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New opportunities in the diagnosis of asthma with sensitization to *Aspergillus* spp.

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BACKGROUND: Diagnosis of asthma with sensitization to *Aspergillus* spp. is becoming increasingly important due to the severe, uncontrolled course of the disease and the possibility of the formation of allergic bronchopulmonary aspergillosis.

AIM: To evaluate the possibility of using the basophil activation test using flow cytometry for the diagnosis of asthma with sensitization to *Aspergillus* spp.

MATERIALS AND METHODS: 118 patients with asthma were examined. The levels of total IgE and specific IgE to aeroallergens were determined in the blood serum by the enzyme immunoassay. Basophil activation was studied by flow cytometry using the Allergenicity kit (Cellular Analysis of Allergy, Beckman-Coulter, USA). The allergen *Aspergillus fumigatus* (Alcor Bio, Russia) was used to stimulate basophils.

RESULTS: The first group consisted of 57 patients with asthma without sensitization to *Aspergillus* spp. The second group included 36 patients with asthma with sensitization to *Aspergillus* spp. The third group consisted of 25 patients with allergic bronchopulmonary aspergillosis. The number of basophils activated by the *Aspergillus fumigatus* allergen in patients with asthma with sensitization to *Aspergillus* spp. and allergic bronchopulmonary aspergillosis was significantly higher than in the asthma group and amounted to 8.1 [5.2; 20.9]% and 84.6 [75.7; 94.0]%, respectively ($p < 0.001$). The stimulation index in the study groups ranged from 0.7 to 72.6. The optimal diagnostic point (cut off) for identifying patients with asthma with *Aspergillus* spp. sensitization there was an stimulation index value of more than 2.4, and for patients with allergic bronchopulmonary aspergillosis — 15.95. Among all patients with sensitization to *Aspergillus* spp. a positive correlation was established between the level of specific IgE to *Aspergillus* spp. and the percentage of basophils activated by the allergen *Aspergillus fumigatus* ($r = 0.792$, $p < 0.001$) and stimulation index ($r = 0.796$, $p < 0.05$).

CONCLUSIONS: The basophil activation test can be used as an additional diagnostic method for asthma with sensitization to *Aspergillus* spp. and allergic bronchopulmonary aspergillosis.

Keywords: *Aspergillus* spp.; asthma; basophil activation test.

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Новые возможности в диагностике бронхиальной астмы с сенсибилизацией к *Aspergillus* spp.

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Обоснование. Диагностика бронхиальной астмы с сенсибилизацией к *Aspergillus* spp. приобретает все большее значение в связи с тяжелым, неконтролируемым течением заболевания и возможностью формирования аллергического бронхолегочного аспергиллеза.

Цель исследования — оценить возможность применения теста активации базофилов с использованием проточной цитометрии для диагностики бронхиальной астмы с сенсибилизацией к *Aspergillus* spp.

Материалы и методы. Проведено обследование 118 больных аллергической бронхиальной астмой. Уровни общего иммуноглобулина E (IgE) и специфических IgE к аэроаллергенам определяли в сыворотке крови иммуноферментным методом. Активацию базофилов изучали методом проточной цитометрии с помощью набора Allerginicity kit (Cellular Analysis of Allergy, Beckman-Coulter, США). Для стимуляции базофилов в работе использовали аллерген *Aspergillus fumigatus* («Алкор Био», Россия).

Результаты. Первую группу составили 57 больных бронхиальной астмой без сенсибилизации к *Aspergillus* spp. Во вторую группу вошли 36 больных бронхиальной астмой с сенсибилизацией к *Aspergillus* spp. Третью группу составили 25 больных аллергическим бронхолегочным аспергиллезом. Количество базофилов, активированных аллергеном *Aspergillus fumigatus*, у больных бронхиальной астмой с сенсибилизацией к *Aspergillus* spp. и аллергическим бронхолегочным аспергиллезом было достоверно больше, чем в группе пациентов с бронхиальной астмой, и составило 8,1 [5,2; 20,9] % и 84,6 [75,7; 94,0] % соответственно ($p < 0,001$). Индекс стимуляции в исследуемых группах варьировал от 0,7 до 72,6. Оптимальной диагностической точкой (cut off) для выявления больных бронхиальной астмой с сенсибилизацией к *Aspergillus spp.* было значение индекса стимуляции более 2,4, а для больных аллергическим бронхолегочным аспергиллезом — 15,95. Среди всех больных с сенсибилизацией к *Aspergillus* spp. установлена положительная корреляционная связь уровня специфических IgE к *Aspergillus* spp. с долей активированных аллергеном *Aspergillus fumigatus* базофилов ($r = 0,792$, $p < 0,001$) и индексом стимуляции ($r = 0,796$, $p < 0,05$).

Заключение. Тест активации базофилов может быть использован в качестве дополнительного метода диагностики бронхиальной астмы с сенсибилизацией к *Aspergillus* spp. и аллергического бронхолегочного аспергиллеза. Тест необходим для подтверждения микогенной сенсибилизации в случаях противоречивых или отрицательных результатов кожных тестов и специфических IgE, а также при отсутствии возможности проведения исследований *in vivo*.

Ключевые слова: *Aspergillus* spp.; астма; тест активации базофилов.

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BACKGROUND

Bronchial asthma (BA) is one of the most common diseases of the respiratory system among the adult population, with a high socio-economic significance. At present, an increase in the prevalence of severe forms of the disease has been noted worldwide [1], with the BA with sensitization to *Aspergillus* spp. being the most significant [2, 3].

According to previous studies, up to 50% adults with insufficiently controlled asthma, despite maximum doses of inhaled glucocorticoids, are sensitized to *Aspergillus* spp. [4]. The recognition of the important role of molds in the pathogenesis of BA has led to the emergence of "severe asthma with fungal sensitization" (SAFS). This is a group of BA patients with an uncontrolled course of the disease and sensitization to fungal antigens, the absence of bronchiectasis, mucus accumulations and a total IgE level of less than 1000 IU/ml [5].

According to calculated data, the number of severe asthma patients with mycogenic sensitization can reach 6.5 million people worldwide and 231,000 people in the Russian Federation, respectively [6, 7].

In addition, BA patients sensitized to *Aspergillus* spp. constitute a risk group in developing the severe chronic lung disease allergic bronchopulmonary aspergillosis (ABPA); although ABPA affects around 5 million people globally, the disease is not readily detectable [6]. The progression of ABPA results in fibrosis, respiratory failure and patient disability [8, 9].

To confirm immediate hypersensitivity to *Aspergillus* spp. *in vivo* methods have been used however, contradictory results have been obtained. In this regard, in recent decades much attention has been paid to *in vitro* methods, with proven safety for the patient, specificity, and the possibility of standardization.

Along with the high demand for systemic glucocorticoids, severe BA sensitized to *Aspergillus* spp. has been associated with frequent life-threatening conditions and a high death risk [10, 11], making it possible to classify the timely diagnosis of this phenotype as one of the urgent problems of modern medicine.

The aim of the study was to evaluate the use of the test for activation of basophils with the *Aspergillus fumigatus* allergen *in vitro* for the diagnosis of BA sensitized to *Aspergillus* spp.

MATERIALS AND METHODS

The study included 118 adult patients with allergic asthma. The total immunoglobulin E (IgE) and specific IgE levels to fungal, household and epidermal allergens in the blood serum were determined by enzyme immunoassay using a test system (Polignost LLC, Russia) and a panel of biotinylated allergens (Alkor Bio, Russia).

Basophil activation test was performed by flow cytometry using the Allerginicity kit (Cellular Analysis of Allergy, Beckman Coulter, USA). The level of basophils was assessed using markers $CD3^{-}CRTH2^{+}$ (CRTH2 chemoattractant receptor). The number of activated basophils was determined by increasing CD203c expression on cells after *in vitro* stimulation. For this purpose, peripheral whole blood samples were stained with a triple cocktail of monoclonal antibodies CRTH2-FITC / CD203c-PE / CD3-PC7 in the presence of a buffer solution (negative control), or a monoclonal antibody to IgE (positive control), or an *A. fumigatus* allergen (Alkor Bio, Russia) for 15 min at 37°C in the dark. The optimal concentration of the allergen was established in previous studies [12]. Lysis of erythrocytes was performed with the lysis fixing reagent included in the Allerginicity kit. At least 500 basophils were counted in each sample using a Navios Beckman Coulter flow cytometer (USA). The spontaneous activation of basophils was assessed; the proportion of $CD3^{-}CRTH2^{+}CD203c^{++}$ cells from the total number of basophils, in a sample with a buffer solution, made it possible to differentiate the expression levels of resting cell molecules compared with the state of cell activation. Counting the number of activated basophils after incubation with anti-IgE antibodies was necessary to confirm the ability of basophils to non-specific activation in order to exclude false negative reactions and increase the specificity of the method.

The diagnosis was made in accordance with the recommendations set out in the "Global strategy for the treatment and prevention of bronchial asthma" (Global Initiative for Asthma, GINA, 2020) [1]. To detect mycogenic sensitization, a criterion proposed by experts from the International Society for Human and Animal Mycology (ISHAM) was used: a positive skin prick test (≥3 mm) and / or determination of the level of specific IgE to a fungal allergen in blood serum corresponding to class I and above (≥0.35 IU / ml). The diagnosis of ABPA was established based on the criteria of Agarwal et al. (2013) [13].

The data obtained were processed using the STATISTICA 10 and SPPS Statistics 23 software system. Data were presented as a median and lower and upper quartile [Me (Q_1; Q_3)]. To assess the differences between independent samples, the Kruskal–Wallis rank analysis of variance and the nonparametric Mann–Whitney test were used. The relationship of indicators was assessed using the Spearman's correlation coefficient. Differences were considered statistically significant at $p < 0.05$. To assess the diagnostic significance of the stimulation index in detecting mycogenic sensitization, a receiver-operator characteristic (ROC) analysis was performed, with the calculation of the area under the ROC curve – AUC (area

under curve), which is one of the quantitative assessments of the diagnostic efficiency of the studied indicator. The ROC curve is plotted on the X and Y axes of the frequency of true positives (sensitivity) and false positives (specificity) for each split point. The maximum value of the sum of sensitivity and specificity was used to select the optimal value of the separation point (threshold value).

RESULTS AND THEIR DISCUSSION

Based on the results of the clinical and instrumental examination, BA patients were divided into three groups. The first group consisted of 57 BA patients without sensitization to Aspergillus spp., with an average age of 50 ± 15 years (women – 80.7%). The second group included 36 BA patients with sensitization to *Aspergillus* spp., with an average age of 49 ± 14 years (women – 77.8%). According to the criteria of Agarwal et al., 25 patients who developed ABPA against the background of BA were identified. The average age of patients in the third group was 45 ± 16 years (women – 64%). The groups did not differ by sex and age.

All patients underwent an *in vitro* test of activation of basophils with the allergen *Aspergillus* fumigatus, using flow cytometry. Results are shown in Table.

Spontaneous activation of basophils in BA patients with sensitization to *Aspergillus* spp. and in the comparison groups did not differ within the respective groups and ranged from 0.6 to 8.3%. The degree of IgE-mediated activation of basophils did not differ between groups and ranged from 29.6 to 96.9%.

The number of basophils activated by the Aspergillus fumigatus allergen in BA patients with sensitization to *Aspergillus* spp. and ABPA was significantly higher than in the group of patients with BA, and amounted to 8.1 [5.2; 20.9]% and 84.6 [75.7; 94.0]%, respectively ($p < 0.001$).

It is accepted to take into account the level of basophil activation not only by the number of cells in which the expression of the CD203c marker increased in response to incubation with the allergen, but also by the stimulation index (SI). The SI is calculated as the ratio of the proportion of activated basophils in the test with the allergen to the proportion of basophils with spontaneous activation in the negative control. The SI in the group of BA patients with sensitization to *Aspergillus* spp. was 4.0 [2.5; 11.2]. The indicator significantly differed from the comparison groups, and occupied an intermediate position between the indicators in patients with BA and ABPA ($p < 0.001$). It should be noted that the maximum values of the SI reached in the group of patients with ABPA was 27.7 [21.1; 48.5].

Examples of histograms of the basophil activation test in BA patients with and without sensitization to *Aspergillus* spp. are shown in Fig. 1.

To assess the diagnostic significance of the basophil activation test in detecting sensitization to *Aspergillus* spp., we performed ROC analysis with the calculation of the area under the curve. The SI ranged from 0.7 to 72.6 in patients with sensitization to *Aspergillus* spp., and from 0.7 to 4.2

Table. Results of immunological examination of patients with asthma, *n* = 118

Таблица. Результаты иммунологического обследования больных бронхиальной астмой, *n* = 118

Parameters	Group 1	Group 2	Group 3	Significance level (p)
	BA (n = 57)	BA with sensibilization to *Aspergillus* spp. (n = 36)	ABPA (n = 25)	
Level of specific IgE to *Aspergillus*, IU/ml	0.02 [0.00; 0.05]	0.90 [0.56; 1.27]	2.20 [1.15; 7.13]	$p_{1-2} < 0.001$ $p_{1-3} < 0.001$ $p_{2-3} < 0.001$
Spontaneous activation of basophils, %	2.6 [1.8; 4.4]	2.3 [1.5; 3.1]	2.3 [1.5; 4.3]	$p_{1-2} = 0.12$ $p_{1-3} = 0.59$ $p_{2-3} = 0.45$
IgE-mediated activation of basophils, %	71.9 [60.0; 81.7]	74.2 [63.1; 87.3]	74.5 [67.0; 88.1]	$p_{1-2} = 0.34$ $p_{1-3} = 0.21$ $p_{2-3} = 0.72$
Number of activated basophils, %	3.6 [2.3; 5.5]	8.1 [5.2; 20.9]	84.6 [75.7; 94.0]	$p_{1-2} < 0.001$ $p_{1-3} < 0.001$ $p_{2-3} < 0.001$
Stimulation index	1.2 [1.0; 1.5]	4.0 [2.5; 11.2]	27.7 [21.1; 48.5]	$p_{1-2} < 0.001$ $p_{1-3} < 0.001$ $p_{2-3} < 0.001$

Note. BA, bronchial asthma; APBA, allergic bronchopulmonary aspergillosis; IgE, immunoglobulins E.

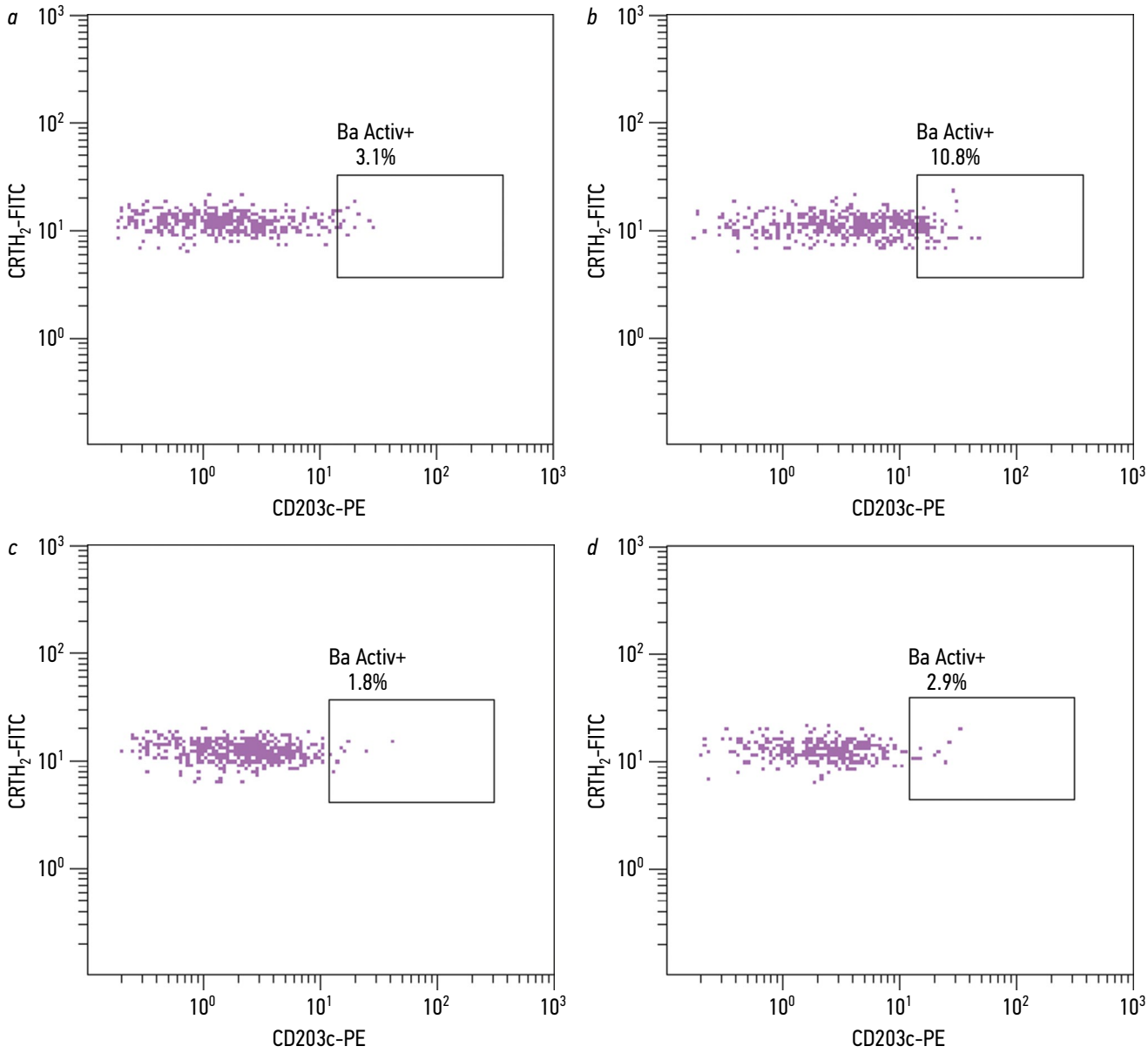

Fig. 1. Basophil activation test in patients with asthma. Patients C. (*a*, *b*) and patients D. (*c*, *d*) at the final basophil gaiting ($CD3^-CRTH2^+CD203^{++}$) after the spontaneous (*a*, *c*) and specific *Aspergillus fumigatus* (*b*, *d*) activation. High percentage of the activated basophils (*b*) confirms the sensibility

Рис. 1. Тест активации базофилов у больных бронхиальной астмой. Пациент С. (*a*, *b*) и пациент Д. (*c*, *d*) на этапе итогового гейтирования базофилов ($CD3^-CRTH2^+CD203^{++}$) после спонтанной (*a*, *c*) и специфической *Aspergillus fumigatus* (*b*, *d*) активации. Большая доля активированных базофилов (*b*) подтверждает наличие сенсибилизации

in patients without. AUC was 0.883 (95% CI 0.809–0.956), sensitivity was 86.9% (95% CI 76.2–93.2), and specificity was 94.7% (95% CI 85.6–98.2) ($p < 0.001$). These results indicated the high specificity and sensitivity of the method, and the SI value of more than 2.4 was the optimal cut off point for detecting mycogenic sensitization, with a high level of reliability in BA patients.

At the next stage, in patients with sensitization to *Aspergillus* spp., the SI value for the diagnosis of ABPA was determined. AUC was 0.887 (95% CI 0.800–0.972), sensitivity was 96.0% (95% CI 80.5–99.3), and specificity was 80.6% (95% CI 65.0–90.3) ($p < 0.001$). Thus, an SI value of more than 15.95 was the optimal cut off point for identifying ABPA patients.

Among all patients with sensitization to *Aspergillus* spp., a positive correlation was established between the level of specific IgE to *Aspergillus* spp. and the proportion of basophils activated by the *Aspergillus fumigatus* allergen ($r = 0.792$, $p < 0.001$) and IS ($r = 0.796$, $p < 0.05$). The data obtained confirmed the relationship of the basophil activation test with the standard determination of the level of IgE to fungal allergens, and has made it possible to use it in the diagnosis of immediate hypersensitivity to *Aspergillus* spp. in BA patients.

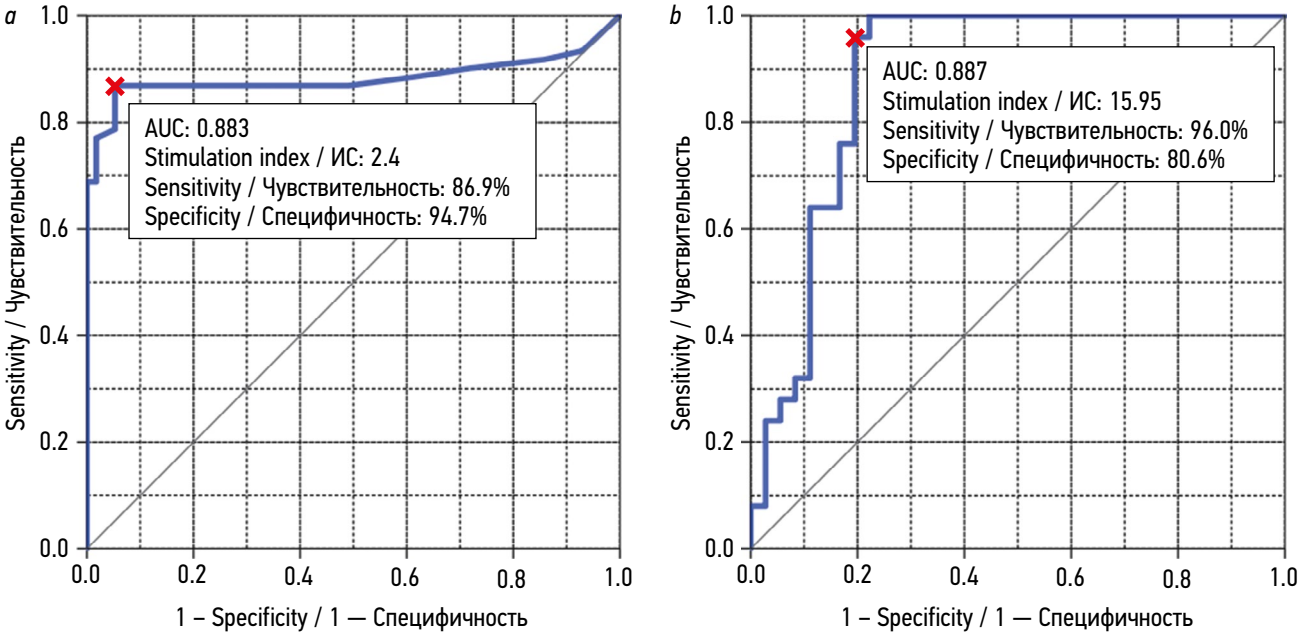

Fig. 2. ROC curves illustrating the optimal cut off points of the index stimulation to detect sensitization to *Aspergillus* spp. patients with asthma (*a*) and ABPA (*b*)

Рис. 2. Кривые ROC, иллюстрирующие оптимальные точки отсечения (cut off) индекса стимуляции (ИС) для выявления сенсибилизации к *Aspergillus* spp. у больных бронхиальной астмой (*a*) и аллергическим бронхолегочным аспергиллезом (*b*)

DISCUSSION

The confirmation of hypersensitivity to *Aspergillus* spp. is an important diagnostic stage in severe asthma patients with sensitization to Aspergillus spp. and ABPA. Currently known laboratory and instrumental methods do not always meet clinical needs; for example, the inhalation test with *Aspergillus* spp. has been associated with the risk of developing fatal bronchospasm and is not recommended for clinical use [13, 14]. While there are a number of contraindications in provocative and skin tests, *in vitro* diagnostic methods have been of particular relevance [15]. However, results obtained are not always reliable and reproducible in different laboratories. It is known that IgE, the level of which is determined in all diagnostic algorithms, is characterized by an insignificant content in the blood serum. In addition, immunoglobulins in this class may be absent in circulation, but fixed on target cells – basophils and mast cells [16].

One of the most promising areas for *in vitro* allergy diagnostics is the test of basophil activation by specific allergens using flow cytometry [17–19]. The test has the advantage of expanding patient indications, safety and standardization.

In recent years, the role of basophils in immune regulation and allergic response has been overestimated. It was found that cells that stimulate Th2-cell differentiation, by the secretion of cytokines and antigenic presentation, are involved in developing IgE-mediated chronic allergic inflammation, and play a key role in IgG-mediated systemic anaphylaxis [20].

In addition to peripheral blood basophils and tissue mast cells being the primary effector cells in IgE-mediated allergic reactions, they may also be involved in other allergic and non-allergic responses, which are based on other reaction mechanisms (activation of complement, non-IgE- mediated stimulation and direct cytotoxic effects). Thus, the study of the functional activity of basophils is of great diagnostic value [21].

The principle of the basophil activation test is that when an allergen comes into contact with IgE molecules fixed on the basophils, a cascade of enzymatic reactions is triggered, leading to degranulation and the appearance of additional molecules on the cell surface. Currently, the most studied and promising markers of basophil activation in allergy diagnostics are CD63 and CD203c [22–24]. The identification of basophils, and the assessment of the expression of activation and degranulation markers is carried out using a flow cytometer.

In this study, we used the CD203c marker (neural cell surface differentiation antigen, E-NPP3), a glycosylated type II transmembrane molecule belonging to the pyrophosphatase / phosphodiesterase ectonucleotide family – enzymes that catalyze the hydrolysis of oligonucleotides, phosphatase nucleosides, and NAD. Among cells in hematopoiesis, surface E-NPP3 is represented exclusively on basophils [25]; in small quantities, it is determined on resting cells. After cell activation, the level of CD203c increases by 350% [26]. Thus, the test of the activation of basophils using flow cytometry is an available and

promising method for laboratory diagnosis of immediate hypersensitivity.

Currently, data on the use of the basophil activation test in the diagnosis of insect, food, pollen, drug allergies, and chronic urticaria have been published [17–19, 27, 28]. The basophil activation test is especially useful in patients with mycogenic allergy, since currently diagnostic fungal allergens for skin testing have not been registered in the Russian Federation.

Studies to identify sensitization to *Aspergillus* spp. have been carried out in patients with cystic fibrosis, with the help of the test of activation of basophils. Gernez et al. showed that the basophil activation test allowed for the differentiation of airway colonization and sensitization to *Aspergillus* in this group of patients. A number of studies have been published in which the basophil activation test, in combination with the determination of specific IgE to *Aspergillus* and total IgE, contributed to the timely detection of ABPA in patients with cystic fibrosis [29–31]. These data are consistent with the results of our previous studies [12].

The results obtained in the course of this study indicate that the test can be used as an additional method for diagnosing BA with sensitization to *Aspergillus* spp. and ABPA. The test can be performed to confirm mycogenic sensitization in cases of conflicting or negative skin tests and specific IgE results, or in the absence of an *in vivo* test.

An important advantage of this method is that the activation test of basophils with the Aspergillus fumigatus allergen is carried out in less than 2 hours; it requires a small volume of peripheral whole blood. This can be done using the same blood sample used for other immunological studies, which significantly reduces discomfort for the patient. In addition, obtaining quantitative results makes it possible to use the basophil activation test as a tool for the differential diagnosis of BA sensitized to *Aspergillus* spp. and ABPA. Timely detection of these diseases of the respiratory tract associated with sensitization to *Aspergillus* spp. is of great importance for determining further therapeutic strategies.

CONCLUSION

1. In BA patients with sensitization to *Aspergillus* spp., mycogenic sensitization was confirmed using the basophil activation test.
2. The SI value for detecting BA sensitized to *Aspergillus* spp. was 2.4, and – 15.95 for ABLA.
3. Basophil activation test can be considered for confirming mycogenic sensitization in case of conflicting or negative results of skin tests and specific IgE, and in the absence of of conducting an *in vivo* study.

Conflict of interest. The authors declare no conflict of interest.

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