The diagnostic usefulness of the basophil activation test (BAT) with annexin V in an allergy to *Alternaria alternata*

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med.* 2018;27(12):1737–1744

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Funding sources
None declared

Conflict of interest
None declared

Received on July 3, 2017
Reviewed on October 8, 2017
Accepted on October 12, 2017

Abstract

Background. The basophil activation test (BAT) is an effective diagnostic tool in mold allergy, which is still not sufficiently known.

Objectives. The aim of our study was to assess the degree of annexin V binding to the surface of the basophil cell membrane after stimulation with anti-immunoglobulin E (anti-IgE) and *Alternaria alternata* allergenic extract.

Material and methods. *Alternaria alternata* allergic patients (n = 32) and healthy volunteers (n = 33) were evaluated using skin prick tests (SPT), quantification of specific IgE (sIgE) and the BAT. Basophil activation was detected as a percentage degree of annexin V binding to the surface of the basophil cell membrane.

Results. Receiver operating characteristic (ROC) curve analysis yielded a threshold value of 4.95% of activated basophils when the tested group and control group were studied, with a sensitivity and specificity of 100% (area under curve (AUC) = 1; p = 0.00000) for 100 SBU/mL *Alternaria alternata* allergen extract. The threshold value was 10.28% with a sensitivity of 93.8% and specificity of 100% (AUC = 0.98958; p = 0.00000) for 10 SBU/mL mold extract, and 9.37% with a sensitivity of 90.3% and specificity of 100% (AUC = 0.96307; p = 0.00000) for 1 SBU/mL *Alternaria alternata* allergen extract. The method was least efficacious in anti-IgE stimulation, where the threshold value was 5.48% with a sensitivity of 90.6% and specificity of 30.3% (AUC = 0.46780; p = 0.67039).

Conclusions. The BAT with annexin V and sIgE measurement against *Alternaria alternata* increase the capability of a diagnostic laboratory for detecting mold sensitization. Both methods may certainly replace SPT, which are currently routinely used in allergy diagnosis. Annexin V may be considered a new basophil activation marker with an efficacy comparable to that of CD63 or CD203c.

Key words: *Alternaria alternata* allergy, basophil activation test, specific immunoglobulins E, flow cytometry, receiver operating characteristic curve

DOI
10.17219/acem/78563

Introduction

The basophil activation test (BAT) is a modern and promising research tool in the field of medical immunology. It was introduced to flow cytometry in 1994 by Sainte-Laudy et al.[1] The assessment of basophil activity during stimulation with an allergen or other causative agent is an in vitro method that raises special interest among scientists. The BAT uses various markers for the identification and activation of basophils. Currently, the gold standard in this field is the evaluation of CD63 expression as a de novo molecule after antigen activation.[2,3] The CD63 antigen was used in studies with various inhalant allergens: mites, grasses,animal dander,in allergy to insect venom and to drugs.[4–14] CD203c is the second antigen used in scientific research as a double marker of both identification and activation of basophils, occurring constantly on its surface.[15–17] Other antigens, such as CD13, CD45, CD107a, and CD164, are also well-known and used in scientific experiments.[18,19] However, no objective studies have so far compared the properties and diagnostic efficacy of most of these markers. Although the BAT has been used in allergology for over 20 years, work continues on improving the protocols for basophil identification. The lack of standardization of experiments using various antigens remains the main problem, causing discrepancies and fundamental differences in test results. In the area of laboratory research, a constant search continues for new and effective markers which could improve modern allergy diagnostics. Cytometry studies may provide an opportunity to develop mold allergy detection, including *Alternaria alternata* hypersensitivity. Although *Alternaria* allergies are fairly frequent, with an occurrence of 3–20% in Europe and 47% in the Polish population, knowledge about molecular mold allergy detection is still insufficient.[20]

In this paper, we investigated if annexin V may be considered a new basophil activation marker, useful in *Alternaria alternata* allergy detection.

Basophil activation was demonstrated by using the phenomenon of basophil membrane reorganization under the influence of applied stimuli. In this process, phosphatidylserine, as the main constitutive phospholipid, is displaced from the cytosolic to the basophil external membrane site. Using annexin V bound to fluorochrome, which is the ligand for phosphatidylserine, it was proven that a colored complex forms, which may be depicted in a flow cytometer. The only fluorescent cells were those bounded to annexin V, i.e., activated basophils.

Our studies are a reference to the experiments conducted by Sainte-Laudy and Ouk, who demonstrated changes in the conformation of the basophil membrane after activation with specific and nonspecific stimuli.[21]

Material and methods

Patients and controls

A total of 32 patients (17 males and 15 females) aged from 17 to 42 years (median: 25 years) with seasonal allergic rhinitis and positive skin prick tests (SPT) to the *Alternaria alternata* allergen mix (Allergopharma, Joachim Ganzer KG, Reinbek, Germany) were included in the study. Control group consisted of 33 healthy volunteers (9 males and 24 females) aged from 19 to 54 years (median: 23 years) with no allergic symptoms and with negative SPT results. All the procedures were performed in accordance with the ethical standards of the Helsinki Declaration. The study was approved by the Ethics Committee of Wroclaw Medical University, Poland. Informed consent was obtained from all the enrolled individuals.

Skin prick tests

Skin prick tests were performed according to the standard procedure, using the panel of inhalant allergen extracts (Allergopharma, Joachim Ganzer KG). The kit of reagents contained *Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus fumigatus*, and *Penicilium notatum* extracts at a concentration of 10,000 SBU/mL, histamine hydrochloride 1.7 mg/mL and sodium chloride 9 mg/mL were used as a positive and negative control. The SPT results were read after 15 min and considered positive if the wheal diameter was >3 mm.

Specific immunoglobulins E measurement

Specific immunoglobulins E (sIgE) against the *Alternaria alternata* allergen mix m6 were determined by the ImmunoCAP FEIA system measurements (Thermo Fisher Scientific Inc., Uppsala, Sweden) according to the manufacturer's instruction. The detection range was 0.35–100 kU/L. Values ≥0.35 kU/L were considered positive.

Basophil activation test protocol

Blood specimens were collected into K-EDTA venipuncture tubes (Sarstedt AG & Co, Nümbrecht, Germany) and used for cell stimulation in BD Falcon Round Bottom Tubes (BD Biosciences, San Diego, USA). Testing samples were performed for each patient as the patient background (Pb), positive (stimulation) control (Pc) with Polyclonal Rabbit anti-Human IgE antibody at a final concentration of 10 µg/mL (Dako Denmark A/S, Glostrup, Denmark) and with the *Alternaria alternata* allergen mix used in SPT at the 3 final concentrations of 100, 10 and 1 SBU/mL, identified as C_1, C_2 and C_3.

Sample preparation and analysis

At the beginning of the experiment, 50 µL of stimulation control anti-Human IgE and 50 µL of corresponding *Alternaria alternata* allergen solution was added to 100 µL of stimulation buffer and marked as Pc, C_1, C_2, and C_3 probe. Only 150 µL stimulation buffer was added to the background probe Pb. In the next step, 50 µL of the patient's whole blood was added to each tube and gently mixed. To analyze basophil activation, cells were stained with 5 µL of annexin V-FITC (BD Biosciences) and 5 µL of anti-CCR3-PE (R&D Systems, Minneapolis, USA), and the samples were incubated for 15 min at 37°C. Then, stimulation was terminated by adding 2 mL of Lysing Solution (BD Biosciences) after which the mixing tubes were incubated at room temperature for 10 min. After centrifugation (5 min, 500 g), supernatants were decanted and cell pellets were resuspended in 300 µL of Cell Wash (BD Biosciences) and gently mixed. A total amount of 100,000 cells were acquired per sample using the FACScan flow cytometer (BD Biosciences).

The data was analyzed using CellQuest flow cytometry analysis software (BD Biosciences) according to the manufacturer's instructions. Basophils were identified following the $CCR3^{high}/SSC^{low}$ BAT protocol (Fig. 1).

The percentage degree of annexin V binding to the surface of the basophil cell membrane after stimulation with anti-Human IgE and *Alternaria alternata* allergenic extract was defined as basophil activation. The calculation of the percentage of annexin V binding expression was detected as brightly fluorescent fluorescein isothiocyanate (FITC) (Fig. 2). The activated cells were identified by comparison of their number to the total amount of basophil population gated (Fig. 1). The results were presented after subtracting patient background values.

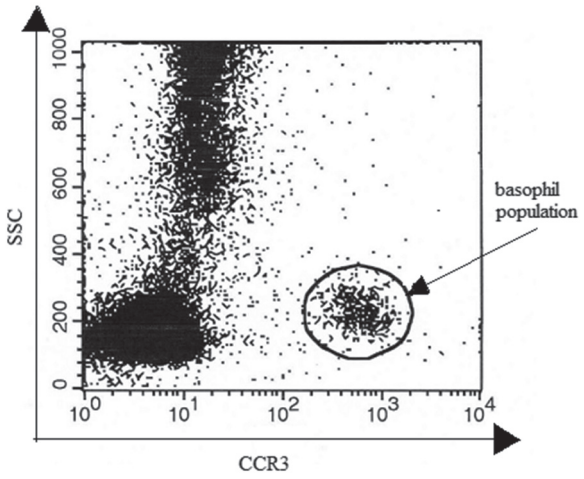

Fig. 1. Basophil population gating

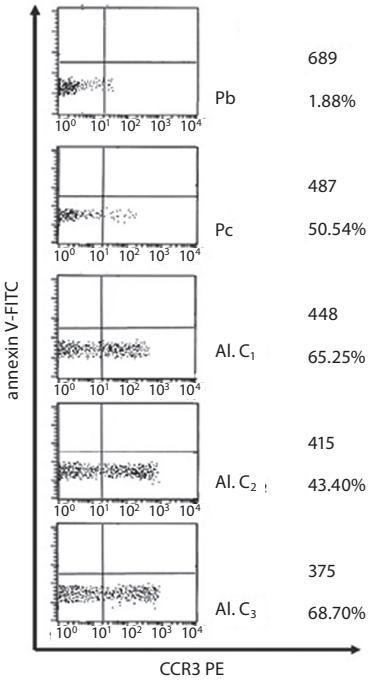

Fig. 2. The number and percentage of activated basophils in each probe in selected individuals in the study group

FITC – fluorescein isothiocyanate; Pb – patient background; Pc – positive control; Al. – allergen; C_1, C_2, C_3 – the concentrations of the *Alternaria alternata* allergen mix (100, 10 and 1 SBU/mL, respectively).

Statistical analysis

Statistical analysis was performed with the use of STATISTICA v. 12.5 software (StatSoft, Kraków, Poland). The distribution of data was performed using the Shapiro-Wilk test. Due to the lack of a normal distribution, the nonparametric Mann-Whitney test was used for the comparison of 2 independent samples. Probability values $p < 0.05$ were considered significant. The optimal cut-off values for allergen stimulation, sensitivity and specificity were determined using receiver operating characteristic (ROC) curve analysis.[22]

Results

Skin prick tests

The results of the SPT with histamine hydrochloride in the study group (n = 32) ranged from a 3 mm to a 6 mm wheal diameter (median: 4 mm). In the control group (n = 33), the results were within the 3–5.5 mm range (median: 3.5 mm). There were no statistically significant differences in SPT with histamine hydrochloride between the groups (p = 0.12721).

The results of SPT with *Alternaria alternata* extract in the control group were completely negative and reached 0 mm for each volunteer, while in the study group the results ranged from 3.5 mm to 11 mm (median: 6.5 mm).

Specific immunoglobulins E

Specific immunoglobulins E concentration to the *Alternaria alternata* allergen mix m6 in the control group (n = 33) were within the range of 0–0.06 kU/L (median: 0.01 kU/L). In the case of patients with mold allergy (n = 32), the results obtained were in the range of 0.002–28.2 kU/L (median: 5.47 kU/L).

Specific IgE concentrations acquired in kU/L were qualified for assigned classes from 0 to 6 according to the manufacturer's instruction. A result was considered positive if the concentration of sIgE was ≥0.35 kU/L, which was interpreted as a low result in class 1.

In the study group (n = 32), 27 positive and 5 negative results of sIgE to *Alternaria alternata* were obtained (Table 1).

Table 1. Specific immunoglobulins E (IgE) concentration to *Alternaria alternata* in the study group (n = 32) by grade of CAP classes

sIgE concentration [kU/L]	CAP class	Study group (n = 32)
>100	6	0
From 50 to <100	5	0
From 17.5 to <50	4	3 (9.38%)
From 3.5 to <17.5	3	15 (46.88%)
From 0.7 to <3.5	2	7 (21.88%)
From 0.35 to <0.7	1	2 (6.25%)
<0.35	0	5 (15.63%)

sIgE – specific immunoglobulin E.

Flow cytometry studies

Basophil number

The number of basophils identified in the whole tested population (n = 65) ranged from 108 to 872 cells (median: 488).

Basophil activity

The activity of basophils in each probe was determined as the percentage of active cells of the whole identified basophil population.

Patient background

The median of Pb in the tested population (n = 65) was 1.75% (min 1.33%, max 1.98%; standard deviation (SD): 0.14%). The mean value of Pb increased by a value of 2 SD (X + 2 × SD) was adopted as the cut-off point and reached 2.04%.

Positive control

In the study group (n = 32), the percentage degree of annexin V binding to the surface of the basophil cell membrane after stimulation with anti-Human IgE was 14.26% (min 2.95%, max 76.01%). The results of Pc in the healthy volunteers were slightly higher than in the patients. After anti-Human IgE stimulation, the median of basophil activation reached 21.69% (min 2.08%, max 83.68%), but the difference was not statistically significant (p = 0.66025).

Stimulation using *Alternaria alternata* extract

In the group of *Alternaria alternata* allergic patients (n = 32), the median percentage of basophil activation at the highest concentration, C_1 = 100 SBU/mL, was 20.42% (min 6.73%, max 74.70%). At the intermediate concentration of mold allergen extract, C_2 = 10 SBU/mL, the median expression of annexin V binding reached 21.89% (min 3.14%, max 85.92%). Stimulation at the lowest concentration of *Alternaria* extract, C_3 = 1 SBU/mL, gave the highest median of basophil activation, 29.41% (min 2.31%, max 88.55%). In the control group, the results of the BAT were considerably lower. The highest *Alternaria alternata* extract concentration (C_1) resulted in a median at the level of 2.31% (min 1.04%, max 5.48%). Stimulation by the C_2 concentration resulted in a median of basophil activation equal to 2.46% (min 1.51%, max 4.83%). In the case of mold allergen extract at the C_3 concentration, the median of annexin V binding to the basophil surface reached 2.54% (min 1.06%, max 5.96%). There were statistically significant differences in basophil activity between the 2 tested groups in C_1, C_2 and C_3 probes ($p < 0.05$). The cut-off value for anti-IgE, and allergen stimulation positivity and specificity was determined on the basis of ROC curves and reached 5.45% for anti-IgE, 4.95% at the C_1 concentration, 10.28% at the C_2 concentration, and 9.37% at the C_3 concentration (Fig. 3).

Sensitivity and specificity

Based on ROC curves, it was demonstrated that the method achieved its lowest effectiveness for the Pc sample. The sensitivity and specificity evaluated together were 120.9%.

The highest sensitivity and specificity of the BAT was achieved for the highest concentration of *Alternaria alternata* extract, C_1 = 100 SBU/mL. In this case, the sensitivity and specificity evaluated together were 200%.

The analysis of ROC curves for the allergen stimulated probes was carried out on the basis of the calculated significance level (p) and area under ROC curve (AUC). A comparison of the results is demonstrated in Table 2.

The dependence of activated basophil percentage on the concentration of *Alternaria alternata* allergen extract

It has been shown that in both tested groups and in the control group, the highest mean value and median of activated basophils was achieved by stimulation with the lowest concentration of *Alternaria alternata* extract, C_3 = 1 SBU/mL (Fig. 4).

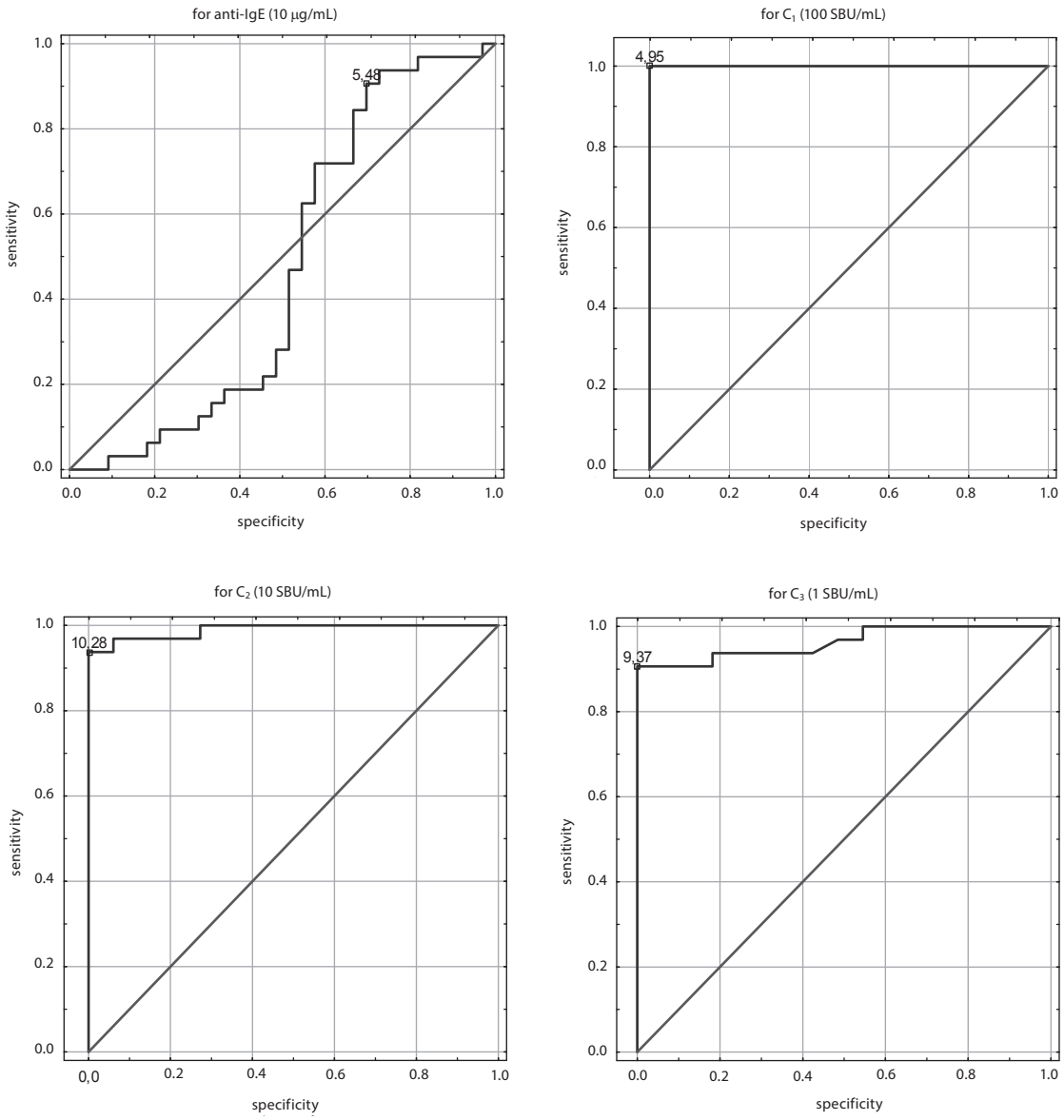

Fig. 3. Receiver operating characteristic (ROC) curves with cut-off value for basophil stimulation with anti-immunoglobulin E (anti-IgE) and *Alternaria alternata* allergen extract

C_1, C_2, C_3 – the concentrations of the *Alternaria alternata* allergen mix (100, 10 and 1 SBU/mL, respectively).

Table 2. The comparison of sensitivity, specificity, p-value, and AUC for the allergen-stimulated probes

Probe	Sensitivity [%]	Specificity [%]	The sum of sensitivity and specificity [%]	p-value	AUC
Pc	90.6	30.3	120.9	0.67039	0.46780
C_1	100	100	200	0.00000	1
C_2	93.8	100	193.8	0.00000	0.98958
C_3	90.6	100	190.6	0.00000	0.96307

Pc – positive control; C_1, C_2, C_3 – the concentrations of the *Alternaria alternata* allergen mix (100, 10 and 1 SBU/mL, respectively); AUC – area under receiver operating characteristic curve.

Discussion

The aim of this study was to evaluate and optimize the diagnostic usefulness of the BAT in *Alternaria alternata* allergies. We also investigated if annexin V could be considered a new basophil activation marker and if it might replace the antigens commonly used in cell tests, such as CD63 or CD203c.

The studies were performed in a group of 65 people, of whom 32 patients were sensitized to *Alternaria alternata*; they had positive results of SPT and presented clinical symptoms. The remaining 33 healthy volunteers

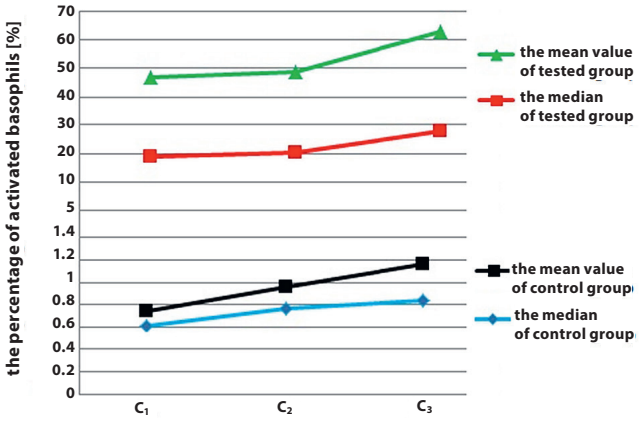

Fig. 4. The distribution of the mean value and median of basophil activity in the tested and control groups (n = 65)

C_1, C_2, C_3 – the concentrations of the *Alternaria alternata* allergen mix (100, 10 and 1 SBU/mL, respectively).

had totally negative results of the SPT and did not present symptoms of allergy-based diseases.

The results of specific IgE measurement directed against the *Alternaria alternata* allergen extract m6 were interesting. Out of 32 patients allergic to *Alternaria* mold with positive SPT results, as many as 5 persons had a sIgE anti-m6 result <0.35 kU/L, i.e., below the lower limit of concentration at which the result is considered positive and qualified as class 1 sIgE. In the tested group, in more than 15% of allergic patients, the concentration of sIgE was in class 0 and the remaining more than 84% of patients were in classes 1–4. The highest percentage of patients sensitized to *Alternaria* had a high level of IgE, qualified as class 3, and they accounted for over 46% of the studied group. After a comparison between the results of SPT and sIgE values, it was observed that the sIgE class is not correlated to the size of the wheal, the diameter of which is interpreted in SPT. In some respondents, the SPT result with the *Alternaria alternata* extract was 12 × 10 mm, in others it was 6 × 7 mm or 5 × 5 mm, but in each case, the sIgE value was qualified as class 3. Moreover, there were allergic patients in the tested group for whom the results of the SPT reached 8 × 7 mm, while the sIgE value was qualified as class 0. In the whole control group, the result was negative, as the concentration of sIgE against *Alternaria alternata* was <0.35 kU/L. It was proven that the concentration of sIgE anti-m6 in the studied group was significantly higher than in the control group, where the min, max and median were equal to 0 mm (p = 0.00000). The results of *Alternaria alternata* sIgE quantification demonstrated a sensitivity of 90.6% and a specificity of 100%. Currently, in order to increase the sensitivity and specificity of IgE measurement, shifting of the lower concentration of sIgE from 0.35 kU/L to 0.1 kU/L is contemplated. However, according to the instructions for interpreting the results of the ImmunoCAP FEIA system (Thermo Fisher Scientific Inc.), the value of 0.35 kU/L is still regarded as the threshold.

The first parameter analyzed in the cytometry studies conducted was the number of cells identified as basophils. Bühlmann Laboratories AG (Schönenbuch, Switzerland) notes that in cell tests, the number of gated basophils should be within the range of 200–600. In the experiment, the median of identified basophils (median: 488) in each sample in the tested and control group (n = 65) was contained in the range specified by the manufacturer, but in some unstimulated and stimulated samples, less than 200 or more than 600 of basophils were collected.

The main objective of the cytometry study was to demonstrate that the binding of annexin V depends on the activation of basophils after stimulation with anti-IgE and the *Alternaria alternata* allergen extract. The displacement of phosphatidyl serine as a ligand for annexin V from the cytosolic to the basophil external membrane site occurs only under the influence of applied stimuli.

The median of basophil activity in unstimulated samples, Pb, was similar in the tested group (1.77%) and in healthy volunteers (1.75%). These results are comparable to the values obtained by De Weck et al., who indicated that the basophil activity at rest is generally below 5%.[16]

No statistically significant differences between the groups studied were observed after anti-Human IgE stimulation (p = 0.66025). The positive control stimulation gave median results equal to 14.26% in the tested group vs 21.69% in the control group.

Receiver operating characteristic curve analysis was performed to determine the cut-off value of the percentage of activated basophils between *Alternaria alternata*-sensitized patients and controls. The positive control stimulation with anti-IgE gave unsatisfactory results, because the specificity reached only 30.3% and the sensitivity was 90.6% (AUC = 0.46780; p = 0.67039). Among the participants there were 6 persons with a negative reaction to anti-IgE below the cut-off value of 5.48%. The threshold was established at 4.95% for C_1 = 100 SBU/mL mold extract concentration with a sensitivity and specificity of 100% (AUC = 1; p = 0.00000). The analysis of the data obtained with C_2 = 10 SBU/mL allergen extract defined a cut-off value of 10.28% activated basophils with a sensitivity of 93.8% and a specificity of 100% (AUC = 0.98958; p = 0.00000). Sensitivity reached 90.3% with a specificity of 100% concerning C_3 = 1 SBU/mL *Alternaria alternata* allergen extract with a threshold of 9.37% (AUC = 0.96307; p = 0.00000). In all healthy volunteers (n = 33), the results of stimulation with the mold allergen extract were negative.

In the BAT, the dependence between the degree of IgE-dependent activation and the value of the concentration of a specific stimulus was variable between individuals. It was assumed that each allergen is characterized by the curve of the dependence of its concentration on the degree of basophil activation. Kleine-Tebbe et al. demonstrated that in the initial phase of the experiment, an increase in the concentration of the allergen causes an increase of basophil activation. Then there is a plateau on the curve,

after which a further increase in the concentration of the allergen causes a decrease of basophil activation.[23] It has been proven that in the tested and control group, the highest percentage of activated basophils was observed at the lowest concentration of the mold allergen extract C_3 = 1 SBU/mL (Fig. 4). In the tested group, the basophil activation in each of the 3 concentrations of the *Alternaria alternata* extract was in the range from a dozen to several dozen percent, while in the control group it extended slightly above or below 1%. The mold allergen extract caused basophil activation only in people allergic to *Alternaria*.

Currently, only a few works describe the BAT performed with mold allergens. Not all researchers have utilized standardized and calibrated allergens in cell tests. Although they are free from preservatives and other cytotoxic additives, their use is expensive. In our studies, we used the *Alternaria alternata* allergen extract intended for SPT. Therefore, a comparison of the results of our studies with the effects of the experiments presented in the literature is possible only to a limited extent. In the paper presented by Mirković et al., a CD203c marker was used for *Aspergillus fumigatus* allergy detection and for allergic bronchopulmonary aspergillosis diagnosis in cystic fibrosis patients.[24] However in this study, the sensitivity and specificity of the BAT were not appointed. It has been proven that the combination of BAT with CD203c and routine measurement of total and specific IgE increases the correct classification of patients into subgroups of unsensitized persons, allergic to *Aspergillus* and aspergillosis-affected.

The results obtained may be compared to the results of experiments with other allergens and basophil activation markers. Sanz et al., in a protocol of BAT using anti-IgE/anti-CD63, mite allergen *Dermatophagoides pteronyssinus* and grass fodder *Lolium perenne* obtained a sensitivity equal to 93.3% and a specificity of 98.7%.[25] González-Muñoz et al. also conducted research in the direction of flow cytometry to detect mite allergy, using anti-CD123/anti-CD63. For allergens at a higher concentration (16 µg/mL) they received sensitivity and specificity of 100%, and in the case of a lower concentration (1.6 µg/mL), sensitivity reached 83% and specificity did not change.[26]

The BAT with annexin V, anti-CCR3 and the *Alternaria alternata* allergen extract presented in this paper has a sensitivity and efficiency comparable to CD63, which is considered the gold standard in the in vitro cell tests used in allergology. It also has a higher diagnostic value than CD203c. Undoubtedly, the advantage of the flow cytometry study with annexin V is the cost, which is much lower than in the case of other markers used in research work. Developing experiments using this protein is therefore encouraging. Further work using the BAT with different markers and mold allergens is necessary in order to validate the method.

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