diseases is emphasized [32].

Similarly, increase of elastase concentration measured in nasal lavage was found in patients with allergic rhinitis after stimulation with a specific allergen. Simultaneously higher concentration of analysed protease was observed in patients in comparison with healthy persons even in the case of lack of allergen stimulation. This suggests participation of neutrophils in the process of chronic rhinitis also beyond the pollen season [33].

More studies bring similar results proving that neutrophilic granulocyte stimulation with an allergen in patients with asthma resulted in increased elastase release, but it was observed only when the reaction was specific. Other antigens did not trigger a similar reaction. Also allergen stimulation of neutrophils that derive from healthy persons was not associated with increased concentrations of analysed elastase [34]. Studies of Wallaert *et al.* showed that in patients with allergic hypersensitivity to food and without symptoms of bronchial asthma, neutrophilic infiltration occurs in the airways and is associated with increased IL-8 concentration. The results of this study may confirm the conception that posits a similar immune response to allergic factors for all mucous membranes, though cells and mediators responsible for this process still remain unknown [35].

To sum up, it can be supposed that both elastase and reactive oxygen metabolites released from neutrophilic granulocytes play an important role in diseases with active inflammation caused by allergic stimulation in patients with allergic type of hypersensitivity to food. A great part of the literature is devoted to participation of eosinophilic cells in allergic reactions to food, but on the basis of our studies it is also possible to indicate increased activity of neutrophilic granulocytes and indirectly involvement of non-specific mechanisms of organism defence. This is confirmed by analysis of selected indicators of effector functions of peripheral blood neutrophils.

## Conclusions

1. Basal and stimulated neutrophils in patients with food allergy show significantly higher ability to generate reactive oxygen metabolites.
2. There were noted increased concentrations of neutrophil elastase measured in a complex with its inhibitor in the serum of analysed patients.

3. The proven increased neutrophil activity may play a significant role in the inflammatory process caused by allergenic stimulation in patients with food allergy, simultaneously indicating that non-specific mechanisms of organism defence participate in these reactions.

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