

Original article

Comparison of two-flow cytometry methods for basophil degranulation in patients sensitized to grass pollen

Background: Two-flow cytometry methods for quantification of degranulated basophil after allergen-specific activation were discussed. The methods are discerned by used membrane receptors – FcεRI or IL-3Rα. Our goal was to evaluate the diagnostic potential of the methods and to correlate them to allergen-specific IgE detection and skin prick test (SPT).

Methods: Patient's and control's groups were studied with Bulgarian grass pollen allergen B1 simultaneously by flow cytometry kits: Basotest and BD FastImmune test. Allergy diagnosis was based on clinical history and SPT. The determination of specific IgE was performed by ELISA - RIDASCREEN^R.

Results: There were no significant differences between the patient's results from Basotest and FastImmune ($P > 0.05$). A significant correlation between values, analyzed by Basotest and by FastImmune was found ($r = 0.88$). The sensitivity and specificity of the Basotest, FastImmune, specific IgE and SPT were 85%, 72%, 92% and 92% sensitivity and 100%, 92%, 100% and 85% specificity respectively. The efficiency was between 82% and 97%. There were a significant correlation between the specific IgE and flow cytometry tests: tau = 0.92 (Basotest) and tau = 0.71 (FastImmune) and a moderate significant correlation between the SPT and the *in vitro* tests: tau = 0.26 (Basotest) and tau = 0.31 (FastImmune).

Conclusion: The successful use of the Bulgarian grass pollen allergen B1 and both flow cytometry tests was presented. These methods could be as specific tools for IgE-mediated diagnosis especially FastImmune in the case of low IgE receptor expression.

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Flow cytometry detection of basophil degranulation upon allergen activation is a new technique, applicable for complete allergy diagnosis (1–5). Two approaches for quantification of degranulated basophils after stimulation with IL-3 and activation with allergen were discussed in this paper. These two-flow cytometry methods are distinguished by different specific membrane receptors, expressed on the activated basophils. Basophils could be identified through high-affinity IgE receptor (FcεRI) which is involved in mediation of allergic responses: α subunit binds free IgE, while β and γ chains are implicated in the signal transduction and bear tyrosine-based kinase activation motifs (6, 7). The second approach is based on a method described by Olweus et al. (8) for dendritic cells identification, using lineage markers CD123 and HLA-DR. The other CD123⁺ but HLA-DR[−] distinctive cell population is the basophil population (9, 10). CD123 is an IL-3 receptor α subunit, member of class I cytokine receptor from the immunoglobulin supergene family. CD123 is a marker for basophil identification and the level of its expression is independent of the allergy status

of the donors, and is less variable, than IgE receptor expression (10). Determination of the percentage of degranulated basophils by both flow cytometry methods was based on CD63 surface expression. This is a 53-Kda glycoprotein, presented on cytoplasmic granules of resting basophils and upon cell activation is moved to cellular surface (1). CD63 molecule is expressed on the outside membrane of < 5% of resting basophils. After allergen activation, the cytoplasmic granules are induced to fuse with the plasma membrane and to release inflammatory mediators, leading to high density expression of CD63 receptors on the activated basophils (3).

Patients with atopic allergy to grass pollen were investigated in this study, simultaneously by both of the flow cytometry methods. The percentage of CD63 membrane expression on peripheral blood basophils, after activation with specific Bulgarian allergen, was analyzed. The aim was to assess the diagnostic potential of the *in vitro* methods and to compare their sensitivity, specificity and efficiency with well-established tests – *in vitro* serum allergen-specific IgE detection and *in vivo* skin prick test (SPT).

Materials and methods

Studied patients and allergen

Fifty-six patients and 14 healthy volunteers were studied with a Bulgarian allergen B1 (Bul Bio NCIPD, Sofia, Bulgaria). This is a mixed grass pollen allergen (*Dactylis glomerata*; *Festuca* sp.; *Lolium perenne*; *Secale cereale*; *Phleum pratense*; *Poa* sp.; *Holcus lanatus*; *Agrostis alba*; *Bromus* sp.; *Alopecurus* sp.; *Agropyron repens*; *Arrhenatherum elatius*; *Zea mays*; *Deschampsia caespitosa*). Allergy diagnosis was based on clinical history and SPT. Skin prick test was performed with 1000 PNU/ml of B1. A wheal more than 3 mm in diameter was regarded as a positive reaction. The control group was with a negative SPT to B1 allergen. For the *in vitro* flow cytometry tests we used previously determined working concentrations of the B1 allergen: 3000 and 30 PNU/ml (11). The data and the distribution of the investigated individuals are presented at Table 1.

Specific IgE determination

The quantitative determination of specific IgE in serum was performed by enzyme immunoassay RIDASCREEN[®] (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's recommendations. The specific IgE was measured, using early grass pollen allergen-gxl (*Dactylis glomerata*; *Phleum pratense*; *Festuca pratensis*; *Lolium perenne*; *Glyceria aquatica*), coated on the surface of the cellulose discs. Values of specific IgE below 0.35 UI/ml were considered as negative.

Basophils activation tests

Two *in vitro* kits for flow cytometry quantitative determination of basophil's degranulation were used: Basotest (OPREGEN Pharma, Heidelberg, Germany) and BD FastImmune test (BD, San Jose, CA, USA). Sample preparations were performed according to the kit's instructions. Peripheral blood was collected in a vacutainer tube with sodium heparin (BD). The probes were stored at room temperature and processed within 24 h of sampling.

For Basotest, after the stimulation step (100 µl heparinized whole blood with 20 µl stimulation buffer for 10 min, at 37°C) each probe was activated with the same volume of the allergen, N-Formil-Met-Leu-Phe (fMLP) for the positive control or washing solution for the negative control, for 20 min at the same conditions. Degranulation was stopped by transferring the sample tubes to an ice bath for 5 min. Twenty microliters of the premixed antibodies CD63/FITC and aIgE/PE were added for direct staining in the dark, on ice, for 20 min. The whole blood samples were lysed and fixed with 2 ml lysing solution for 10 min, at room temperature. After washing, cell

pellet was stored in 200 µl washing solution in an ice bath, up to 2 h before analysis.

For BD FastImmune the stimulation and the activation of the blood samples were performed simultaneously. Twenty microliters of the allergen into stimulation buffer with IL-3 (10 ng/ml final concentration) were added to 100 µl of blood in 37°C water bath, for 15 min. For the negative and positive controls stimulation buffer or diluted fMLP (2 µM concentration) were used respectively. Degranulation was stopped as described above. The staining was proceeded with 20 µl of the three-colored combination: CD63 FITC/CD123 PE/Anti-HLA-DR PerCP in the dark, on ice, for 20 min. The samples were lysed with 2 ml lysing solution at room temperature, for 10 min and after washing, were resuspended in 0.3 ml 0.5% formaldehyde.

Flow cytometry analysis

The samples were analyzed by flow cytometer FACSCalibur (BD, San Jose, CA, USA) with a 488 nm argon laser. At least 1000 basophils were acquired for each sample.

The basophils with IgE^{high} expression were identified by analytical gate R1 in SSC vs FL2 dot plot in case of Basotest kit. Basophil degranulation was visible in F1 vs F2 dot plot (R1). The percentage of CD63 + IgE^{high} + cells was estimated by F1 histogram after analysis of the negative control samples and set up the markers (11).

For BD FastImmune basophils were defined by two analytical gates: R1 in dot plot SSC vs FL2 with a threshold on FL2 (SSC^{low} CD123 + cells) and R2 in dot plot FL2 vs FL3 on R1 gated cells (CD123 + and HLA-DR-cells). The degranulated basophils were identified as CD63 + CD123 + HLA-DR- cells in FL1 vs FL2 dot plot (R1 and R2) and the percentage was measured by FL1 histogram, where the markers were set by the negative control samples (10).

Statistical analyses

Statistically significant differences between the groups were evaluated by Mann-Whitney *U*-test and descriptive statistics with box-whisker plots were performed. Spearman's rank test was used for evaluating the correlations between paired data from patients investigated simultaneously with both *in vitro* flow cytometry tests. The results from SPT, specific IgE determination, Basotest and FastImmune were compared by Kendall test, to assess whether the results from the *in vivo* tests correlate with those from the *in vitro*. A *P*-value < 0.05 was considered statistically significant. Receiver operating characteristic (ROC) curve analysis for determination of the optimal cut-off (percentage of CD63⁺ basophils), corresponding to the best sensitivity, specificity and efficiency was performed (5, 12). It was calculated as follows:

$$\text{Sensitivity} = \frac{\text{true-positive results}}{\text{true-positive results} + \text{false-negative results}}$$

$$\text{Specificity} = \frac{\text{true-negative results}}{\text{true-negative results} + \text{false-positive results}}$$

$$\text{Efficiency} = \frac{\text{sensitivity} + \text{specificity}}{2}$$

Results

Distribution of healthy volunteers and patients, analyzed with *in vitro* flow cytometry tests

A control group of 14 persons and 56 patients with convincing clinical history and positive SPT to mix grass

Table 1. The data and the distribution of patients and controls by different methods

	Basotest	FastImmune	Specific sIgE
Controls			
Number	14	14	13
Male	6	6	6
Female	8	8	7
Age (years)	41 (26–67)	41 (26–67)	39 (26–67)
Patients			
Number	56	33	36
Male	23	18	20
Female	33	15	16
Age (years)	26 (7–51)	27 (7–51)	27 (12–51)

pollen B1 (Table 1) were studied. All healthy volunteers (14 of 14) and 89% (50 of 56) of the patients were analyzed with Basotest. One patient (2%) had a positive SPT but no peripheral basophils were detected with the Basotest. Five patients (9%) were with high negative control and were dropped off the analysis.

The same controls and 33 persons from the patient's group were also examined with BD FastImmune test. All healthy volunteers (100%) and 91% (30 of 33) of the patients were analyzed. Three patients (9%) were with high value of the negative control and were excluded from the analyses.

Comparison and correlation of the Basotest and FastImmune results in allergy patients and controls

The average percentages of degranulated basophils were calculated. The obtained values from Basotest and FastImmune after *in vitro* allergen activation were compared and were presented in Fig. 1.

The percentages of degranulated basophils of the controls were very similar: 3–8% (for the negative) and 37–49% (for the positive). The mean values of CD63⁺ cells, obtained after activation with 3000 and 30 PNU B1 in the controls and patient's groups, were also compared. Higher percentages of CD63⁺ cells were observed by Basotest than by FastImmune (Fig. 1). The average values 54% and 50% with Basotest vs 42% and 46% with FastImmune in case of pollen allergy were calculated. All detected mean values in the patient's groups were significantly higher than in the controls and were above the 10% accepted threshold. However there were no

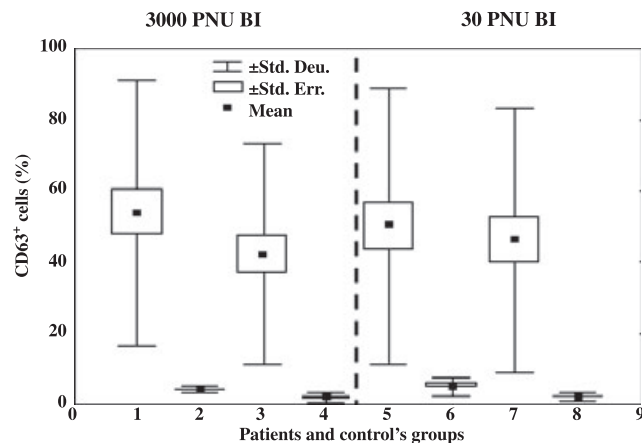


Figure 1. Comparison between results obtained with Basotest and FastImmune after allergen's activation. 1 and 5 – patients, activated with 3000 and 30 PNU/ml B1 and analyzed with Basotest; 2 and 6 – controls, activated with 3000 and 30 PNU/ml B1 and analyzed with Basotest; 3 and 7 – patients, activated with 3000 and 30 PNU/ml B1 and analyzed with FastImmune; 4 and 8 – controls, activated with 3000 and 30 PNU/ml B1 and analyzed with FastImmune.

significant differences between the patient's results from Basotest and FastImmune with both concentrations of the allergen (Mann–Whitney $P > 0.05$).

The data obtained by Basotest and FastImmune was analyzed by Spearman's rank test to evaluate the correlation. A significant positive correlation between the percentages of degranulated basophils, analyzed by Basotest and by FastImmune in patients with B1 atopic allergy was found: Spearman $r = 0.88$; $P < 0.05$ for 3000 PNU B1 and $r = 0.85$; $P < 0.05$ for 30 PNU (Fig. 2A).

Specific IgE

The concentration and EAST-class of specific IgE was determinate at 36 of the patients and they were classified as follows: 2 class 6; 5 class 5; 11 class 4; 5 class 3; 5 class 2; 2 class 1 and 6 class 0. Twenty of controls were class 0 and 1; only one person was 2.

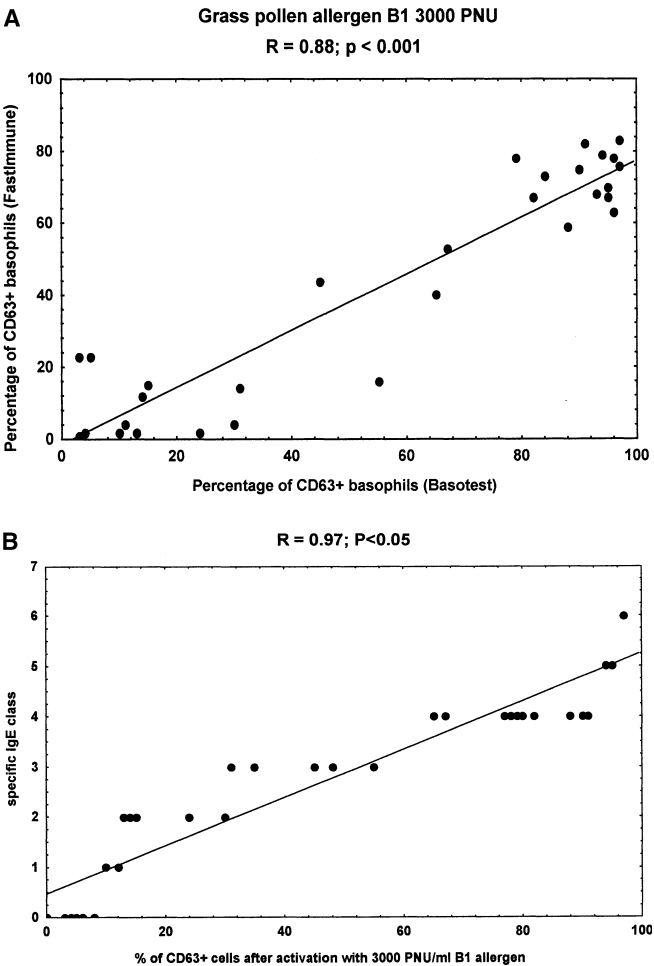


Figure 2. Correlation between results obtained with *in vitro* flow cytometry methods and other tests (A) Correlation between the percentages of degranulated basophils after activation with B1 allergen determined by Basotest and FastImmune. (B) Correlation between results obtained with Basotest and specific IgE determination.

Table 2. Sensitivity, specificity and efficiency of *in vivo* and *in vitro* tests

Assay	Cut-off	Sensitivity (%)	Specificity (%)	Efficiency (%)	P-value
Basotest	>9.5	85	100	93	<0.0001
FastImmune	>9.5	72	92	82	<0.0001
SPT	>2.5	100	85	93	<0.0001
Specific IgE determination	>0.35	92	92	92	<0.0001

Sensitivity, specificity and efficiency of *in vivo* and *in vitro* tests for determination of pollen allergy

The sensitivity, specificity and efficiency of the *in vitro* methods were calculated by ROC curve analyses. The received data were compared with the results from the SPT and specific IgE (Table 2).

The estimated threshold for both flow cytometry tests was 10% of CD63⁺ cells. The sensitivity and specificity of the Basotest, for patients with pollen allergy were 85% and 100%. Relevant results for FastImmune were shown 72% sensitivity and 92% specificity. Similar values with other *in vitro* test – assessment of specific IgE and with *in vivo* SPT were obtained: 92% and 100% sensitivity and 92% and 85% specificity.

The efficiency of the tests was calculated as stated in *Materials and methods* section. All values were high – between 82% and 97%, independent of the methods.

Correlation between *in vivo* and *in vitro* tests

The results from flow cytometry tests, *in vitro* allergen-specific determination of IgE and *in vivo* SPT were compared using Kendall tau test in both patient's groups (Table 3).

There was a significant positive correlation between the specific IgE and the Basotest's results of patient's group – Kendall $P < 0.05$, tau = 0.92 for 3000 PNU B1; tau = 0.70 for 30 PNU B1 (Fig. 2B). We also obtained a significant positive correlation between the other flow cytometry test FastImmune and the IgE assessment – Kendall $P < 0.05$, tau = 0.71 for 3000 PNU B1; tau = 0.57 for 30 PNU.

The *in vitro* tests results from patients with pollen allergy were compared also with the *in vivo* test results. A

Table 3. Kendall correlations between *in vivo* and *in vitro* tests

	SPT	Specific IgE determination
Specific IgE determination	0.26	–
Basotest 3000 PNU B1	0.26	0.92
Basotest 30 PNU B1	0.28	0.70
FastImmune 3000 PNU B1	0.31	0.71
FastImmune 30 PNU B1	0.16	0.57

The Kendall tau values are statistically significant ($P < 0.05$).

significant correlation existed between the SPT and the *in vitro* tests results. Moderate values of positive correlation between the SPT and the specific IgE determination, Basotest and FastImmune were found (Table 3).

Discussion

Basophils are a part of the effector cells in IgE-associated immune responses despite their insignificant amount in the blood leukocytes (<0.2%). In the last decade, the quantitative determination of cell degranulation, based on CD63 membrane expression prompted the development of allergy diagnostic flow cytometry methods. This study is the first comparative investigation on the two tests used for *in vitro* allergen-induced activation of basophils – Basotest and FastImmune. The basic differences between these methods are based on the way of stimulation and activation, and the markers used for basophils identification – FcεRI or IL-3Rα.

From the patients' group one person was with undetectable basophils through IgE receptors and could not be analyzed with Basotest. That patient was analyzed with another flow cytometry method – FastImmune. The basophil's surface marker used in this test was CD123, which did not depend on allergy status of the patient. By this way we were able to uncover basophils and to analyze patients in case of FcεRI low density or αIgE receptor's bridging and internationalization (9, 10). It is important to note, that FastImmune results from the negative and positive controls of the patient were in defined ranges; despite the positive SPT value, percentages of the CD63⁺ cells with both concentrations of the allergen were below the 10% thresholds and the determined quantity of specific IgE was low (<0.35 UI/ml). A possible explanation for the differences of obtained results from the *in vivo* and *in vitro* tests is the difference in the specificity of detected IgE antibody by the used methods – fixed on the skin mast cells or on the basophils, or serum IgE and the various nonantibody-mediated pathways for basophil activation (11, 13).

No significant difference in the percentages of degranulated basophils in the control group with both of the tests was shown, independently from the used concentrations (Fig. 1). However the calculated values in the patients group after *in vitro* activation with FastImmune were different. Lower percents of CD63⁺ cells were obtained when basophils were determined by the CD123⁺HLA-DR[−] phenotype. This might be due to the differences in the stimulation and activation conditions. With FastImmune a simultaneous stimulation with IL-3 and activation with allergen are performed. Independently of this, all patients' data from the Basotest and FastImmune are considerably above the control and 10% threshold limit value was accepted. No false negative cases with the FastImmune analyses were found.

A correlation of the paired data from the flow cytometry determination of basophil's degranulation by the two methods between the patient's groups was performed with Spearman's rank test (Fig. 2). An excellent significant positive correlation was ascertained, irrespective of the allergen concentration.

For the comparison of the data from all of the methods Kendall's tau test was used. A positive correlation between the *in vitro* tests, with high value of the tau coefficient and a moderate correlation between the *in vivo* and *in vitro* tests in the patient's group with pollen allergy were found (Table 3).

Our results complement the findings of the other researches, regarding the correlation between the different tests for allergy diagnosis. Some authors reported a significant positive correlation by Spearman's rank test between the basophil activation test and the skin test, determinations of sIgE, histamine release and allergen-induced sulphidoleukotriene quantification in case of allergy to *D. pteronyssinus* and *Lolium perenne* (14). Another group which studied muscle-relaxant allergy also reported a correlation between the *in vivo* and basophil tests (13). The same results in the case of food allergy were represented by Moneret-Vautrin et al. – correlation between sIgE, basophil test and LTC4 release (15).

However some groups reported different results. No significant correlation between the results from the Basotest and serum-specific IgE level was found in the case of Cupressaceae pollen allergy (12) or venom allergy (16). The explanation for the absence of correlation is the absence of relationship between the amount of circulating specific IgE and basophile release ability. However, a parallelism was found between the kinetics of CD63 upregulation and the release of histamine or LTC4, and a strong correlation between the basophil activation test and histamine or LTR release was shown.

To evaluate and compare the diagnostic accuracy of the *in vitro* and *in vivo* assays a ROC analyses have been performed (5, 17). The sensitivity and specificity may vary among the different subpopulations and thus depend on the composition of the population under study (17). In this investigation a group of Bulgarian population was studied and the sensitivity, specificity and efficiency of the two-flow cytometry tests, by using the specific Bulgarian allergen – B1 were analyzed.

A very high sensitivity with the Basotest, by ROC curve analysis, was received (Table 2). The value was comparable with the relevant data from the *in vivo* test (SPT) and from the *in vitro* – specific IgE determination test, in the case of pollen allergy. The sensitivity from the FastImmune is lower than other test's value – 72%.

Our results correspond to other studies, which also present high percentages of the sensitivities of the flow cytometry tests – allergies to pollen (3, 12, 14), house dust mite (14, 18), Hymenoptera venoms (5, 16, 20), foods (15, 19), latex (2) and drugs (1, 13, 21, 22).

Both flow cytometry tests however are very specific independent of the used allergen concentrations: Basotest – 100% and FastImmune – 92%. These results exclude the possibility of potential false-positive results from IL-3 priming or platelet adherence on activated basophils (23).

Despite of the fact, that the traditional skin test remains the gold standard for IgE antibody detection, the cell-based *in vitro* methods Basotest and FastImmune offer numerous advantages like speed, precise quantitation and absolute safety (23). This study demonstrated the successful use of the Bulgarian allergen B1 and both flow cytometry tests, as specific tools for IgE-mediated pollen allergy diagnosis. These *in vitro* methods could be efficient instruments, to follow the effect of immunotherapy on the modulation of the allergic response, especially FastImmune in the case of low IgE receptor expression.

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