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Respiratory Viruses and Proinflammatory Cytokines Imbalance in Adults and Children with Bronchial Asthma

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Abstract

Complications of acute respiratory viral infections include pneumonia, development and exacerbations of bronchial asthma (BA). Respiratory infections with human rhinoviruses, respiratory syncytial virus, adenovirus, influenza viruses A and B, parainfluenza virus's type 1 and 3 were detected in 36.2 ± 7.1 % of clinical samples of adult patients and in 54.5 ± 10.9% of samples from children with BA using RT-PCR. High frequencies of mixed respiratory infections (35.7 ± 13.3 in adult patients and 16.7 ± 11.2 among children) along with detection of antibodies against respiratory viruses in blood sera suggested immunodeficits.

Disbalance of the IFN gene expression in the absence of IFNβ gene expression, IFNα RNA detection in 58.3% samples and IFNλ RNA - in 42.9% samples together with low levels of the IFN-stimulated antiviral protein MxA RNA and IFNα/γ functional deficiency were found in the induced sputum of patients with BA. Concentrations of proinflammatory cytokines and especially interferon (IFN) γ ($P<0.001$) significantly exceeded control values of healthy donors. Additional growth of the tumor necrosis factor (TNF) α and interleukin (IL) 6 production was revealed for patients with the BA exacerbations. Increased levels of the proinflammatory cytokines were accompanied by the normal concentrations of regulatory IL10. Additionally, amounts of TGFβ responsible for induction of Th17 cells for clearing extracellular pathogens and tissue inflammation was decreased. Induction of the proinflammatory cytokines caused by the respiratory viruses and the IFN imbalance can cause allergic inflammation.

Keywords Bronchial asthma; Chronic obstructive pulmonary disease; Respiratory viruses; Interferon's; MxA protein; Reverse transcription; Real-time PCR; ELISA

Introduction

Asthma is a multifactorial disease characterized by chronic airway inflammation, bronchial hyperresponsiveness, smooth muscle contraction, hypertrophy and hyperplasia of smooth muscle, hypersecretion of bronchial mucus, activation of mast cells, eosinophils, lymphocytes, epithelial cells, macrophages, disruption of the bronchial epithelium, and production of free radicals such as expired nitric oxide (eNO) and 8-isoprostane [1-3]. Currently, the disease is widely spread among 4-10% of the world population; in Russia in approximately 7 million [3]. Environmental conditions, industrial pollutions, allergens, respiratory viral and bacterial infections may cause its development and exacerbations. Viral infections are known to be the main cause of BA development and following exacerbations. Airway inflammation resulting from acute respiratory viral infections (ARVI) may cause allergic manifestations with subsequent asthma [4-7]. Respiratory syncytial virus (RSV) has been first associated with an increased risk to develop asthma [4] but recently other respiratory viruses and especially human rhinoviruses (hRV) have been proposed to be involved in BA pathogenesis [6,7]. Moreover, asthmatic patients have a higher susceptibility to hRV infection due to enhanced expression of intercellular adhesion molecule (ICAM-1) that is known to serve as receptor for the major group of the human rhinovirus A (hRV A) [7]. Besides that influenza A and B viruses (IV), parainfluenza virus (PIV) and adenovirus (AdV) infections can also contribute to BA [4-5].

Virus-infected cells secrete a broad range of IFN which confer resistance to yet uninfected cells by triggering the synthesis of antiviral factors [3,7-9]. Impaired IFNβ and IFNλ production has been demonstrated in bronchial epithelial cells from asthmatic adults upon exposure to hRV [7]. Both innate response including IFNα/β, TNFα, IL1α/β, IL6, IL8, IL11 and adaptive immunity, apoptosis of infected cells, monocytes, macrophages, lymphocytes and granulocytes are involved in inflammation [8-13]. Th2-polarized immunity [2,14] and low T-cell immunity, natural killer activity, IFN types I and II production, phagocytic activity of neutrophils are typically in patients with BA [11-13].

Our aim was to analyze the respiratory viruses and cytokines in clinical samples of adult patients and children with BA.

Materials and Methods

Sampling

Clinical samples including blood sera and lymphocytes, as well as induced sputum were examined for 3 groups of patients:

1. 19 patients from the Department of Allergy of the Moscow Clinical Hospital №57 with BA exacerbations (average age 41.36 ± 3.8, the period of the disease more than 10 years);

2. 28 patients with BA and chronic obstructive pulmonary disease (COPD) without exacerbations from the same Moscow Clinical Hospital №57 (average age 57.22 ± 10.5, the period of the disease more than 10 years);

3. 22 samples of mononuclear blood cells of children of 4-6 years old with exacerbations of BA.

Detection of Respiratory Viruses in Clinical Samples

Viral RNA was detected by means of reverse transcription with subsequent quantitative real time PCR (RT2-qPCR) with fluorescent hydrolysis probes. Total RNA were isolated from 50 μl of clinical samples by using "Proba-NK" kit ("DNA-technology", Russia). Then the reverse transcription was performed using "Reverta-L" kit ("AmpliSens", Russia). Real time PCR was performed using "ORVI-AmpliSens" ("AmpliSens", Russia) with heterologous FITC-labelled internal control.

IgG antibodies against the influenza virus A and B; the parainfluenza virus; RSV; adenovirus were detected in pair blood sera in the first days and after 2-3 weeks of hospitalization by ELISA using test systems of Research Institute of Influenza, Saint Petersburg, Russia.

Cytokine gene expression

RT-qPCR to detect mRNA of IFNα, β, λ and anti-viral protein MxA was performed according to [15]. Quantitations of genome-equivalents were based on Lukyanov-Matz equation and on calibration curve with the standards.

Besides that IL4, IL6, IL8, IL10, IFNα, IFNγ, TNFα were detected using ELISA kits «Vector-Best» (Russia) and TGFβ1 ("Bender MedSystems", Austria).

IFN status including circulating IFN; level of IFNα production by leukocytes infected with Newcastle disease virus; level of IFNγ production by lymphocytes induced by 10 μg/ml FGA («Difco»), level of spontaneous IFN production *in vitro* were assayed in whole heparinized blood. Units/ml of IFN was calculated as reverse dilutions protecting 50% of cellular monolayers from the virus-induced cytopathic effect [13].

Statistical analysis

Statistical comparisons were carried out using the Student's t-test. In the text and tables mean values are represented with the Standard Error of the Mean (SEM) and percentages with the Standard Error of the Percentage (SEP) [16]. Correlation analysis was performed using Statistica 7.0 (StatSoft Inc.). In all cases, p-values <0.05 were considered to be significant.

Results

High annual rate of ARVI with bronch muscle contraction in 70% of patients with BA might suggest both chronic infections and immunodeficits. Among respiratory viruses the hRV were detected in 28.6 ± 8.7% of samples of induced sputum of the adult patients with BA and COPD and in 36.4 ± 10.5% of lymphocytes of children with BA (Table 1). Infections with RSV, the adenoviruses and influenza viruses were less frequent (Table 1). In spite of different rates of the respiratory viruses during a year and their annual fluctuations the hRV appeared to be the most frequent respiratory infection both in adult and children with BA.

	RT-PCR detection rate of ARI (% ± SEP)			ELISA detection of antibodies (% ± SEP)
Viruses	adult patients with BA +COPD	adult patients with BA	children with BA	adult patients with BA
Human rhinoviruses	28.6 ± 8.7	0	36.4 ± 10.5%	0
Respiratory syncitial virus	3.6 ± 3.6	0	18.2 ± 8.4%	15.8 ± 8.6
Adenoviruses B, C and E	3.6 ± 3.6	26.3 ± 10,4%	0	15.8 ± 8.6
Influenza virus A, B	0	10,5 ± 7,2%	0	42.1 ± 11.6
Parainfluenza virus 1, 2, 3 and 4	0	0	0	26.3 ± 10.4%
Coronaviruses OC43, E229, NL63, HKUI	0	N/A	0	N/A
Human metapneumovirus	0	N/A	0	N/A
Bocavirus	0	N/A	0	N/A
In total	35.6 ± 9.2	36.8 ± 12.2	54.5 ± 10.9	73.7 ± 10.4
Mixed infection	0	0	16.7 ± 11.2 (metapneumovirus, parainfluenza virus, RS virus)	35.7 ± 13.3 (adenoviruses, respiratory syncitial virus, influenza virus, parainfluenza virus)
SEP: Standard Error of Percentage				

Table 1: Detection of ARI in clinical samples from patients with bronchial asthma.

Viral loads varied in a range from a few up to 105 genome-equivalents per 1 ml of sputum. Other respiratory viruses including parainfluenza virus type 1, 2, 3 and 4; coronaviruses OC43, E229, NL63, HKUI, human metapneumovirus and bocavirus were not found in all the clinical samples of different groups of patients despite their availability in Moscow region during the period of observation. Nevertheless, detection of antibodies in blood sera of adult patients with BA allowed us to reveal IgG against 4 respiratory viruses such as the RSV, the adenoviruses, the influenza and parainfluenza viruses (Table 1).

Besides that high rate of mixed infections in 16.7 ± 11.2 of children lymphocytes and in 35.7 ± 13.3 of sera of adult patients (Table 1) as well as persistent infection with herpes simplex virus in 53% of adult

patients with BA may serve as additional evidences of their immunodeficits.

Cytokine gene expression was analyzed at levels of RNA transcription and protein production. IFNα RNA (10^3-10^6 molecules in 1 ml of sputum) was detected in 58.3% and IFNλ RNA (10^6-10^9 molecules in 1 ml of sputum) –in 42.9% of the samples of patients with BA-COPD in the absence of IFNβ RNA. RNA of the IFN-stimulated antiviral protein MxA was revealed in 28.6 ± 8.7% samples (10^1-10^2 molecules in 1 ml of sputum) (Figure 1).

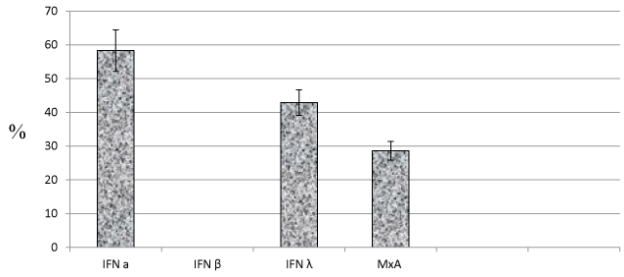

Figure 1: RT2-qPCR detection rate (%) of the gene expression in the induced sputum samples of patients with BA-COPD.

Groups of patients	BA	Cytokines (pg/ml)							
		proinflammatory					anti-inflammatory		
		TNFα	IL6	IL8	IFNα	IFNγ	TGFβ	IL10	IL4
Adults	Remission	0.9 ± 0.3	1.7 ± 1.0	14 ± 6.8	0.2 ± 0.1	72.9 ± 14.9 ***	5775 ± 345 ***	1.1 ± 0.9	51 ± 26 ***
	Exacerbation	331 ± 184 ***	91 ± 44.2 ***	N/A	N/A	104.9 ± 32.7 ***	N/A	N/A	19.3 ±15.1 **
	BA+COPD Remission	1.2 ± 0.2	4.0 ± 1.1	49.7 ± 12	2.5 ± 0.98	2.1 ± 0.6	14660 ± 2034 *	2.97 ± 1.7	0.3 ± 0.1
Children	Exacerbation	N/A	N/A	N/A	15.5 ± 4.3 ***	27.4 ±12.3 ***	N/A	N/A	20.4 ± 8.5 ***
Control		0.4 ± 0.2	1.7 ± 0.2	4.6 ± 0.5	1.4 ± 0.6	0.1 ± 0.1	21120 ± 3245	1.4 ± 0.5	0.4 ± 0.2
Notes: *, **, ***, statistical significance p<0.05; 0.01; 0.001, respectively.									

Table 2: Cytokines in sera of BA patients

Proinflammatory cytokines IFNγ and IL8 were found by means of ELISA in all samples; Whereas IL6, IFNα, IL4, TGFβ1 - in 41-44% and TNFα - in 11% of induced sputums. Concentrations of proinflammatory cytokines and especially IFNγ (p<0.001) essentially exceeded corresponding values of control group of healthy donors (Table 2).

However, the functional activities of IFNα (125.9 ± 32.7 units/ml) and IFNγ (12.6 ± 4.6) were decreased in comparison with healthy volunteers (388.6 ± 75.9 and 50.3 ± 8.4, respectively (p<0.01) (Figure 2). Spontaneous IFN synthesis was revealed in leukocytes of one third part of the patients *in vitro* in complete absence of its production in healthy people.

Moreover, the BA exacerbations were associated with the TNFα and IL6 additional growth (Table 2). Increased levels of proinflammatory cytokines were accompanied by normal concentration of regulatory IL10 (Table 2). Decreased amounts of TGF β which together with IL6 induces Th17 cells responsible for clearing extracellular pathogens and tissue inflammation permitted to exclude an induction of Th17 lymphocytes.

Discussion

Asthma pathogenesis with involvement of many cytokines remains unsolved public health concern [1-3,13]. Despite the evident correlation between BA symptoms and ARVI especially with the hRV or the RSV both in the adult patients and in the children (Table 1) a certain conclusion about a role of the respiratory viruses in BA development, exacerbations or as a consequence of binding of the hRV with the ICAM-1 receptor with enhanced level of the corresponding gene expression is hardly possible. Antiviral protection of BA patients appeared to be attenuated due to the disbalance of IFN of types I, II and III (Table 2 and Figure 2). For IFNα high levels of RNA and normal concentrations of the protein in adult patients did not correspond to its low activity (Figure 2) perhaps because of disfunctions of some IFN-stimulated genes (ISG). The discrepancy between IFNγ significantly enhanced concentrations in all the studied groups of BA patients compared to the control samples (Table 2) and its decreased activity in leukocytes of patients with BA (Figure 2) was even more evident [13]. IFNβ RNA was not found whereas IFNγ RNA with high concentrations (106-109 genom-equivalents/ml) was detected in 42.9% of the samples of patients with BA-COPD. Airway inflammation in patients with BA was characterized by an exaggerated

activation of proinflammatory cytokines and especially TNFα and IFNγ causing activation of T helper type 1 lymphocytes [1]. Concentrations of regulatory cytokines were not elevated (Table 2) in the different groups of the patients with various BA forms. Without the necessary regulation the airway inflammation could not be suppressed. Statistically significant growth of IL6 along with TGFβ decline could not induce Th17 lymphocytes responsible for physiological tissue inflammation [14].

Antiviral response depends on the ability of infected cells to produce IFN which activates transcription of numerous ISG. However, disproportionately strong or chronic IFN expression is a common cause of inflammatory and autoimmune diseases [15-17]. Both IFN and ISG are regulated at the different gene expression levels. Di-methylation of histone H3 at lysine 9 (H3K9me2) catalyzed by histone methyltransferase G9 was described as an epigenetic suppression of the IFN and ISG expression [17,18]. The epigenetic mechanisms are essential for the induction and differentiation of T-cells [19]. IFN, ISG and the differentiation of the immune cells can confer resistance to pathogenic RNA viruses.

IFNα/β was shown to block Th2 response by suppressing the transcription factor GATA3 thus inhibiting allergic inflammatory processes by blocking granulocyte activation and IL-4-mediated B cell isotype switching to IgE [20]. According to our data (Table 2 and Figure 2) the absence of IFN β mRNA and functional deficit of IFN α are associated with BA.

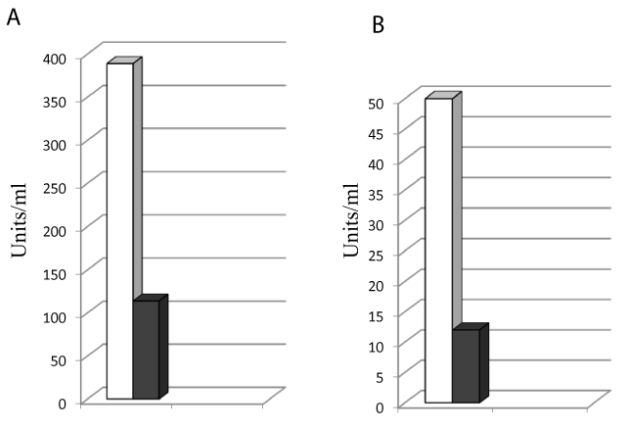

Figure 2: The production of IFNα (A) and IFNγ (B) in leukocytes of patients with BA (dark columns) and healthy volunteers (white columns).

IFNγ induces JAK/STAT-associated but not NF-κB signaling pathways in airway epithelial cells causing their insensitivity to glucocorticoids which are used for treatment of patient with severe BA [21]. Our observations about significant growth of IFNγ RNA transcription revealed a risk of development of the patient's insensitivity to glucocorticoids.

IFNλ inhibits viral replication, upregulates cytotoxic responses to virally infected cells, induces the proliferation of Foxp3-expressing regulatory T cells and inhibits the production of Th2 cytokines IL5 and IL13 *in vitro*. High levels of IFNλ mRNA in patients with BA coincide with previously described reverse correlation between the severity of allergic BA and IFNλ gene expression [22].

Viral respiratory infections are the main causative agents of the BA onset and exacerbations. IFN of type I and II, together with IL27, restrict Th2 cytokines by means of the transcriptional activator ISGF3 [23]. Th1 immune responses with reduced Th2 and IL17 previously described for the broncholalveolar lavage cells isolated from patients with BA [24] completely match with our observations (Figure 1 and Table 2).

Taken together, the inflammation induced by the respiratory viruses together with proinflammatory cytokine gene expression without suppression with anti-inflammatory cytokines could cause BA.

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