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The usefulness of CD203c expression measurement on basophils after activation with grass pollen and *Dermatophagoides pteronyssinus* antigens. Preliminary study

Abstract

Introduction: 'Gold standard' in the diagnosis of atopic disease are skin prick tests and specific IgE evaluation. Well-established *in vitro* tests, such as the histamine release test, the leukotriens release test and the flow cytometric basophil activation test can be very helpful in diagnostics, especially when the skin prick test is contraindicated.

The aim of this study was to evaluate the usefulness of antigen CD203c expression, as a marker of basophil activation by grass pollen or *D. pteronyssinus* antigens.

Material and methods: Peripheral blood from 13 allergic patients and nine healthy volunteers was analysed. Basophils activation was measured by the breakdown of antigen CD203c expression with Allergen Kit (Beckman Coulter), using Cytomics FC 500 flow cytometer (Beckman Coulter).

Results: The sensitivity was 92,3% and specificity of test was 100%. $50.95 \pm 15.7\%$ of basophils (median 49.7%, 1.91–72.42%) were activated after grass pollen stimulation in atopic patients sensitised to this allergen, in comparison to 1.91% (0.00–7.96%) in control patients ($p = 0.002$). The percentage of activated basophils after *D. pteronyssinus* antigens stimulation was $40.6 \pm 25.2\%$ in allergic patients, compared to only $2.51 \pm 1.96\%$ of basophils from non-atopic controls ($p = 0.0003$). Basophils from 21 patients responded after anti-IgE stimulation ($44.1 \pm 18.9\%$), and none of the analysed samples was activated after PBS stimulation ($2.03 \pm 1.19\%$, $p < 0.0001$).

Conclusions: These results demonstrate that basophil activation test based on antigen CD203c expression is very accurate in the diagnosis of atopic diseases.

Key words: CD203c, basophils, atopic disease, grass pollen, *D. pteronyssinus*

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Introduction

One quarter of the Polish population suffers from allergic rhinitis [1]. The frequency of atopic diseases has been increasing since the last century [2–4]. The 'gold standard' for atopic disease diagnostics are the skin prick test and evaluation of specific IgE concentration [5]. Grass pollen, house dust mites, nuts, drugs and insect venom are the allergens which may cause a life-threatening allergic response, including anaphylaxis [6, 7]. *In vivo*

diagnostics (skin prick test and provoking test) of allergy in those cases may have negative output. The best solution may be *in vitro* tests, which are safe for patients.

Basophils and mast cells are the main effector cells responsible for IgE-dependent reactions. High affinity receptor for IgE immunoglobulines (FcεRI) are present on the surface of those cells. When receptors bind IgE cross-linked with allergens, intracellular chains cross over and the cell degranulates. A large panel of mediators, including cytoki-

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nes and histamine, is released into extracellular space due to the degranulation process. In addition, changes in the expression of basophil surface molecules are observed: some antigens appear *de novo* (CD63, CD107a), while others increase their expression (CD13, CD203c). Histamine and cytokines release, as markers of basophils activation, can be measured with *in vitro* methods. Unfortunately, these methods are time-consuming, laborious and sometimes their results are not in line with the clinical picture. As cell surface antigens can be detected with monoclonal antibodies conjugated with fluorochromes, not such a time-consuming method as mentioned above, the flow cytometric test can be an alternative [6, 7].

The CD203c antigen is one of the most important markers of basophil activation. This protein (ectonucleotide pyrophosphatase/phosphodiesterase 3, E-NPP3, PD- β , B10, gp130^{RB13-6}) belongs to the type I phosphodiesterase/nucleotide pyrophosphatase family [7]. Its physiological function is unknown. The CD203c was found exclusively on basophils and mast cells. Its expression increases after basophils degranulate and is a reliable marker of a basophil's activation in IgE-dependent process. Resting basophils are characterised by low expression of this protein and the expression rapidly increases when activated. It indicates that intracellular magazines of CD203c antigens are present in cytoplasm [8, 9].

The aim of this paper is to evaluate CD203c antigen expression on basophils in atopic asthma patients after *in vitro* stimulation with *Dermatophagoides pteronyssinus* and grass pollen antigens.

Material and methods

Patients

Thirteen individuals suffering from atopic disease (aged 6–16 years), which was confirmed with a skin prick test, shortlisted for specific immunotherapy, served as the studied group. Nine healthy subjects with no history of allergy to house dust mites or grass pollen and no sensitisation to those allergens' extracts (negative skin prick test and absence of specific IgE) served as the control. Atopic and control subjects were matched for age and sex. Atopic individuals were patients of the Department of Pediatric Pneumology and Allergology, Medical University of Warsaw. None of the analysed subjects was treated with antihistamine drugs or oral corticosteroids. 1 ml blood taken by venipuncture in tubes containing EDTA was collected from the 22 participants. Tests were performed before specific sublingual immunotherapy. The experiments were approved by the Ethics Commis-

sion of the Medical University of Warsaw. Blood was collected with parents' approval.

Basophil's activation tests

All tests were carried out within two hours of blood collection. Anticoagulated blood was used for complete blood count analysis. A residual sample (400 μ l) was used for basophil's activation test. CD203c induced expression was evaluated using the Allergenicity Kit (Beckman Coulter) according to the manufacturer's instructions. Allergens (Stalergenes, France) used for the test were prepared in 1:500 dilution of concentration used for skin prick tests in phosphate buffered saline (PBS).

Briefly, EDTA-anticoagulated peripheral blood aliquots (100 μ l) stained with 20 μ l of mixture of monoclonal antibodies (CRTH₂-FITC, CD203c-PE, CD3-PC7) and Activation solution (100 μ l) were stimulated (37°C) for 15 min with 20 μ l of optimal dilution of allergens; antibody directed against the high affinity IgE receptor (Fc ϵ RI) (Beckman Coulter) was used as a positive control and PBS as a negative control. After incubation, the reaction was stopped with Stop solution. Erythrocytes were lysed with Lysing solution for 10 minutes at room temperature (RT). Suspension was centrifuged (5 min, 300 g) after lysing, washed with PBS, once more centrifuged and resuspended in 500 μ l Fixative solution. Leukocytes were analysed using a five-colour flow cytometer (Cytomics FC500, Beckman Coulter). In forward scatter/side scatter dot plot basophils were localised in gate A (Fig. 1). During acquisition, basophils were selected as CD203c positive/CRTH₂ high/CD3 negative population using FL1/FL2 and SS/FL5 dot plots. In negative control, the threshold for positivity was set at less than 5% of activated cells according to the literature data [3, 7]. In positive control, the sensitivity for IgE dependent reaction was verified. The threshold for po-

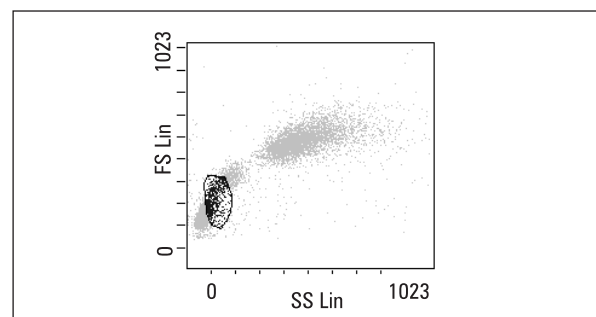


Figure 1. Location of the basophil's population at FS/SS cytogram of peripheral blood. Cells are placed in "E" region between lymphocytes and monocytes populations

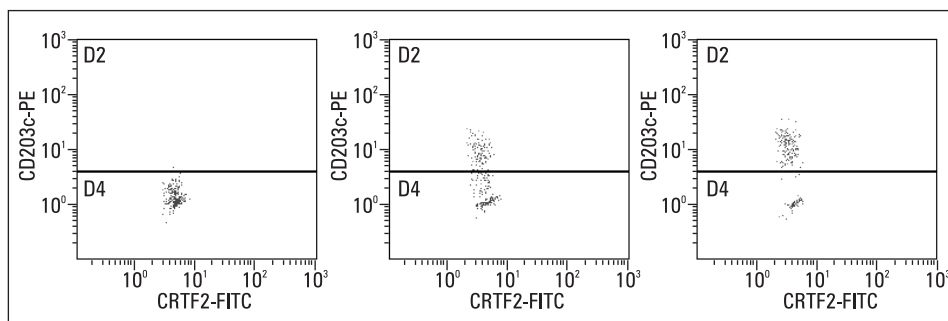


Figure 2. Basophil's activation after grass pollen antigens stimulation in atopic patient. Gating was set with CD3/CRTF2/CD203c protocol. Resting basophil are presented at the first cytogram, AntyIgE stimulated at the second and grass pollen antigens activated at the third

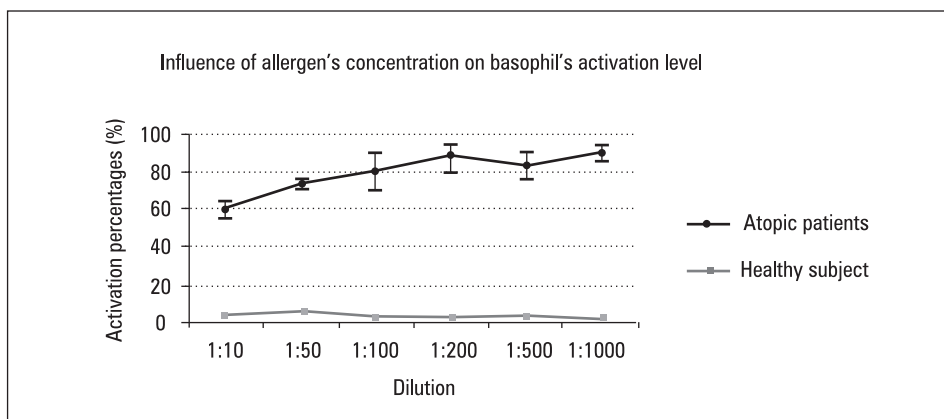


Figure 3. Influence of analyzed allergens concentration (1:10–1:1000) on basophil activation level in atopic and nonatopic subjects

sitive reaction was settled at less than 15% of activated cells, according to literature data [7, 10] and investigator studies results (Fig. 2).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U test for unpaired data. A p value of less than 0.05 was considered significant. The sensitivity was calculated as a ratio between the number of atopic patients with positive result of basophil activation test and the total number of atopic individuals. The specificity was calculated as a ratio between the number of healthy individuals with negative basophil activation test and the total number of healthy subjects.

Results

In the pilot study, the stimulatory properties of glycerol (diluent for allergens) was examined. The percentage of activated basophils preincubated in glycerol solution was 4.8%, in comparison to 3.85% in saline. This demonstrates that glycerol does not cause basophil activation. Different allergens dilution was examined as well (1:10, 1:50,

1:100, 1:200, 1:500 i 1:1000) to select optimal dilution. Preliminary study was performed with blood from six adult volunteers, including three allergic to grass pollen and house dust mites and three non atopic individuals. None of the examined dilution caused unspecific basophils activation in healthy individuals, and all of them caused basophils activation in atopic individuals (Fig. 3).

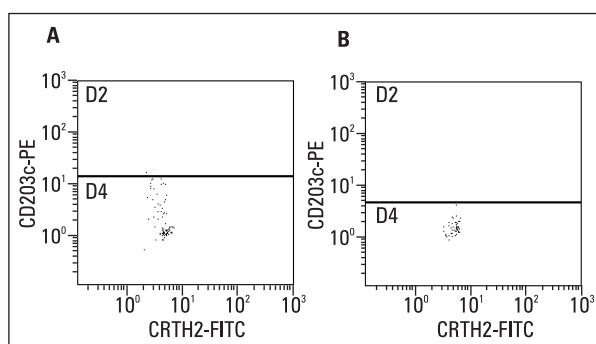
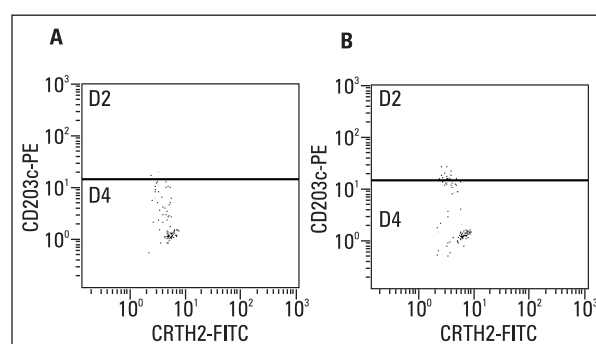
In the study group six (27%) of the studied individuals were sensitised to grass pollen, two (9%) were sensitised to *D. pteronyssinus* antigens, five (23%) were sensitised to both allergens and nine (41%) were non-atopic. Results are shown in Table 1.

Basophil's activation test was negative in one atopic patient and all nine nonatopic individuals. The test was positive in 12 atopic patients. According to these data sensitivity of the test was 92.3% and specificity was 100% for both of the allergens. Sensitivity of the test for grass pollen allergen's stimulation was 100% and for *D. pteronyssinus* antigens sensitivity was 87.5%.

Basic CD203c antigen expression differed between analysed individuals. In 12 atopic patients, basic activation level was similar (mean fluorescence 6.46 ± 2.27) but in one case (number 10) this

Table 1. Percentages of activated basophils after PBS, anti-IgE, grass pollen antigens and *D. pteronyssinus* antigens stimulation. Patients 1–13 are atopic, patients 14–22 — healthy subjects

Patient	% of activated cells in negative control	% of activated cells in positive control	% of activated cells after grass pollen antigens stimulation	% of activated cells after <i>D. pteronyssinus</i> stimulation
1	2.67	46.79	63.87	62.72
2	2.7	43.67	43.49	39.74
3	0.99	48.31	46.49	2.36
4	3.71	56.12	57.4	20.07
5	1	22.73	52.91	3.67
6	1.28	22.53	39.68	50.63
7	2.45	37.96	56.02	24.88
8	2.34	50.71	72.42	1.75
9	1.46	42.31	35.75	–
10	1.69	15.8	20.99	1.32
11	5.04	46.82	71.48	4.57
12	0.87	57.89	–	78.71
13	1.42	4.47	1.91	7.63
14	3.85	89.02	–	4.38
15	2.49	20.83	0.97	0.96
16	3.23	50.23	3.36	1.89
17	0.44	43.17	0.48	1.9
18	1.87	49.77	0.89	0
19	2.22	75.93	2.24	3.56
20	1.38	42.8	0	0.47
21	0.01	53.05	7.96	1.12
22	1.56	48.32	6.49	7.23

**Figure 4.** The spontaneous basophil's activation level from atopic patient with high CD203c expression before allergen stimulation (A) and from atopic patient with low CD203c expression (B)**Figure 5.** High level of spontaneous basophil activation (A) causes low percentage of activated cells after IgE stimulation (B)

level was incomparably high (mean fluorescence 19.3) (Fig. 4). When threshold for negative control was set at < 5%, activation level in positive control and tube with allergen were rather low (respectively 15.8% and 21%) (Fig. 5), compared with average results, respectively $45.9 \pm 17.1\%$ for positive control and $50.9 \pm 20.7\%$ for allergen stimulated sample.

Basophils from 21 individuals increased CD203c expression after anti-IgE stimulation ($44.1 \pm 18.9\%$), none of the analysed samples increased CD203c antigen expression after incubation with PBS ($2.03 \pm 1.19\%$), $p < 0.0001$.

Among atopic patients there was one case of non-responder to allergens solution (*D. pteronyssinus*).

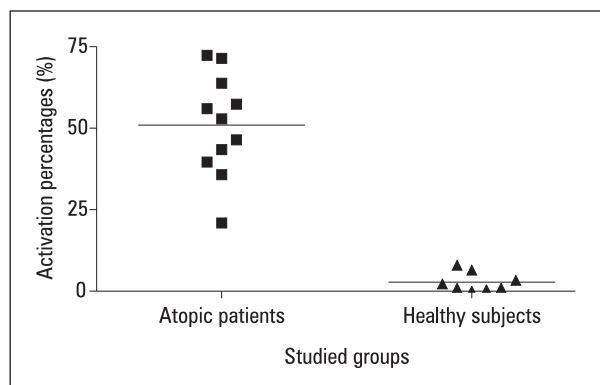


Figure 6. Comparison of percentages of basophils activated with grass pollen antigens in atopic and healthy subject, $p = 0.0003$

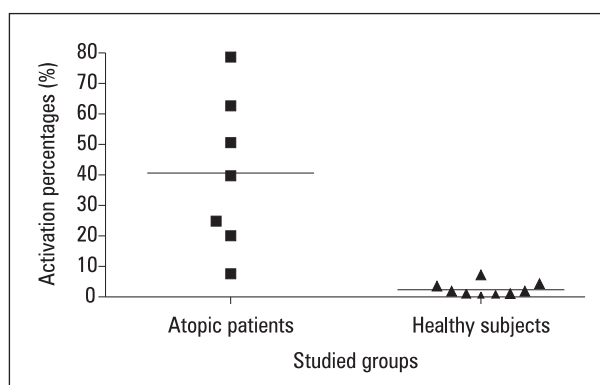


Figure 7. Comparison of percentages of basophils activated with *D. pteronyssinus* antigens in atopic and healthy subject, $p = 0.0002$

sinus) (number 13), despite the positive result of specific IgE evaluation.

There was a significantly relevant difference ($p = 0.0008$) between test results from non-sensitised and sensitised to grass pollen groups. 114–880 basophils were analysed (median 247 cells). Mean percentage of activated basophils after grass pollen antigen's stimulation for sensitised individuals was $46.86 \pm 20.9\%$ (median 49.7%, range 1.91–72.42%), for non sensitised median was 1.91% (0.00–7.96%) (Fig. 6).

79–1145 basophils (median 256 cells) were analysed after stimulation with *D. pteronyssinus* allergens. The mean percentage of activated basophils after *D. pteronyssinus* antigens' stimulation in samples from sensitised individuals was $40.6 \pm 25.2\%$, whereas from non-sensitised it was $2.39 \pm 2.29\%$, $p = 0.0002$ (Fig. 7).

Discussion

The aim of the study was to evaluate the expression of CD203c on basophils from children sensitised to grass pollen or house dust mites and

from healthy individuals. Recent data suggests that CD203c is a specific marker for human basophils and is up-regulated in response to FcεRI cross-linkage [6]. A number of previous studies compared the usefulness of CD203c and CD63 antigen expression in evaluation of basophils activation after specific allergen's stimulation [3, 7, 10, 11]. However, the CD63-based test appears to have several limitations.

Firstly, CD63 is not a basophil-specific marker (its expression was detected on blood platelets). Secondly, the test based on CD63 evaluation is less specific and less sensitive with regard to most tested allergens, including grass pollen and house dust mite. It was reported that specificity and sensitivity of basophil activation test after inhalant allergens stimulation was around 90%, and correlation factor of tests based on CD203c or CD63 was 0.76 [8]. Sensitivity of the test based on CD203c expression in diagnosis of latex allergy was 75% in comparison to 50% of test based on CD63 expression [7]. Sensitivity of the test based on CD203c in diagnosis of wasp venom hypersensitivity was higher than test based on CD63 (respectively 97% vs. 89%) [10]. Whereas the inverse tendency was observed in anaesthetic drug allergy, sensitivity of the test based on CD203c was 36% comparing to 79% for the test based on CD63 [2].

In the present paper we assessed CD203c antigen expression usefulness in grass pollen and house dust mite allergy, because of high prevalence and frequency of atopic reactions caused by those allergens. Sensitivity and specificity of the test using grass pollen allergen was 100%. Sensitivity of the test based on *D. pteronyssinus* antigens was 100%, specificity was 87.5%. The sensitivity accords with what the literature describes [3], but specificity differs from results obtained by other authors [7, 10–12]. Differences can result from a limited number of examined patients and various allergen's match.

One patient did not respond to anti-IgE and to allergen stimulation, despite a positive result from specific IgE evaluation. Other authors have also reported cases of non-responders. Around 5% of the population are non-responders [13]. Non-responsiveness of basophils to FcεRI stimulation is ascribed to impaired intracellular signal transduction [14]. Some authors suggest that it can be a result of decreased Syk tyrosine kinase expression as well as impaired Fyn and Lyn kinases expression. It is proven that non-responsiveness is not dependent on FcεRI chain structure [14].

Interestingly, in one case high constitutive expression of CD203c and low post anti-IgE or al-

lergen stimulation, were observed. Basophils with constant high expression of basophil activation markers are responsible for chronic symptoms of atopic disease. It might be a result of chronic basophil's activation or initial reaction to immunotherapy [15]. Increased constitutive CD203c antigen expression might be also the result of previous drug intake, such as corticosteroids or antihistamine treatment [10]. It is postulated that inflammatory cytokines, like IL-3, can provoke increase of basophil's activation [13]. Significantly, none of the tested individuals was taking any listed drugs nor was in the active phase of the inflammatory process.

Increased CD203c expression might be explained by the time of year when the test was performed. The patient was sensitised to grass pollen and the blood sample was taken at the beginning of April, when grasses start to dust. There is a probability that analysed cells had previous contact with grass pollen antigens and were prestimulated. However, the patient did not present any symptoms of allergic response.

In conclusion, obtained results indicate that CD203c expression can be a useful marker of basophil activation to grass pollen and *D. pteronyssinus* antigens in sensitised individuals. The test might be useful as a diagnostic tool in atopic individuals with a contraindication to the skin prick test or when typical tests cannot give an obvious response (e.g. food allergy).

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