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## Determination of immunological stress and oxygen free radical formation in white blood cells during allergen immunotherapy

### Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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### Summary

#### Background:

Airway inflammation represents the basis of respiratory allergic disease and is generally associated with increased oxidative stress. As a consequence of successful treatment leading to hyposensibilization and remission of symptoms, decrease of reactive oxygen formation is expected.

#### Material/Methods:

This preliminary study evaluates the production of oxygen free radicals in white blood cells and changes in basic immunological parameters in a cohort of 50 patients (27 females and 23 males, age 14–48 years) with upper airway allergic inflammation caused by pollens, before and during specific immunotherapy.

#### Results:

We found an unexpected significant increase in the free radical concentration during and after treatment in comparison to values before the treatment and to the control group. Statistical analysis also found significant increase of IgG3 after initial treatment and also 1 year after allergen immunotherapy. Although there were similar trends in the elevated ROS and elevated IgG3, these were not statistically significant.

#### Conclusions:

We observed changes in oxidative mechanisms in white blood cells of patients treated with AIT. Allergen immunotherapy works at a multilayer level and influences airway inflammation as well as the protective antimicrobial defense in treated patients. Further studies for understanding the mechanisms involved in oxidative stress as well as for laboratory monitoring of therapeutic approaches in allergic diseases are needed.

#### key words:

reactive oxygen species • allergy • allergen immunotherapy • monitoring

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## BACKGROUND

Oxidative stress is associated with many human diseases, especially with those including a significant inflammatory component. Airway inflammation is a typical example of such an association [1–3]. Anti-inflammatory treatment could therefore influence the oxidative stress intensity in some respiratory diseases (rhinitis, asthma, obstructive sleep apnea, chronic obstructive pulmonary disease) and contribute to its convalescence.

In allergy accompanied by immunological inflammation there are several potential sources of reactive oxygen species (ROS), mainly the respiratory burst associated with immune cell activation, arachidonic acid metabolism and NADPH oxidase [4–6]. ROS are also important second messengers generated in response to many types of environmental stress [7,8].

Allergen immunotherapy (AIT) is the only treatment that may affect the natural course of allergic disease and it may prevent the later development of asthma in patients with allergic rhinitis. AIT alleviates the allergic symptoms and also suppresses the inflammatory reaction associated with natural exposure to these allergens.

Efficacy of AIT in patients with respiratory allergy is affected by the absence of validated measured criteria.

Oxidative stress occurs as a consequence of excessive formation of ROS, including superoxide, hydrogen peroxide and hydroxyl radicals or inadequate antioxidant defense. Decreased antioxidative defense in patients with respiratory allergy have been published previously by several authors [9–11].

As a result of successful treatment leading to hyposensibilization and remission of symptoms, a decrease of reactive oxygen formation is expected. In this preliminary study we followed the production of oxygen free radicals in white blood cells and changes in basic immunological parameters in a cohort of patients with upper airway allergic inflammation before and during specific immunotherapy.

## MATERIAL AND METHODS

### Patients

Fifty patients (27 females and 23 males, aged 14–48 years) with respiratory allergy caused by pollens (seasonal allergic rhinitis, seasonal allergic rhinoconjunctivitis and seasonal stable bronchial asthma) were enrolled in this study (Table 1). The beginning of the study was after the pollen season. Patients with clinically manifest symptoms of bronchial asthma, atopic dermatitis or combination of these diagnoses were excluded. Eight healthy subjects (4 females, 4 males, aged 23–48 years, without any allergic condition) served as controls. All included patients were non-smokers.

All patients had pollen allergy diagnosed according to their history, clinical evaluation, positive skin prick tests and the diagnosis was confirmed by specific IgE assay positive for at least 1 principal allergen. Before specific allergen immunotherapy all of them received symptomatic treatment in an outpatient unit for at least 1 year.

**Table 1.** Basic characteristics of patients.

Number of patients	50
Average age (years)	23.4
Range (years)	14–48
Sex (m/f)	23/27
Seasonal allergic rhinitis	37
Seasonal allergic rhinitis with chronic cough	8
Seasonal allergic rhinitis with stable bronchial asthma	5

### Treatment protocol

Subcutaneous allergen immunotherapy started at the end of the pollen season (middle October) with standard initial treatment regimen lasting 15–17 weeks. Treatment regimen was arranged as a prospective open randomized study. After the initial stage, therapy was continued with maintenance doses applied every 4–6 weeks for the entire period of the study. Peripheral blood samples were taken outside the pollen season (start – middle October (M0), end of initial treatment – before the pollen season – January/February (M3), end of study – middle October next year – end of pollen season (M12)). Effectiveness of the therapy was evaluated by the visual analogue scale according to Wewers and Lowe [12]. The patients mark their own perception of respiratory symptom severity (nasal allergic symptoms, asthma symptoms) in arbitrary units (0 = no complaint, 10 = intolerable complaints) on a 10-point horizontal scale.

The study was approved by Institutional Ethics Committee of the Medical School, Safarik University in Kosice and all patients gave informed consent.

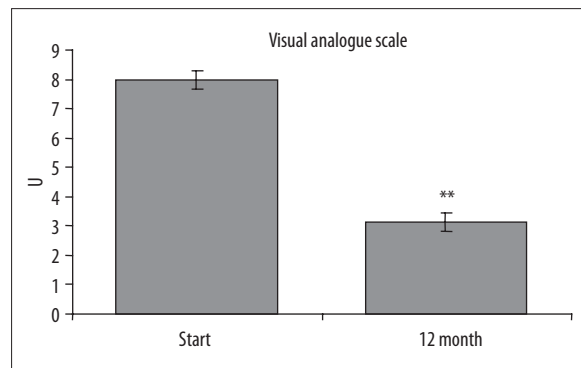
### Estimation of ROS in white blood cells

White cell lysates were prepared according to the standard dextran sedimentation method as described in Procházková and John [13] with slight modifications (60 min sedimentation on dextran instead of 45 and a longer red cell lysis time as compared with the original protocol).

Oxygen free radical concentration from white cell lysates was assayed before the beginning of the therapy, after the standard initial treatment period (3 months) and at the end (1 year) of the treatment by an indirect colorimetric method [14], commercially available as Free Radicals from Sevapharm (Czech Republic). The method is based on the ability of chlorophyllin (sodium-copper salt of chlorophyll) to transfer and accept electrons from the sample, resulting in an increase of the absorbance at 450 nm. The results are calculated according to a calibration curve with known concentrations of  $\text{Fe}^{2+}$  (an electron donor) and expressed as  $\text{mmol/l Fe}^{2+}$ .

### Other assays

Immunoglobulins A, G, M, total IgE, subclasses IgG were analyzed with Roche kits on Cobas Integra 800, IgG subclasses



**Figure 1.** Visual analogue scale in M12 ( $p < 0.01$ ). (U – arbitrary units – see text).

with the Binding Site kits (Great Britain) on a Cobas Mira S analyzer and specific IgE with EAST Hycor kits on Hytec 288 (Hycor Biomedicals, Germany).

**Statistical methods**

Statistical analysis was performed using the STATSDIRECT (version 2.5.2) software package. In addition to basic descriptive statistics, Shapiro-Wilk test, paired T-test, one-way ANOVA and methods of linear regression and correlation were used.

**RESULTS**

Specific immunotherapy led to an overall and significant decrease of symptoms characteristic of upper airway allergic inflammation, measured by the visual analogue scale (Figure 1). We found a significant increase in the free radical concentration during and after treatment in comparison to values before the treatment and to the control group (Table 2). However, from the broad dispersion of the data in the 2<sup>nd</sup> and 3<sup>rd</sup> measurement, it is obvious that the increase was not general. In Table 3 there is an evaluation of the data according to the increase or decrease of the free radical concentration by more than 0.5 units.

Total IgE levels were increased in the third month after beginning the therapy and had a tendency to return to the initial levels thereafter. Concentrations of IgM, IgA, IgG and

**Table 2.** Free radical concentration in white blood cells (n=50).

Group	FR, $\mu\text{mol/l}$	Significance
Controls (n=8)	1.68±0.65	
Before treatment (M0)	1.60±0.34	
After 3 <sup>rd</sup> month (M3)	2.02±0.80	M0/M3 – $p < 0.05$
After 1 year (M12)	2.92±0.61	M0/M12 – $p < 0.01$ M3/M12 – $p < 0.05$

**Table 3.** Changes in free radical concentration during and after immunotherapy (n=50).

Change during treatment	M3	M12
Increase $> 0.5 \mu\text{mol/l}$	24 (48%)	28 (56%)
No change	19 (38%)	17 (34%)
Decrease $> 0.5 \mu\text{mol/l}$	7 (14%)	5 (10%)

subclasses of IgG were in the normal range in all patients during the whole treatment period and did not show any significant changes, except of IgG3 (Table 4). Statistical analysis found a significant increase of IgG3 after initial treatment and also 1 year after allergen immunotherapy. Linear correlation (before and after the treatment) in ROS and IgG3 was statistically significant ( $p < 0.01$ ). Although there were similar trends in the elevated ROS and elevated IgG3, these were not statistically significant.

**DISCUSSION**

Although allergic mechanisms such as the importance of Th1 and Th2 cells, cytokine regulation of the immune response and specific inhibition or ablation of pathogenic immune responses by means of tolerance induction are well studied, relatively little is known about mechanisms of oxidant/antioxidant metabolism that can modify the course of allergic diseases. Allergen immunotherapy is the only standard therapy that influences the basic mechanisms of allergy.

**Table 4.** Basic immunological parameters (n=50, \*\*  $p < 0.01$ , \*  $p < 0.05$ ).

Parameter	M0 (g/l)	M3 (g/l)	M12 (g/l)
IgE	299.6±64.4	326.9±73.2**	250.6±58.5**
IgM	1.34±0.69	1.29±0.63	1.33±0.67
IgA	2.38±0.88	2.36±0.87	2.35±0.89
IgG (total)	11.15±2.42	11.44±2.21	11.54±1.79
IgG1	5.71±1.41	5.62±1.35	5.8±1.12
IgG2	4.21±1.59	4.15±1.49	4.1±1.5
IgG3	0.77±0.32	0.87±0.33**	0.88±0.41*
IgG4	0.46±0.34	0.46±0.33	0.41±0.24

However, the specific mechanisms by which allergic inflammation is attenuated by this type of therapy are still unclear.

Direct assessment of free radical production under common clinical laboratory conditions is not possible. The colorimetric method is based on the ability of chlorophyllin to accept electrons, with subsequent change of its absorbance. According to Votruba et al. [14] the results of this simple and indirect method are in good agreement with direct electron paramagnetic resonance (EPR) measurements. The method is designed to carry out measurements from blood serum or urine, but according to the manufacturer, measurements from other types of biological material are also possible. This is the first report on the application of the colorimetric free radical assay in white cell lysates in general and specially for monitoring therapy effectiveness in allergic patients.

We observed changes in oxidative mechanisms under the AIT, and in concordance with the previously published report [10] we postulate that AIT works at multilayer levels and that it influences allergic inflammation.

In this preliminary study we observed an unexpected increase of the free radical formation in about half of the patient treated by specific immunotherapy. However, this increase is not necessarily in conflict with the apparent better clinical outcome as judged from the results of the visual analogue scale. The notion that disease is always associated with increased free radical production and decreased antioxidant system activity, and that successful therapy ameliorates these changes, is a mechanistic simplification. Mates et al [4] in a similar study observed an increase of 3 antioxidant enzyme activity (manganese, zinc-copper superoxide dismutase and catalase) in white cells of patients with airway allergic disease but a decrease of another key antioxidant enzyme, selenium glutathione peroxidase. Another interesting possibility arises from the observation of Boldogh et al. [15] and Dharajiya et al. [16] who found that reactive oxygen species could be generated through NADPH oxidase present in pollen. Both treatment regimens used in this study were realized by chemically modified pollen particles, and the possibility of persisting enzyme activity cannot be excluded.

We have no reasonable explanation for subgroups with "no change" and "decreased" in Table 3. Such results need further investigation in a larger and more exactly defined prospective study. Hypothetically, oxidative stress is thought to be associated with many environmental and physiological factors such as genetics, weight, antioxidant micronutrient supplementation, age, passive smoking and unknown conditions and comorbidities. From this point of view it is evident that our patient group is not homogenous. On the other hand, as can be seen from the basic features of the treated group, our patients were quite young (average age of 24 years) and apart from their allergic disease they are in good general condition. Even so, the relationship between allergen immunotherapy and oxidative stress might be influenced by these variables.

Total IgE levels rose immediately after the onset of AIT, followed by a gradual decrease as the therapy was continued. The first mention of such change was already suggested by

Sherman, Stull, and Cooke in 1940 and was confirmed after the discovery of IgE [17].

IgG 3 subclass deficiency is closely associated with recurrent upper and lower respiratory infections, particularly in patients with respiratory allergy [18,19]. IgG3 is the predominant subclass involved in primary immune response to viral antigens, to *M. Catarrhalis* and *S. pyogenes* [20,21], as well as atypical pathogen infections and the exacerbations of asthma symptoms [19]. We did not find IgG3 deficiency, but the statistically significant increase after allergen immunotherapy could support several clinical findings that patients after such treatment better tolerate respiratory infections, not only due to suppression of allergic Th2 inflammation, but also due to changes in immune regulation (increased ROS in leukocytes, increased IgG3). Increased ROS support phagocytic activity of leukocytes, and IgG3 binding to human alveolar macrophages improves a possible benefit of IgG3 antibodies in antibody-mediated phagocytosis of pathogens [22]. In addition, while allergen immunotherapy is thought to normalize the Th1/Th2 balance by depressing Th2, such a Th1 immune response should be associated with production of the IgG2a, IgG2b and IgG3 [23].

## CONCLUSIONS

The results of our study show that oxidant/antioxidant metabolism could modify the course of allergic diseases. We observed changes in oxidative mechanisms in white blood cells of patients treated with AIT. Allergen immunotherapy works at multilayer levels and influences airway inflammation as well as the protective antimicrobial defense in treated patients. Due to the limited number of patients in the study, we are not able to confirm an additive effect of our results for creating laboratory monitoring criteria for allergen immunotherapy. Further investigation of the mechanisms involved in oxidative stress for laboratory monitoring of therapeutic approaches in allergic diseases is needed.

## REFERENCES:

1. Pires KM, Valença SS, Resende ÂC et al: Grape skin extract reduced pulmonary oxidative response in mice exposed to cigarette smoke. *Med Sci Monit*, 2011; 17(8): BR187-95
2. Volná J, Kemlink D, Kalousová M et al: Biochemical oxidative stress-related markers in patients with obstructive sleep apnea. *Med Sci Monit*, 2011; 17(9): CR491-97
3. Szlagatys A, Korzon M, Malczyńska T et al: The effect of anti-asthmatic treatment on oxidative stress intensity and antioxidative capacity in blood of children with asthma. *Med Sci Monit*, 2003; 9(Suppl.4): 52-55
4. Mates JM, Segura JM, Perez-Gomez C et al: Antioxidant enzymatic activities in human blood cells after an allergic reaction to pollen or house dust mite. *Blood Cells Mol Dis*, 1999; 25(2): 103-9
5. Mates JM, Perez-Gomez C, Blanca M: Chemical and biological activity of free radical 'scavengers' in allergic diseases. *Clin Chim Acta*, 2000; 296(1-2): 1-15
6. Bowler RP, Crapo JD: Oxidative stress in allergic respiratory diseases. *J Allergy Clin Immunol*, 2002; 110(3): 349-56
7. Gutteridge JM: Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin Chem*, 1995; 41(12): 1819-28
8. Valko M, Leibfritz D, Moncol J et al: Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem*, 2007; 39(1): 44-84
9. Zhang SF, Cheng LL, Huang DN: Effect of kebimin decoction in treating allergic rhinitis and on blood levels of nitric oxide and superoxide dismutase. *Zhongguo Zhong Xi Yi Jie He Za Zhi*, 2004; 24(2): 118-20

10. Lukan N, Racz O, Mocnejova I, Tkac I: Monitoring antioxidant enzymes in red cells during allergen immunotherapy. *J Physiol Biochem*, 2008; 64(2): 143–48
11. Sadowska-Woda I, Bieszczad-Bedrejczuk E, Rachel M: Influence of desloratadine on selected oxidative stress markers in patients between 3 and 10 years of age with allergic perennial rhinitis. *Eur J Pharmacol*, 2010; 640(1–3): 197–201
12. Wewers ME, Lowe NK: A critical review of visual analogue scales in the measurement of clinical phenomena. *Res Nurs Health*, 1990; 13(4): 227–36
13. Procházková J, John C: Vybrané diagnostické metody lékařské imunologie. Praha: Avicenum, 1986: 197–98 [in Czech]
14. Votruba M, Stopka P, Hroudová J et al: A simple method for quantitative estimation of free radicals in serum. *Klin Biochem Metab*, 1999; 7(3): 96–101
15. Boldogh I, Bacsí A, Choudhury BK et al: ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. *J Clin Invest*, 2005; 115(8): 2169–79
16. Dharajiya NG, Choudhury BK, Bacsí A et al: Inhibiting pollen reduced nicotinamide adenine dinucleotide phosphate oxidase–induced signal by intrapulmonary administration of antioxidants blocks allergic airway inflammation. *J Allergy Clin Immunol*, 2007; 119(3): 646–53
17. Lichtenstein LM, Ishizaka K, Norman PS et al: IgE antibody measurements in ragweed hay fever. Relationship to clinical severity and the results of immunotherapy. *J Clin Invest*, 1973; 52(2): 472–82
18. de Moraes Lui C, Oliveira LC, Diogo CL et al: Immunoglobulin G subclass concentrations and infections in children and adolescents with severe asthma. *Pediatr Allergy Immunol*, 2002; 13(3): 195–202
19. Kim JH, Park HJ, Choi GS et al: Immunoglobulin G subclass deficiency is the major phenotype of primary immunodeficiency in a Korean adult cohort. *J Korean Med Sci*, 2010; 25(6): 824–28
20. Goldblatt D, Scadding GK, Lund VJ et al: Association of Gm allotypes with the antibody response to the outer membrane proteins of a common upper respiratory tract organism, *Moraxella catarrhalis*. *J Immunol*, 1994; 153(11): 5316–20
21. Ferrante A, Beard LJ, Feldman RG: IgG subclass distribution of antibodies to bacterial and viral antigens. *Pediatr Infect Dis J*, 1990; 9(Suppl.8): S16–24
22. Naegel GP, Young KR Jr, Reynolds HY: Receptors for human IgG subclasses on human alveolar macrophages. *Am Rev Respir Dis*, 1984; 129(3): 413–18
23. Germann T, Bongartz M, Długoska H et al: Interleukin-12 profoundly up-regulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses *in vivo*. *Eur J Immunol*, 1995; 25(3): 823–29