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

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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-α1-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 α1-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
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-α1-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 ±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 ≥ 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 ±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 clott 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 ≥ 2 were regarded as a positive result.

Evaluation of neutrophil oxygen metabolism was performed with the chemiluminescence method (CL) intensifying with luminol (5-amino-2,3 dihydrophthalazine-1,4-dione, Sigma, dissolved in 0.4% NaOH solution up to the concentration 28 μmol/ml. Luminol is a compound that evolves into the aroused 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 ±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 × 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 \frac{(\text{Hb} \%) / \text{(WBC \[thousands/μl\]}} \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 α1-IP (EL-α1-IP) was measured in the serum with the ELISA enzymatic method using commer-
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 and elastase concentration in the serum are presented in Table 1 and graphically in the Figures 1-4 (together with probability values).

Similarly, in the group of patients statistically significantly higher elastase concentrations were obtained in the study between complex (EL-α-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. Neutrophil 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.

### Table 1. Results of measurements and chemiluminescence ranges of blood granulocytes depending on used stimulators

<table>
<thead>
<tr>
<th>Analysed patients</th>
<th>Chemiluminescence CL (RLU total [40 min])</th>
<th>Elastase EL-α-IP [ng/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BS</td>
<td>fMLP</td>
</tr>
<tr>
<td>Analysed group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 30)</td>
<td>x = 1.24</td>
<td>x = 1.69</td>
</tr>
<tr>
<td></td>
<td>SD = 0.76</td>
<td>SD = 0.79</td>
</tr>
<tr>
<td>Control group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 10)</td>
<td>x = 0.34</td>
<td>x = 1.14</td>
</tr>
<tr>
<td></td>
<td>SD = 0.14</td>
<td>SD = 0.64</td>
</tr>
</tbody>
</table>
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 reactive oxygen metabolites that can be associated with the phenomenon of pre-reactivation 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.
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 poisons 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 allergic stimulation in patients with food allergy, simultaneously indicating that non-specific mechanisms of organism defence participate in these reactions.

References