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Exacerbation of asthma and neutrophil-dominated airway inflammation in patients with cold-induced airway hyperresponsiveness

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ABSTRACT

Background. Neutrophils can play a significant role in the formation of bronchial inflammation in asthma exacerbation in patients with cold-induced airway hyperresponsiveness (CIAHR).

Aim. To evaluate the role of neutrophils in the dynamics of the inflammatory pattern of bronchi in the exacerbation of asthma in patients with CIAHR.

Materials and methods. In 31 patients (average age (37.2 ± 2.7) years) with persistent bronchial asthma (BA) with moderate exacerbation and previously established CIAHR during cold air isocapnic hyperventilation (CAIH) (– 20 ºC, 3 min), the level of asthma control (Asthma Control Test (ACT), score) and external respiration (forced expiratory volume in the first second (FEV_1), forced expiratory flow between 25% and 75% of the vital capacity ($FEF_{25–75}$)) were assessed; induced sputum (IS) was examined initially and after 24 weeks of follow-up. At the time of the examination, the patients were additionally prescribed prednisone orally (at a maximum dose of 30 mg) for the first 10 days in order to stop the exacerbation, and then they continued treatment with a combination of budesonide / formoterol (640 / 18 μg per day) for 24 weeks.

Results. At the time of the initial examination, the ACT score was 17.0 (13.0; 19.5), FEV_1 was 89.1 ± 3.9%, and the number of neutrophils in the sputum was 55.9 ± 5.6%. At the end of treatment, the ACT score was 22.0 (17.0; 24.5) (p = 0.037), FEV_1 was 96.2 ± 2.9% (p = 0.038), the number of neutrophils in IS decreased, but remained high enough (40.0 ± 5.5%; p = 0.048); and the number of eosinophils did not change. A linear regression equation was made reflecting the relationship between the initially high number of neutrophils in the sputum, other cellular elements in the sputum, the level of asthma control, and the degree of severity of the bronchial response after a bronchoprovocation test with CAIH.

Conclusion. Asthma exacerbation in patients with CIAHR is associated with an increase in the neutrophil pool of the bronchial inflammatory infiltrate and correlates with the degree of severity of the airway response to bronchoprovocation with cold and the level of asthma control.

Key words: bronchial asthma, cold-induced airway hyperresponsiveness, exacerbation, neutrophil-dominated airway inflammation, pattern of bronchial inflammation, asthma control.

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Обострение астмы и нейтрофильный сегмент воспаления бронхов у пациентов с холодовой гиперреактивностью дыхательных путей

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РЕЗЮМЕ

Введение. Нейтрофилы могут играть значительную роль в формировании бронхиального воспаления при обострении бронхиальной астмы (БА) у больных с холодовой гиперреактивностью дыхательных путей (ХГДП).

Цель. Оценить роль нейтрофилов в динамике воспалительного паттерна бронхов при обострении БА у пациентов с ХГДП.

Материалы и методы. У 31 больного (средний возраст (37,2 ± 2,7) лет) персистирующей БА со среднетяжелым обострением и ранее установленной холодовой гиперреактивностью дыхательных путей при проведении стандартной изокапнической гипервентиляции холодным (–20 ºС, 3 мин) воздухом (ИГХВ) оценивали уровень контроля БА (Asthma Control Test, ACT, баллы), функцию внешнего дыхания (объем форсированного выдоха за первую секунду ($ОФВ_1$), максимальная объемная скорость выдоха на уровне 25–75% форсированной жизненной емкости легких ($МОС_{25–75}$)), исследовали индуцированную мокроту (ИМ) исходно и через 24 нед наблюдения. На момент обследования больным с целью купирования обострения в течение первых 10 дней дополнительно назначался преднизолон перорально (в максимальной дозе 30 мг), затем 24 нед они продолжали лечение комбинацией будесонид/формотерол (640/18 мкг/сут).

Результаты. На момент первичного обследования ACT составил 17,0 (13,0; 19,5) баллов, $ОФВ_1$ 89,1 ± 3,9%, число нейтрофилов в мокроте 55,9 ± 5,6%. В конце лечения уровень контроля над астмой составил 22,0 (17,0; 24,5) (p = 0,037), $ОФВ_1$ 96,2 ± 2,9% (p = 0,038), количество нейтрофилов в ИМ снижалось, но оставалось достаточно высоким (40,0 ± 5,5%; p = 0,048); число эозинофилов не изменялось. Построено уравнение линейной регрессии, показавшее зависимость между исходно высоким количеством нейтрофилов в мокроте, другими клеточными элементами мокроты, уровнем контроля над болезнью и степенью выраженности реакции бронхов после проведения острой бронхопровокационной пробы ИГХВ.

Заключение. Обострение у больных бронхиальной астмой с холодовой гиперреактивностью дыхательных путей ассоциируется с увеличением нейтрофильного пула бронхиального воспалительного инфильтрата и коррелирует со степенью выраженности реакции на холодовую бронхопровокацию и уровнем контроля над заболеванием.

Ключевые слова: бронхиальная астма, холодовая гиперреактивность дыхательных путей, фаза обострения, нейтрофильный сегмент воспаления, паттерн воспаления бронхов, контроль астмы.

Конфликт интересов: Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. *Исследование выполнено при финансовой поддержке Российского фонда фундаментальных исследований (грант № 18-54-53019).*

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным комитетом по биомедицинской этике ДНЦ ФПД (протокол № 120/1 от 25.10.2017).

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INTRODUCTION

The clinical syndrome of cold-induced airway hyperresponsiveness (CIAHR), associated with constant exposure to such an ecologically conditioned trigger as low ambient air temperature, is diagnosed in the majority (60–80%) of patients with bronchial asthma (BA) of any severity [1]. In addition to other disturbing factors, exposure to low temperatures, associated with cold-induced bronchospasm, is an inducer of airway oxidative stress, which is interpreted as a typical pathological process that initiates the development and progression of various lung diseases [2].

Taking into account the fact that clinical manifestations of BA are modulated by chronic inflammation [3], a priority role in studying the mechanisms of inactive control and exacerbations of the disease can be assigned to the leading cellular effectors of inflammation – eosinophils and neutrophils [4], generating reactive oxygen species (ROS) and other mediators of oxidative stress, which are presented as signaling molecules that regulate the expression of proinflammatory cytokines [2]. An increased level of proinflammatory cytokines is potentially capable of activating a cascade of inflammatory reactions that determine the severe clinical course of BA [5].

It is known that with steroid-resistant asthma in patients receiving high doses of systemic corticosteroids, a high level of neutrophils in the bronchoalveolar lavage is identified, whereas in patients not receiving systemic corticosteroids, eosinophilia predominates [6]. During acute exacerbations and fatal BA attacks, neutrophil-dominated inflammation proceeds in the airways [6]. Neutrophilia of the bronchial infiltrate contributes to aggravation of clinical manifestations of BA, limits the possibility of achieving the disease control, and is accompanied by a decrease in airway patency and an increase in the frequency of airway response to a cold stimulus [4, 7].

Based on the study of bronchial biopsy specimens obtained from patients with severe exacerbations of BA and endotracheal intubation for respiratory failure, it was found that at the stage of exacerbation the bronchial mucosa is intensively infiltrated by eosinophils and, to a greater extent, by neutrophils [8]. This does not exclude the fact that such patients have airway neutrophilia before the exacerbation – due to the previous severe course of BA against the background of treatment with high doses of inhaled corticosteroids (ICS) [9].

The role of the morphological and functional status of neutrophils in the manifestation of the inflammatory pattern in the bronchi during BA exacerbation in patients with CIAHR has not been clarified. Since the response of the bronchi to cold exposure is clearly associated with a low level of asthma control and difficulties in reducing a cold-induced bronchospasm [10], the possibility of activation of neutrophil-initiated oxidative stress during the exacerbation could result in loss of control and an increase in BA severity. The solution to this problem is associated with the search for methods of appropriate pharmacotherapy for the neutrophil component of inflammation.

The aim of this work was to assess the dynamics of changes in the neutrophil component of the inflammatory pattern in the bronchi during exacerbation and without exacerbation of BA in patients with CIAHR.

MATERIALS AND METHODS

The study included patients (n = 31, average age 37.2 ± 2.7 years) of both sexes (14 men, 17 women) with an established diagnosis of persistent asthma (disease duration ≥ 2 years) and clinical symptoms of exacerbation, according to the criteria [3], and previously identified CIAHR during a bronchoprovocation test with cold air isocapnic hyperventilation (CAIH) (3 min; – 20 °C) [1].

Before inclusion in the study, the patients received anti-inflammatory therapy with a combined medication ICS / long-acting beta(2)-agonists (LABA) at a daily dose of < 1000 μg equivalent to beclomethasone for at least 3 months. The therapy was inadequate and irregular for various reasons, mainly economic ones. From the start of the study (1st visit) for the entire follow-up period (24 weeks), treatment of patients with the first-line anti-inflammatory drug budesonide/formoterol (Symbicort® Turbuhaler®) was planned in a stable dosing regimen, at an increased dose of 640 /18 μg per day. To relieve exacerbation symptoms, oral prednisolone therapy (at a maximum dose of 30 mg) was used for 5–10 days [3], and then the treatment with budesonide/formoterol at a stable dose continued for 24 weeks.

The study design included assessment of BA severity and determination of the pulmonary function with analysis of the indices of the forced expiratory flow-volume curve (forced expiratory volume in the first second (FEV_1), forced expiratory flow between 25% and 75% of the vital capacity ($FEF_{25–75}$), forced vital capacity (FVC), the FEV_1/FVC ratio, peak expiratory flow (PEF), maximal expiratory flow at 50% of vital flow capacity (MEF_{50}), maximal expiratory flow at 75% of vital flow capacity (MEF_{75})) by spirometry on an Easy on-PC (NDD Medizintechnik AG, Switzerland) with subsequent registration of parameters after inhalation of a β_2-agonist (salbutamol, 400 μg) initially (1st visit), 10 days after the start of the treatment (2nd visit), and at the end of 24 weeks of the therapy (3rd visit). At the 1st and 3rd visits, asthma control was assessed using the Asthma Control Test (ACT, Quality Metric Inc., 2002). The criteria for complete, good, and insufficient control of the disease were 25, 24–20 and less than 20 points, respectively.

At the 1st and 3rd visits, samples of induced sputum (IS) were collected and studied [11]. The cytosis level was determined by the number of cells contained in 1 μl of sputum. In order to determine the cellular composition, the sputum smears were examined according to the standard technique using optical microscopy, with calculation of at least 400 cells in 100 fields of view in the central and peripheral parts of the specimen. The number of neutrophils, eosinophils, macrophages, lymphocytes, and bronchial epithelial cells counted in the cytological smears was expressed as a percentage; according to the results, cytograms were formed.

Statistical analysis of the obtained results was carried out using the Statistica 10.0 software package (StatSoft, Inc., USA) and the Automated System for Scientific Research program [12]. Compliance of the trait with the law of normal distribution was evaluated using the Kolmogorov – Smirnov and Pearson – von Mises tests. With normal distribution, Student's *t*-test was used. With the distribution of data other than normal, the Wilcoxon test was used. Descriptive statistics of quantitative variables was presented as the mean, a standard error of the mean ($M \pm m$), as well as the median and the interquartile range Me (Q_1; Q_3). In order to establish the type of dependence and build a mathematical model between a random variable and values of several independent variables, stepwise multiple regression analysis was used with creation of a regression equation. For all values, the level of p less than 0.05 was considered statistically significant.

RESULTS

In accordance with the criteria [3], clinical symptoms of moderate exacerbation at the time of the initial examination were present in all patients with BA included in the study and were characterized by increased respiratory discomfort, shortness of breath (92%), an increase in the number of daytime episodes of shortness of breath and their appearance at night (64%), suffocation (56%), cough varying in intensity and nature (88%), wheezing (60%), chest congestion (50%), and an increase in the need for short-acting bronchodilators (94%).

Interviewing patients with subsequent assessment of the severity of the disease using a validated ACT questionnaire showed a low level of asthma control and significant improvement in the bronchial patency (ΔFEV_1) during the bronchodilation test (Table). The clinical success of the treatment, which was greatly determined by patients' adherence to therapy (it was 85% at the final stage of observation), was reflected in the dynamics of ACT (Table). When assessing ACT values in the group as a whole at the 3rd visit, a statistically significant increase in the level of asthma control was observed (Table). At the final stage of monitoring (3rd visit), 62% of patients reported signs of achieving asthma control (ACT score > 20), 22% of patients receiving regular ICS therapy assessed their condition as the one without changes (ACT score < 19), in 16% of patients, a decrease in ACT compared to the initial testing (score less than 15) and clinical signs of repeated exacerbation were observed. 38% of patients continued to experience the need for the use of short-acting bronchodilators.

At the 2nd visit, after 10 days of oral prednisolone (at a dose of 30 mg), there was no change in bronchial patency parameters (Table). A statistically significant improvement in the ventilation function of the lungs with a decrease in the response during the bronchodilation test occurred by the 3rd visit, with long-term regular use of a stable dose of ICS/LABA.

Calculation of the main cellular elements in induced sputum was performed at baseline, before prescribing corticosteroid therapy, and 6 months after ICS treatment. In all patients included in the clinical study, sputum induction was carried out increasingly by means of 7-minute inhalations with 3-, 4- and 5% sodium chloride solution. To perform the cytological study, a sample was selected that had a minimum level of contamination with squamous epithelial cells (less than 20% of squamous epithelial cells from the total number of cells).

Table

Pulmonary ventilation function and the level of asthma control in patients with BA at runtime, *n* = 31

Parameter	1st visit	2nd visit	3rd visit	*p*	p_1
FEV_1, l, *M* ± *m*	3.17 ± 0.22	3.10 ± 0.16	3.43 ± 0.23	0.024	0.022
MEF_{25-75}, l/s, *M* ± *m*	2.66 ± 0.24	2.61 ± 0.24	3.21 ± 0.26	0.013	0.006
FEV_1/FVC, %, *M* ± *m*	72.7 ± 1.7	74.8 ± 1.5	78.0 ± 1.5	0.005	0.008
FEV_1, % of pred., *M* ± *m*	89.1 ± 3.9	88.3 ± 2.3	96.2 ± 2.9	0.038	0.016
MEF_{25-75}, % of pred., *M* ± *m*	64.6 ± 4.3	64.8 ± 3.1	76.0 ± 3.8	0.015	0.006
ΔFEV_1, %, *M* ± *m*	12.3 (6.0; 20.0)	11.0 (5.0; 15.0)	4.5 (2.0; 13.5)	0.018	> 0.05
ACT, points, *M* ± *m*	17.0 (13.0; 19.5)	–	22.0 (17.0; 24.5)	0.037	–

Note. *p* – level of significance of the differences between the 1st and 2nd visits; p_1 – between the 2nd and 3rd visits.

Relevant sputum samples, which allowed to assess changes in the cellular pattern of bronchial inflammation, were obtained in only 26 people. As the results of the data analysis showed, the pattern of bronchial inflammation underwent transformation: from predominantly mixed during exacerbation of BA, with a high number of eosinophils (>2%) and neutrophils (> 1%) in the sputum, to eosinophilic, with a pronounced neutrophil component, without exacerbation of the disease, at the end of the examination period (Figure).

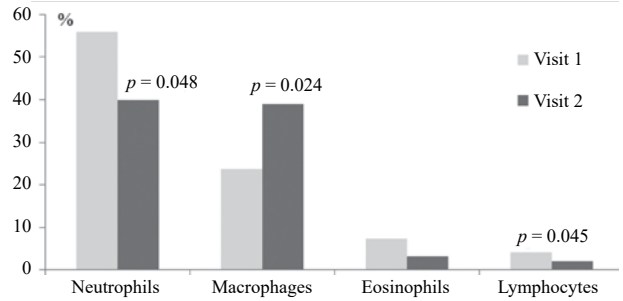

Figure. Cellular composition of induced sputum (%) in BA patients at runtime

If the percentage of eosinophils in the sputum at the 1st and 3rd visits did not differ significantly (2.2 (1.5; 7.8) and 1.6 (0.8; 3.6) %, respectively, $p > 0.05$), the number of neutrophils dramatically decreased from 55.9 ± 5.6% during exacerbation to 40.0 ± 5.5%; ($p = 0.048$) without exacerbation, remaining sufficiently high. Additionally, without exacerbation, the level of lymphocytes decreased (from 4.1 ± 0.79 to 2.1 ± 0.41%; $p = 0.045$) and the level of macrophages increased (from 23.8 ± 2.9 to 39.0 ± 6.1%; $p = 0.024$).

Linear regression analysis was used to build a model that would best show the relationship between the studied indices. When assessing the baseline values of parameters at the time of the initial examination, a relationship was revealed between the initially high number of neutrophils (N) in the sputum, other cellular elements of the sputum (macrophages, M, %), the level of control over the disease (ACT, points), and the severity of the bronchial response (ΔFEV_1) after a bronchoprovocation test with IHCA:

N (%) = 93.0 – 1.4 × M (%) – 0.3 × ACT (points) – 0.25 × ΔFEV1 (%)

The regression is significant with 98.8% probability, explains 71.7% of the dispersion. This dependence disappeared after regular long-term use of ICS/LABA.

DISCUSSION

In our earlier works concerning the relationship between the clinical manifestations of BA and the inflammatory pattern in the bronchi in patients with CIAHR, the negative effect of neutrophils on the achievement of clinical criteria for disease control was repeatedly mentioned [4]. Thus, in patients with persistent mild-to-moderate BA of mixed type, a decrease in the clinical and functional features of BA depended on an increase in the number of neutrophils and a rise in the level of neutrophil peroxidase in the inflammatory pattern of the bronchi [4].

The use of a 24-week treatment regimen with a combination of ICS/LABA in patients with moderate asthma exacerbation did not lead to a controlled decrease in the number of neutrophils in the airways, which was interpreted as a risk factor of the possible loss of the achieved asthma control [7]. The prognostically unfavorable mixed pattern of inflammation in patients with severe uncontrolled asthma in combination with CIAHR was characterized not only by a large pool of neutrophils, but also by a high degree of activity of oxidative enzymes and destruction and cytolysis of bronchial granulocytes, which was clinically manifested through a more severe course of the disease and a more complex problem of asthma control [13].

According to a number of authors, a mixed pattern of inflammation, determined in approximately 10–15% of patients with BA, is combined with more severe symptoms and more frequent exacerbations difficult to treat with ICS, compared to the Th2 pattern of bronchial inflammation [14, 15]. Neutrophilia and a mixed pattern of inflammation are associated

with increased expression of non-Th2 cytokines – IL-17 cytokine and proinflammatory IFNγ [14]. The expression of IL-17 is associated with the *in vitro* and *in vivo* identified phenomena of neutrophil NETosis, which often develops in BA patients according to the so-called non-lytic pathway with the formation of enucleated cytoplasts that induce differentiation of naive $CD4^+$ T-helper lymphocytes ($CD4^+$ Th0) into a subpopulation of T-helpers 17 (Th17) – producers of IL-17 [14, 15].

Enucleated cytoplasts, resulting from disintegration of the nuclear envelope into many vesicles and eruption of decondensed chromatin through the rupture in the neutrophil plasma membrane with resealing of the latter, have a set of rather significant functional properties. If the discarded double-stranded DNA can interact with dendritic cells of the airways *via* TLR2 receptors, which leads to the formation of $CD4^+$ Th2, then activation of dendritic cells by cytoplasts, on the contrary, causes differentiation of Th0 into antigen-specific Th17 [14, 15].

Overproduction of Th17-related cytokines, including IL-17A and IL-17F, is considered to be the main driving force for recruitment and activation of neutrophils through induction of cytokines and chemokines, such as CXCL8, IL-6, G-CSF and GM-CSF, IL-8, and CXCL1 and CXCL5, the expression of which correlates with the severity of BA and bronchial neutrophilia [16]. Cytokines and activated enzymes interacting in Th17 and Th1 inflammatory responses modify the structure of the respiratory tract in BA patients and cause remodeling and an increase in bronchial obstruction, which contributes to a fall in FEV*1* [17]. Differentiation of Th2 cells of the respiratory tract into double positive cells Th2 / Th17 is of particular interest. *In vivo* studies showed that the predominance of double positive Th2/Th17 cells in the bronchoalveolar lavage of BA patients is associated with a high degree of airway obstruction and hyperresponsiveness and an increase in the severity and cortical resistance of the disease [18].

Comparing the data on the accumulation of neutrophils, activation of Th17-associated cytokines, and the possibility of development of non-Th2 inflammation during increasing BA severity with identification of a mixed inflammatory phenotype in patients with CIAHR in the exacerbation phase, it can be assumed that deterioration of the clinical characteristics of BA during exacerbation is associated with stimulation of neutrophil-dominated inflammation. Without exacerbation, despite the decrease, the number of neutrophils in IS in 71% of patients remained quite high (more than 41%), as a result of which a pronounced neutrophil component in the inflammatory pattern was isolated.

Maintenance of relative neutrophilia in the bronchi of BA patients with CIAHR without exacerbation can be explained by the role of budesonide / formoterol therapy in the increase in the functional activity of neutrophils, namely, the antiapoptotic effect of budesonide on neutrophils. The ability of ICS to suppress the cytotoxicity of pulmonary NK cells, leading to a decrease in the intensity of NK-mediated apoptosis in granulocytes, which promotes efferocytosis in macrophages, was proven [13]. Researchers argue that neutrophilia in patients with BA can be observed independently of hormonal therapy, and non-eosinophilic asthma is the phenotype of the disease that is characterized by insensitivity to ICS therapy [17].

The mechanisms responsible for the decrease in the number of neutrophils in the airways of patients with CIAHR without exacerbation include classical NETosis – the process of programmed oxygen-dependent cell death, the purpose of which is to form highly active neutrophil extracellular traps (NETs) in response to irritants, which serve as an important tool for elimination of pathogens and inflammation products [19–21]. The formation of NETs begins with priming of neutrophils, triggering of the NADFH-oxidase enzyme complex and respiratory burst, and generation of ROS, inducing neutrophil elastase and PAD-4, which convert arginine and methylarginine residues into citrulline in histones of the nucleus. As a result, chromatin decondensation occurs with a simultaneous disturbance of the structural integrity of the cytoplasmic granule membranes. When decondensed chromatin (DNA strands, histones) is mixed with enzymes of lysosomal granules, net-like NETs are secreted into the extracellular space [19–21].

Exocytosis of myeloperoxidase (MPO) can take place not only as a result of neutrophil NETosis. As it is known, MPO, when interacting with H_2O_2, catalyzes the oxidation of halides (Cl^-, Br^-, I^-), generating the production of hypohalogenites (active forms of halogens (AFH)), hypohalogenite derivatives (HOCl, HOBr, and HOI), and their ionized forms (hypochlorite, hypobromite, and hypoiodite), resulting in a link between oxidative and halogenated stress [22, 23]. Being a product of azurophilic granules of neutrophils, MPO is secreted into the intercellular environment during cell degranulation associated with a respiratory burst.

It was shown that degranulation of neutrophils in IS of BA patients with CIAHR is capable of intensifying to the level of destruction [4, 7, 13]. Total degranulation of cells, which induces destruction and to the fullest discloses the effector capabilities of neutrophils with a maximally pronounced respiratory burst, is preceded by enzymatic activation in the form of enhanced synthesis and intragranular deposition of MPO, proportional to the needs of bronchial inflammation in AFH, involved in the prolongation and maintenance of CIAHR. Enhanced accumulation of peroxidase reserves in neutrophils, stimulated by accelerated utilization of highly reactive halogen-containing compounds in the bronchial matrix, ends with functional depletion of cells, depletion of the peroxidase-positive granule reserve, intensive destruction, and cytolysis with destruction of the cytoplasm and then the nucleus with cell lysis.

Therefore, a slight decrease in the number of neutrophils in the inflammatory pattern of the bronchi of BA patients with CIAHR without exacerbation was a consequence of ROS-stimulated NETosis, as well as destructive and cytolytic processes, the manifestation of which corresponded to the period of exacerbation associated with the activation of non-Th2 inflammatory response, the prevalence of proinflammatory cytokines, and escalating oxidative stress.

CONCLUSION

It can be concluded that first-line anti-inflammatory therapy, accompanied by the elimination of clinical and functional manifestations of exacerbation and the increase in the level of control over the disease, promoted transformation of the mixed inflammatory pattern in the bronchi into the eosinophilic one. At the same time, the neutrophil component remained quite pronounced without exacerbation, which indicated the limited effectiveness of the proposed therapy in relation to the regulation of neutrophil-dominated inflammation. This is an evidence of the preserved difficulty of comprehensive drug control over inflammation in asthma, in particular, over the pool of neutrophils in the granulocyte population, infiltrating the bronchi of patients with CIAHR.

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Pirogov A.B. – conception and design, analysis and interpretation of data, drafting of the article. Prikhodko A.G. – analysis and justification of the manuscript, critical revision for important intellectual content. Afanaseva E.Yu., Shvetsova Ya.G., Sheludko E.G. – selection and management of patients in the clinical study, collection and processing of biological material, statistical analysis of the obtained material. Zhou X., Li Q. – analysis and interpretation of data. Perelman Yu.M. – critical revision for important intellectual content, final approval of the manuscript for publication.

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