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Bronchial inflammatory profile in interferon-gamma-mediated immune response in asthma patients during airway response to cold stimulus

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Abstract

Objective: To evaluate the inflammatory pattern and the interferon (IFN) -γ in the bronchial secretion of asthma patients in response to acute cold bronchoprovocation. **Material and methods:** We enrolled 42 patients with asthma. We assessed asthma by Asthma Control Test, the lung function by spirometry before and after the bronchodilator test, followed by collecting induced sputum. The next day, we collected exhaled breath condensate (EBC) and conducted a 3-minute isocapnic hyperventilation with cold air (IHCA), followed by collecting spontaneously produced sputum. **Results:** Group 1 included 20 patients with cold airway hyperresponsiveness (CAHR), and group 2 included 22 patients without CAHR. In both groups, a high level of neutrophils in bronchial secretion was observed before and after IHCA. In response to IHCA, the number of epitheliocytes in the sputum decreased to a greater extent in patients of group 1.The baseline epitheliocytes and the concentration of IFN-γ after IHCA had an inverse relationship (r = -0.60; P = 0.017). The baseline IFN-γ in EBC before and after IHCA was lower in group 1. Airway response to cold exposure directly correlated with IFN-γ levels after IHCA (Rs = 0.42; P = 0.014). **Conclusion:** In asthma patients with CAHR, there is a relationship between the persistence of mixed inflammation and the level of IFN-γ in the bronchi. IFN-γ in response to IHCA is decreased with increased cytokine utilization during cold bronchospasm, which is accompanied by the mobilization of neutrophils and the shift in the cytokine spectrum of the respiratory tract towards the T helper cells (Th) 1 immune response.

Keywords

asthma; cold airway hyperresponsiveness; mixed pattern of bronchial inflammation; pro-inflammatory interferon-γ; T helper cells 1 immune response

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1 Introduction

Long-term exposure to low atmospheric air temperatures in the winter is an important factor that negatively affects the human respiratory system. In cold climate areas, asthma in the majority (60%-80%) of patients is accompanied by the development of cold airway hyperresponsiveness (CAHR)[1]. This phenomenon is associated with the lack of asthma control, as well as the activation of a mixed pattern of bronchial inflammation[2], in which patients' sputum contains ≥ 2% eosinophils and ≥ 40% neutrophils[3]. The result of the dominance of neutrophils in the airway infiltrate is the formation of an endotype of neutrophilic asthma that is resistant to controller treatment with corticosteroids[4].

The priority in the development of neutrophilia and a mixed pattern of bronchial inflammation in asthma is given to increasing the expression of such non-T helper cells (Th) 2 type cytokines such as interleukin (IL)-17 and interferon (IFN)-γ[5]. In particular, the molecular cellular mechanisms of the pathogenesis of neutrophilic inflammation correlate with the activation of the Th17 immune response, which is manifested by a high concentration of IL-17A, IL-17F, and IL-8 in the sputum of patients with severe neutrophilic asthma[6]. Cytokine IFN-γ is an important antiviral defense factor and a specific marker of cellular immunity. The leading role of its pro-inflammatory effects is to polarize the immune response by the Th1 type, increase differentiation of immature $CD4^+$Th0 T cells into $CD4^+$Th1 inflammatory T cells, suppress the Th2 helper population in combination with stimulation of antigen processing, and express

surface costimulatory molecules on antigen-presenting cells[7].

The immunoregulatory IL-12 and the powerful IFN-γ inducer IL-18 are the main cytokines that activate T cells. They induce transcription of the IFN-γ gene and enhance the Th1 immune response[7-9].The primary signal for the activation of T-lymphocytes is the binding of the T-cell receptor (TCR) to the antigenmajor histocompatibility complex (AG-MHC), on the cell surface (infected cells in the case of MHC-I, antigen-presenting cells in the case of MHC-II). The binding of the AG-MHC complex to TCR leads to phosphorylation of its intracellular domain and activation of signaling cascades of reactions associated with mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB). At the same time, transcription factors of genes responsible for growth, differentiation, and effector functions of Th1 cells and production of pro-inflammatory cytokines–IFN-γ, tumor necrosis factor (TNF)-α, and IL-2 are mobilized[7]. Th2-type cytokines (IL-4, IL-5, IL-10, IL-13), corticosteroids, immunosuppressants cyclosporine, and FK506 inhibit IFN-γ expression[10-11].

It is known that the high incidence of non-allergic asthma phenotype in patients with CAHR and a mixed pattern of bronchial inflammation and high rates of peroxidase activity, destruction, and cytolysis of neutrophils stimulate the synthesis of pro-inflammatory cytokines through producing respiratory burst and free radicals[2]. These data indicate a certain role of the Th1 immune response in cold bronchospasm. In this study, we aimed to assess the nature of the inflammatory pattern and the activity of the IFN-γ in the bronchial secretion of asthma patients in response to acute cold bronchoprovocation.

2 Material and Methods

A cross-sectional observational study involved 42 patients of both sexes (24 women and 18 men), who had been diagnosed with mild-to-moderate non-allergic asthma (GINA criteria[12]) at the median age of 37.1 (27.0, 47.0) years.

2.1 Inclusion criteria

Age over 20 and under 60; documented clinical diagnosis of persistent asthma[12]; regular controlled therapy during the previous 6 months with inhaled corticosteroids (ICS) at a dose of < 1000 μg/d in terms of beclomethasone; the presence of clinical symptoms of the airway response to cold air.

2.2 Exclusion criteria

Obstructive pulmonary dysfunction (forced expiratory volume in 1 sec [FEV_1] below 70% predicted); the presence of a cold allergy, documented by an allergist during a skin test with an ice cube (according to the Douglas method); concomitant respiratory diseases; clinically significant concomitant diseases from other organs and systems that could affect the further interpretation of the study results; lack of written informed consent from the patient for the study.

2.3 Technical information

The design of the work included testing patients sequentially in a 2-day regimen. On the 1st day, the clinical symptoms of asthma were assessed by questioning patients using the Asthma Control Test questionnaire (ACT, Quality Metric Inc., 2002); the lung function by spirometry using the Easy on PC device (NDD Medizintechnik AG, Switzerland) with the "flow-volume" analysis at baseline and 15 minutes after inhalation of a short-acting β_2-agonist (salbutamol, 400 μg) was evaluated. Next, the induced sputum was collected according to the standard method[13]. On the 2nd day of the study, exhaled breath condensate (EBC) was collected immediately before and 10 minutes after the bronchoprovocation test of isocapnic hyperventilation with cold air (IHCA)[1]. Before the 2nd collection of EBC after the provocation, spontaneously produced sputum was collected first. For all patients, the procedure for taking biological material was standardized in terms of time and sequence of execution. All studies were carried out in the first half of the day, not earlier than 1.5-2.0 hours after a light breakfast.

Functional studies were carried out according to uniform standards[14]. Patients abstained from taking bronchodilators at least 6-24 hours before the proposed testing. For the analysis of spirometry data, the proper ECCS/EGKS values for Caucasians over 18 years of age were used.

The cold airway responsiveness was tested using IHCA with the duration of hyperventilation was 3 minutes at the level of 60% of the maximum voluntary ventilation, the temperature of the inhaled air was -20°C, and the concentration of CO_2 in the inhaled air was 5%. Spirometry control was carried out before the start of cold provocation at the 1st and 5th minutes of the recovery period[1]. The diagnosis of CAHR was made on the condition that the FEV_1 fell by more than 10% after IHCA. In order to relieve cold bronchospasm, a short-acting β_2-agonist (salbutamol, 200 μg) was used. Drug therapy was performed upon completion of the collection of biological material.

Sputum induction was carried out by inhalation of 3%, 4%, and 5% sodium chloride solution using an ultrasonic nebulizer (Omron NE-U-17) in 5-minute sessions under spirometric control. When FEV_1 had decreased by more than 10% of the initial value and/or when a satisfactory sputum sample had been obtained, inhalation was stopped. The cytological examination of sputum

was carried out no later than 2 hours after its receipt. To analyze the cellular composition of sputum, 50 μL was applied to heated (37°C) glass slides. The smears were made by the Kost method and dried in a ventilated thermostat TM-2 (5-10 minutes, 37°C). After fixation (10 minutes) in vapors of 40% formalin solution, the smears were stained in 4%-5% water Romanowsky-Giemsa staining at pH 6.8. The study of micropreparations was carried out according to the generally accepted method[13] using light-optical immersion microscopy based on the percentage of the number of cells counted in smears.

EBC was collected using an ECoScreen II device (VIASUS Healthcare GmbH, Germany) through a mouthpiece attached to a pneumotachograph with a dual valve block, which made it possible to record respiratory parameters. Exhaled vapors condensed at -20°C. The temperature and relative humidity of the ambient air were recorded daily before the study using an electronic thermometer (weather station ea2 bl508 slim) (measurement accuracy of the temperature sensor 0.1°C) and a hygrometer (VIT2, Russia) located next to the device. The fluctuations in values of the indicators were in the range of 24°C-25°C and 55%-65%, respectively. The patients rinsed their mouth cavities twice with distilled water. Next, following quiet breathing for 20 minutes with nasal breathing excluded by applying a nasal clamp, the collection of EBC was carried out. Upon completion, the biological material frozen in a special bag was removed from the apparatus. After defrosting, the liquid condensate was removed using a sterile disposable syringe and immediately placed in a freezer (Sanyo Ultra-Low) at a temperature of -80°C, where it was stored for no more than 2 weeks until biochemical studies were carried out. The concentration of IFN-γ (pg/mL) was determined by enzyme-linked immunosorbent assay (ELISA) on a semi-automatic EIA analyzer Multiskan Fc (Thermo Fisher Scientific Inc., Waltham, USA) using a commercial kit from "Bender Med Systems" (Austria). The main reagent is monoclonal antibodies to interleukins adsorbed on the surface of the wells of a collapsible polystyrene tablet.

2.4 Statistical analysis

Statistical analysis was carried out using the program "Automated system of clinical examination"[15] based on standard methods of variation statistics. The conformance evaluation of the characteristic to the normal distribution law was assessed using the Kolmogorov-Smirnov and Pearson-Mises criteria. If the samples corresponded to the Gaussian type of distribution, the unpaired and paired *t* (Student's) test was used; and if they followed a non-Gaussian distribution, the Kolmogorov-Smirnov test was used. The descriptive statistics of quantitative traits are presented using the arithmetic mean and the standard error of the arithmetic mean (mean ± SE), as well as the median (first quartile [Q1], third quartile [Q3]). In order to determine the degree of connection between two random variables, we used the classical correlation analysis according to Pearson and the nonparametric one according to Spearman. The *P* value of 0.05 was taken as the critical level of significance.

3 Results

3.1 Lung function and asthma control

Despite receiving anti-inflammatory therapy, 76% of patients had low disease control with less than 20 ACT points (average of 17 [15, 19] points). The mean FEV_1 value was 94.5% (85.0%, 105.0%), and the airway response to the administration of a short-acting β_2-agonist ($\Delta FEV_{1bronchodilator}$) was 7.7% (3.1%, 14.55%). All patients enrolled in the present study adequately endured the induction of sputum with saline solutions, the collection of EBC, and the acute cold bronchoprovocation itself. Out of the 42 patients, 20 (48%) had an overreaction of the bronchi in response to cold bronchoprovocation, with a fall in FEV_1 by 10% or more from the baseline value. After the collection of biological material, this group of patients received therapy with a short-acting β_2-agonist (salbutamol, 200 μg).

Further analysis of the afore-presented data was carried out after the distribution of patients into two groups: group 1 (*n* = 20) included those with airway hyperresponsiveness (ΔFEV_1) to a cold stimulus (-16.0% [-18.0%, -12.0%]) and group 2 (*n* = 22) the ones with no response to IHCA (-5.0% [-6.8%, -2.55%], *P* = 0.00001).

Mean values of asthma control in groups 1 and 2 were 16.8 ± 0.6 and 15.7 ± 0.8 ACT points (*P* > 0.05), respectively. Indicators of lung function, including the patency of small bronchi, were significantly higher in patients of group 2 compared with group 1 (Table 1).

Table 1 Lung function and FEV_1 dynamics after administration of salbutamol in asthma patients with different types of airway response to IHCA

Indicators	Group 1 (*n* = 20)	Group 2 (*n* = 22)	*P* value
predicted FVC, %	106.9 ± 2.1	110.3 ± 1.8	>0.05
predicted FEV_1, %	90.1 ± 2.1	98.0 ± 1.9	0.0019
FEV_1/VC, %	70.3 ± 1.3	74.1 ± 1.1	0.0057
predicted MEF_{25-75}, %	60.3 ± 3.0	70.7 ± 3.4	0.032
$\Delta FEV_{1bronchodilator}$, %	10.05 (4.00, 18.70)	7.28 (3.37, 13.05)	>0.05

Values are presented as mean ± SE and median (Q1, Q3). IHCA, isocapnic hyperventilation with cold air; IFN, interferon; FVC, forced vital capacity; MEF_{25-75}, the maximal midexpiratory flow at the level of 25%-75% FVC; $\Delta FEV_{1bronchodilator}$, %, change in the index after inhalation of a short-acting β_2-agonist (salbutamol, 400 μg).

3.2 Results of a cytological study

In the cytological study of sputum in patients of group 1 after IHCA, we observed an increase in cytosis from 2.7 ± 0.5 cells/μL to 3.3 ± 0.5 cells/μL (P = 0.016), in comparison with group 2 (from 2.1 ± 0.3 cells/μL to 2.2 ± 0.2 cells/μL, P > 0.05). The cellular composition of sputum indicated the presence of a mixed pattern of bronchial inflammation, characterized by a significant proportion of neutrophils in both groups either before or after IHCA (Fig. 1).

The average concentration of neutrophils in the general group of patients was 56.4% (50.0%, 62.0%) and had no significant intergroup differences at baseline 56.0% (46.7%, 59.6%) *vs*. 57.6% (50.4%, 64.6%) (P > 0.05) and after IHCA 53.5 (47.7%, 64.3%) *vs*. 58.6% (47.2%, 64.5%) in groups 1 and 2. In response to IHCA, the number of structurally intact cells of the bronchial epithelium (cylindrical ciliated and goblet cells) in the sputum of patients decreased to a greater extent in patients of group 1 (decrease from 8.9% ± 1.2% to 5.7% ± 1.2%, P = 0.025) in comparison with group 2 (decrease from 12.1% ± 1.6% to 9.1% ± 1.0%, P = 0.033; between groups P = 0.011), indicating a destruction of the bronchial epithelium in the former. We also noticed that the initial number of structurally intact epitheliocytes and the concentration of IFN-γ after IHCA had an inverse relationship (r = -0.60; P = 0.017). In addition, a relationship was found between the concentration of neutrophils and macrophages both before (r = -0.81; P = 0.0001) and after IHCA (r = -0.61; P = 0.001), as well as between the initial percentage of neutrophils and eosinophils (r = -0.63; P = 0.0001), which indicated the dominant role of neutrophils in the induction of cold bronchospasm.

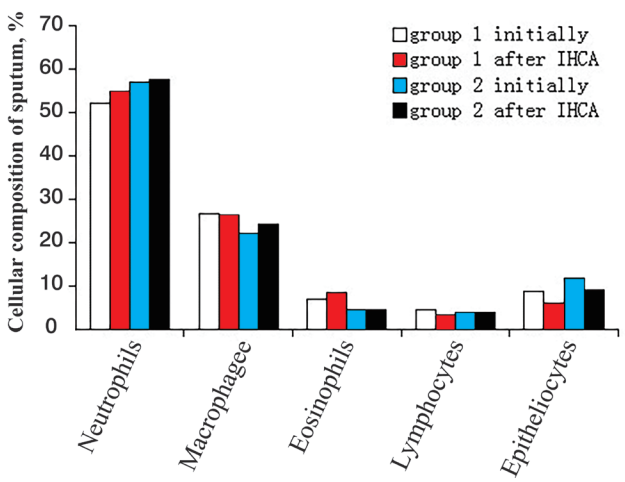

Fig. 1 Cellular composition of sputum in asthma patients with different types of airway response to IHCA

IHCA, isocapnic hyperventilation with cold air.

3.3 Research of IFN-γ in EBC

The baseline levels of pro-inflammatory IFN-γ in EBC of patients of groups 1 and 2 did not differ significantly. After bronchoprovocation with cold air, however, the concentration of IFN-γ remained unaltered in patients of group 1, whereas it doubled in group 2 (Table 2).

The baseline FEV_1 and IFN-γ values were closely related (r = 0.73; P = 0.005) (Fig. 2). The intensity of the bronchial response (ΔFEV_1) in response to bronchial provocation correlated well with the content of IFN-γ after IHCA (Rs = 0.42; P = 0.014) (Fig. 3). In group 1, the value of IFN-γ after the test depended on its baseline value (r = 0.67; P = 0.007) (Fig. 4).

4 Discussion

Neutrophilia of the inflammatory infiltrate in asthma is a sign of an unfavorable clinical prognosis, since, in addition to resistance to corticosteroids, it is also associated with a severe course and frequent exacerbations of the disease[16]. Epithelial destruction is regarded as one of the key links in the pathogenesis of mucociliary dysfunction, an obligate symptom of all chronic inflammatory diseases of the respiratory tract[17]. Destruction of the epithelium is more pronounced in asthma patients with CAHR and is accompanied by activation of neutrophilic inflammation against the background of a decrease in lung function and the level of asthma control[18].

The mixed pattern of inflammation and manifestations of epithelial destruction found in patients of group 1, which could potentiate the development of cold bronchospasm and airway remodeling, might be considered as factors that impose a negative impact on the course of the disease. We have previously shown that patients with a mixed pattern of bronchial inflammation, despite receiving controller therapy with inhaled corticosteroids, are more likely than patients with an eosinophilic pattern to experience respiratory discomfort and have the need to use emergency drugs. They have lower FEV_1 and MEF_{25-75}, a greater increase in FEV_1 in response to bronchodilator and an increased airway response to bronchial provocation with cold air[19]. The incidence of CAHR among patients with a mixed

Table 2 The concentration of IFN-γ in the exhaled breath condensate in asthma patients with different types of airway response to IHCA

Indicators	Group 1 (n = 20)	Group 2 (n = 22)	P Value
Initial	17.3 ± 2.8	21.2 ± 3.4	>0.05
After IHCA	13.6 ± 3.2$^{\#}$	48.7 ± 5.4$^{\#\#}$	0.0024

Values are presented as mean ± SE. FVC, forced vital capacity; IHCA, isocapnic hyperventilation with cold air; IFN, interferon; P, significance level between groups 1 and 2 (unpaired t-test); $^{\#}$, P > 0.05; $^{\#\#}$, P = 0.022.

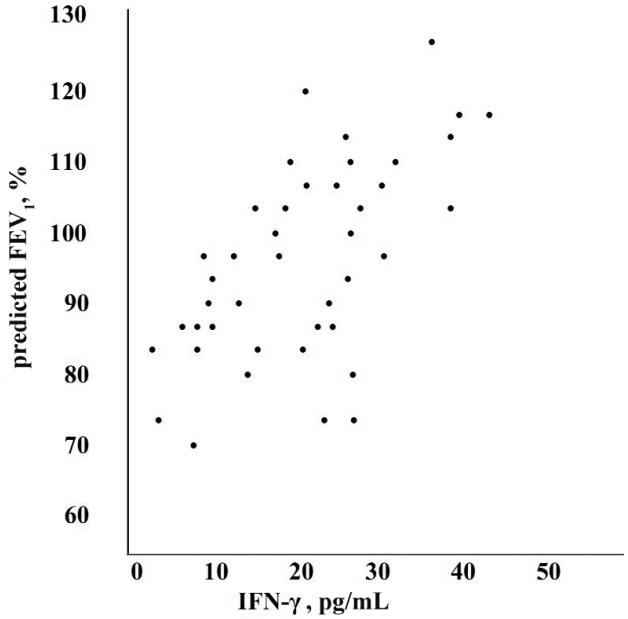

Fig. 2 The correlation between baseline FEV_1 (% predicted) and baseline IFN-γ (pg/mL) in EBC
IHCA, isocapnic hyperventilation with cold air; IFN, interferon; EBC, exhaled breath condensate.

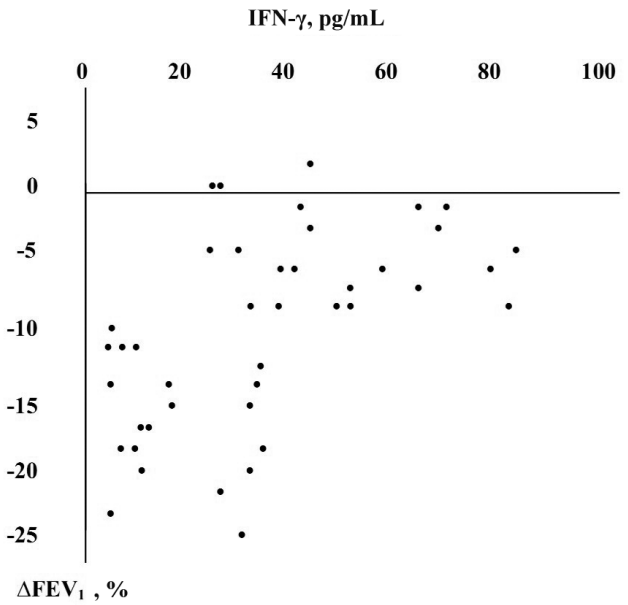

Fig. 3 The correlation between the ΔFEV_1 (%) in response to IHCA and the content of IFN-γ (pg/mL) in EBC after IHCA
FEV_1, forced expiratory volume in 1 sec; IHCA, isocapnic hyperventilation with cold air; IFN, interferon; EBC, exhaled breath condensate.

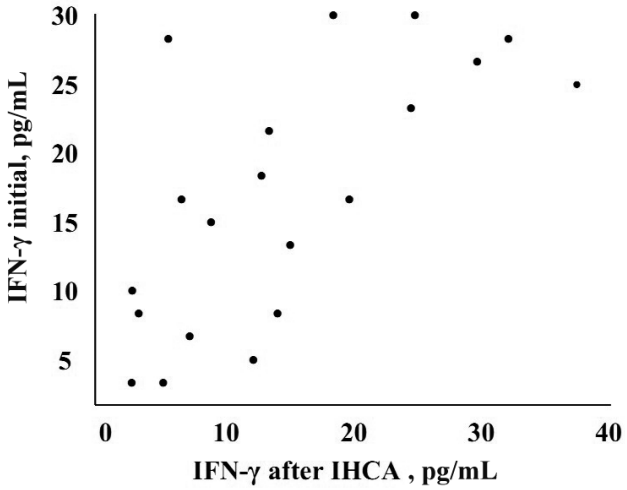

Fig. 4 The correlation between the content of IFN-γ (pg/mL) in EBC before and after IHCA (in group 1)
FEV_1, forced expiratory volume in 1 sec; IHCA, isocapnic hyperventilation with cold air; IFN, interferon; EBC, exhaled breath condensate.

pattern of inflammation is significantly higher than in patients with bronchial eosinophilia (51.4% *vs*. 28.6%, $\chi^2 = 6.15$; $P < 0.05$, respectively)[19].

The bronchial inflammation profile depends on the generation of pro-inflammatory cytokines. Neutrophilia is accompanied by hyperproduction of IL-1β, the inflammatory protein MIP-3 alpha/CCL20 and disturbance of lung function[3]. The decrease in the concentration of IFN-γ in patients of group 1 after IHCA could be explained by increased utilization of this cytokine during bronchospasm associated with the maintenance of neutrophilic inflammation. IFN-γ is able to activate neutrophils[11] and thus participate in cold bronchospasm by mediating the Th1 immune response.

The most studied mechanism of intracellular signal transmission upon IFN-γ binding to the cognate IFN-γR receptor is the Janus Kinases (JAK)/signal transducer and activator of transcription (STAT)-dependent signaling pathway (JAK–STAT). JAK1 and JAK2 activate the latent cytoplasmic transcription factor STAT1, which, when activated, translocates to the nucleus where it induces the transcription of IFN-γ-activated (GAS) genes[7]. IFN-γ-activated STAT1 expression triggers the generation of $CD4^+$Th1 cells[7].

In contrast to STAT1 phosphorylation, the traditional fact for Th2 cell differentiation is the activating effect of the cytokine IL-4 on STAT6 phosphorylation (IL-4/STAT6 variant) with the help of the JAK/STAT system (JAK1- and JAK3-kinases) with the

conversion of the inactive STAT6 monomer to active transcription factor pSTAT6 dimer[20-21]. An increase in its expression is a key process for the induction of the Th2 transcription factor GATA-3, which activates the secretion of Th2 cytokines, inhibits specific Th1-type transcription factors, causing allergic inflammation and asthma components such as airway hyperresponsiveness and remodeling of bronchi[21-26].

It is known that the manifestation of corticoresistant neutrophilic asthma is dominated by the activity of cytokines IL-17A, IL-17F, IL-21, IL-22, and Th17-initiated neutrophilic inflammation, which occurs via the IL-6/STAT3-dependent signaling pathway (and not via IL-4/STAT6 option)[6]. We assumed in asthma patients with CAHR and a mixed pattern of bronchial inflammation, there is a significant activation of the transcription factor NF-κB and Th1 of the immune response modulated by IFN-γ signals. This process is synchronized with a critical decrease in the expression of the GATA-3 transcription factor and a decrease in the activating effect of IL-4 on pSTAT6 expression. As a result, asthma patients with CAHR develop pro-inflammatory effects of IFN-γ, as well as other NF-κB-dependent cytokines responsible for various stages of proliferation, apoptosis and, mainly, the immune response, *i.e.* controlling adaptive cellular homeostasis under conditions of inflammation[27]. With the development of asthmatic inflammation and airway hyperresponsiveness, the pool of neutrophils generates reactive oxygen species and other molecular mediators of cellular oxidation, which regulate the activity of NF-κB and cause the expression of cytokine Th1 genes and escalation of inflammation, which causes a severe clinical course of the disease[26, 28].

Thus, in asthma patients with CAHR, there is a relationship between the persistence of mixed inflammation and the functioning of IFN-γ contained in the bronchi. A decrease in the IFN-γ concentration in response to IHCA develops because of increased cytokine utilization during cold bronchospasm, which is accompanied by the mobilization of neutrophils and a shift in the cytokine spectrum of respiratory pathways towards the Th1 immune response. IFN-γ is one of the main products of $CD4^+$Th1 cells. We suggest that its secretion in patients with CAHR is associated with the neutrophil pool and the escalation of inflammation in the bronchi. The mixed pattern of bronchial inflammation in the IFN-γ mediated immune response is an inducer of worsening lung function and poor asthma control, even in patients treated with long-term anti-inflammatory therapy using ICS/long-acting β_2-agonist (LABA).

The present study has some limitations associated with a small number of participants and groups formed for comparison, although statistically significant differences support the hypothesis of the study.

Conflicts of interests

All authors declare no competing interests.

Author contributions

These authors contributed equally to this work. The manuscript has been read and approved by all the authors, the requirements for authorship have been met, and each author believes that the manuscript represents honest work that has been read and approved by all the authors.

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Ethical approval

The study was performed in accordance with "Ethical Principles for Medical Research Involving Human Subjects" (WMA Declaration of Helsinki, 2013). Patients signed an informed consent to participate in the study in accordance with the protocol permission of the Local Ethics Committee (No. 121 of 10/25/2017).

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