The content of antimicrobial proteins in children with respiratory tract inflammatory diseases

Abstract. Background. Inflammatory diseases of the respiratory tract are often associated with impaired synthesis of antimicrobial peptides, whose representatives are defensins and lactoferrin (LF). The purpose of the study was to establish the concentrations of antimicrobial peptides in the oropharyngeal secretion and plasma in children with inflammatory diseases of the respiratory tract. Materials and methods. The study group included 111 children aged 4 to 17 years from orphanages. The content of antimicrobial proteins of LF, α-defensins 1–3 (human neutrophil peptides (HNP) 1–3), secretory immunoglobulin A in the oropharyngeal secretion, HNP 1–3 in the blood plasma, of interleukin 6 (IL-6) and -10 in the blood serum in children was measured by immunoassay. Results. Analysis of anamnestic data in somatically healthy children from orphanages showed the presence of 4 to 6 episodes of acute respiratory infections throughout the year, the duration of which averaged 5.3 ± 0.4 days. According to the results of the study, the content of α-defensins 1–3 in the blood plasma of the children of the control group was 3583.3 ± 735.4 pg/ml, while in the group of children with recurrent bronchitis, the HNP 1–3 level was almost twice higher — 6576.7 ± 602.8 pg/ml, p < 0.01. When studying the serum cytokine profile, it was found that in children with recurrent bronchitis compared with the control group, the content of pro-inflammatory IL-6, which regulates the inflammatory process and provides mobilization of the inflammatory response, as well as an antigen-specific immune response, had only a tendency to increase (10.1 ± 2.0 pg/ml vs 9.1 ± 1.1 pg/ml in the control group, p > 0.05). Against this background, we observed more than 3-fold increase in the level of anti-inflammatory IL-10, which is a product of type 2 helper cells, responsible for the formation of a humoral antigen-specific immune response, — 4.42 ± 1.00 pg/ml vs 1.41 ± 0.59 pg/ml, respectively (p < 0.05). Conclusions. It was found that sickly children had been characterized by changes of mucosal protection, low content of α-defensins 1–3 in oropharyngeal secretions. The revealed changes indicated the stress of the local defense mechanisms. The investigation has revealed that overproduction of HNP 1–3 in children with recurrent bronchitis against the background of the disturbed balance between pro- and anti-inflammatory cytokines could be one of the causes of recurrent forms of the respiratory tract infection.

Introduction

The leading role in protection of an organism at relationship between bacterium and the owner belongs to polymorphonuclear leukocytes that is caused by products them highly reactive metabolites of molecular oxygen, and also secretion of the proteins posses-sing antimicrobic action [1]. Inflammatory diseases of the respiratory tract are often associated with impaired synthesis of antimicrobial peptides, one of whose representatives are defensins [2]. Effect of antimicrobic peptides leads, mainly, to disturbance of structure and function of a cytoplasmic membrane of microorganisms due to change of transmembrane permeability. This, in turn, causes the progreidementalization of the cell and leads to the death of the microorganism [3, 4]. In the process of development of the immune system, defensins were given a special immunoregulatory role. On the one hand, defensins have antiinflammatory properties due to the induction of interleukin-10 secretion. On the other hand, defensins direct neutrophils, B-cells and macrophages to the inflammatory focus, leading to the release of inflammatory mediators, such as interleukin-8, -6, -10, interferon-γ and leukotrienes B4 [5, 6]. Thus, defensins act...
not only as endogenous antibiotic, they also play an important role in the activation of processes of inflammation, repair and regulation of the adaptive immune response [4, 7]. The violation of the expression of defensins is accompanied by an increased risk of infectious, inflammatory, allergic and autoimmune diseases [7].

Another antimicrobial peptide is LF, which is an iron-binding glycoprotein that acts as a tissue protector against the damaging effects of hydroxyl radicals. It mediates the surface tension reactions on the cell membranes and the repulsive forces between them. The biological role of this effect lies in the retention of neutrophils in the inflammatory focus. According to a number of researchers, LF is a highly sensitive marker of any inflammatory process [8], and testify to the biological role of this peptide in protecting the body [10].

All listed above, as well as the absence of such reports in the literature, served as a prerequisite for the present study, whose goal was to study the concentrations of these antimicrobial peptides in the oropharyngeal secretion and plasma in children with inflammatory diseases of the respiratory tract.

**Materials and methods**

The study group included 111 children aged 4 to 17 years from orphanages. An obligatory criterion for including children in the study was the presence of a state of somatic health and clinical well-being at the time of the survey. In addition, we conducted a comprehensive survey of 68 children with recurrent bronchitis, in the period of acute inflammation, aged in age from 5 to 16 years. The control group consisted of 30 healthy children, representative by age and sex.

Evaluation of the content of antimicrobial proteins of LF (Human Lactoferrin), α-defensins 1–3 (Human HNP1–3), α-defensins 1–3 (Human HNP1–3) in the oropharyngeal secretion in children was carried out using the elisa kit of the test systems HyCult®, Biotech, NTV, BioChimMac, and SIgA in the oropharyngeal secretion — using a reagents IgA secretory — ELISA-BEST; Russia, Novosibirsk, ZAO "Vector-Best". The content of antimicrobial peptides — α-defensins 1–3 (Human Neutrophil Peptides 1–3, HNP1–3) in blood plasma was studied by immunoassay using the commercial kit HNP1–3 (ELISA, Bio Tech Lab-S). Determination of interleukin-6 and -10 content in blood serum was carried out using commercial sets for the enzyme immunoassay interleukin-6 and -10 (DRG, USA).

The obtained results are processed by the variational statistics method using the Statistic for Windows 6.0 program analysis package with the function of calculating the arithmetic mean (M), the standard deviation (σ) and the mean errors (m). To assess the differences in the indicators in the compared groups, the t-test of the Student was used. Distinctions considered reliable at p < 0.05.

**Results**

Analysis of anamnestic data in somatically healthy children from orphanages showed the presence of 4 to 6 episodes of acute respiratory infections throughout the year, the duration of which averaged 5.3 ± 0.4 days, while the children in the control group had no more than two acute respiratory infections a year. Chronic lesions of upper respiratory tract infections have been reported in 85 examined children (84.2 %), among which compensated chronic tonsillitis observed in 69 % of the patients (70 children), adenoid vegetation in 19 % of the patients (20 children). An adenotomy was carried out at 15 (13.5 %) children, tonsillectomies — at 5 (4.5 %) children. Ten (9.0 %) children suffered from acute sinusitis, manifestations of caries in the history were noted in half of our observations.

A characteristic feature of the group of patients with recurrent bronchitis was the prevalence of preschool and primary school age children (47 children — 69.1 %). Frequency of exacerbations of bronchitis fluctuated within 3–7 episodes a year and averaged 5.3 ± 0.3 episodes.

Development of 3–4 episodes of bronchitis per year was observed in 36 (52.9 %) children, 5–6 episodes a year — in 25 (36.8 %) children, and the presence of more than 6 episodes of bronchitis per year was noted in 7 (10.3 %) children with recurrent bronchitis. Exacerbations of bronchitis were mainly provoked by acute respiratory viral diseases. The average duration of exacerbations of bronchitis was 20.6 ± 0.9 days. The acute period of the inflammatory process in 58 (85.3 %) children was 2–3 weeks, in 7 (10.3 %) patients — 4–6 weeks, and in 3 (4.4 %) patients — more than 6 weeks.

Comorbidity of ENT organs were found in most patients with recurrent bronchitis (53 children — 77.9 %), among which 24 (35.3 %) children experienced chronic adenoids, 13 (19.1 %) patients were diagnosed with chronic tonsillitis, and 16 (23.5 %) patients had chronic sinusitis. Hypertrophy of the lymphatic oropharyngeal ring was found in 14 (20.6 %) patients.

According to the results of a microbiological examination of the mucous membranes of the throat and nose of frequent sick children and episodically ill children from the organized collectives, they had similar characteristics of pharyngeal and nasal biocenosis, with a slightly higher coefficient of bacterial presence in children, who often were ill, due to variety of species of normal mucosal microflora and presence of different representatives of opportunistic flora.

It can be assumed that the children surveyed from the organized collectives had approximately the same characteristics of the state of microbial colonization of the throat and nose mucous, but some of them realized a high incidence of acute respiratory illness in the clinic, while the other part had sufficient compensation for local immunity, which is most likely due to properties and features of biofilm, the functional activity of which directly depends on the quality characteristics of normal microflora.

According to the results of present study in the period of clinical well-being in frequently ill children from pharyngeal mucosa were sowed 12 species, from the nasal mucosa — 9 species of bacteria. Thus, in the frequently ill children predominance of growth of streptococci of the viridians group (53.3 %), Staphylocococcus aureus (46.7 %) and gramnegative cocci — bacteria of the genus Neisseria (43.4 %) was registered. Slightly more rarely, in 26.6 % of
cases, opportunistic streptococci were sown from mucous of throat (Strep. haemolyticus and Strep. pyogenes — 13.3 %, respectively). Incidentally was registered growth of epidermal staphylococcus (5 % of observations), diphtheroids (Cor. pseudodiphtheribicum and Cor. xerosis — 5 and 3.3 % respectively) and fungi of the genus Candida (5 %).

In a few cases, the presence of Pseudomonas aeruginosa (1.6 %), bacteria of the genus Enterobacter (1.6 %) and E. coli (1.6 %) were registered in the mucous membrane.

Analysis of the results of crops from mucous membranes in frequently ill children showed that in the seeding of this level the leading role was played by grampositive cocci: epidermal (56.7 %) and golden (22.2 %) staphylococci. Non-toxic corynebacteria diphtheria or diphtheria were sown in 20 % of cases (Cor. pseudodiphtheribicum and Cor. xerosis, respectively, 10 % each). The remaining microorganisms were less common — Strep. viridans (13.3 %), Haemophilus influenza (6.7 %) and Neisseria spp. (3.3 %). In rare cases, it recorded growth of Strep. haemolyticus (1.6 %) and Strep. pyogenes (1.6 %).

Frequently ill children in the overwhelming majority of cases (75 %) have been registered the sowing of at least three or more species of bacteria, among which a high proportion fell on Staph. aureus and other opportunistic bacteria with high growth titers (> 10^4 cfu/ml). The normal flora dominated the mucous membranes of both the throat and nose, but it should be noted that a part of the examined children (20 % of cases) registered high growth titers of normal microflora of throat (Neisseriae spp., up to 10^6 and Strep. viridans up to 10^10) and nose (Cor. xerosis and Strep. viridans up to 10^7), which, in combination with the registration of a high growth titer of opportunistic pathogenic flora, allows to establish the presence of dysbiosis in 83.3 % of cases. This necessitated the study of the state of mucosal immunity.

According to the results of bacteriological examination of smears from the nose, oropharynx and sputum of children with recurrent bronchitis, it was established that the dominant microflora was from Haemophilus genus, which was found in 32 (47.1 %) patients. Among the strains of the pathogen H. influenzae found in 20 (29.4 %) patients, H. haemolyticus — in 4 (5.9 %), H. parainfluenzae — in 3 (4.4 %) and H. parahaemolyticus — in 5 (7.4 %) patients. In addition, 14 (20.6 %) children had S. pneumoniae as an etiological factor, 8 (11.8 %) had Klebsiella pneumonia, and 5 (7.4 %) had Enterococcus faecalis. Other pathogens were much less common, i.e. S. pyogenes was detected in 2 (2.9 %) children. Klebsiella pneumonia — in 3 (4.4 %) and Proteus mirabilis — in 1 (1.5 %) patients.

In 3 (4.4 %) patients, the presence of a fungal-bacterial association was established. Thus, for children with recurrent bronchitis, the primary colonization of the respiratory tract with a haemophilic rod was characteristic, which confirmed the literature data on the significant role of this microorganism in the development and support of inflammation in respiratory tract infections [11].

The results of an immunological study of frequently and episodically ill children in the period of clinical well-being are presented in table 1. According to our data, a significantly low content of antimicrobial protein α-defensin HNP1–3 and SIgA in the period of clinical health in the oropharyngeal secretion was registered in frequently ill children in comparison with the group of episodically sick children (p < 0.05). In 22 frequently ill children, concentrations of secretory immunoglobulin A were 5–10 times lower (SIgA ≤ 46 mg/L, 22 children) of healthy people 370–670 mg/l. The content of lactoferrin in the oropharyngeal secretion in frequently ill children was not significantly different from the episodically sick children.

The results of our evaluation of the content of antimicrobial proteins and secretory immunoglobulin A indicate a decrease in the level of protective immunity of mucous membranes and a strain of local defense mechanisms in children with frequent diseases, which corresponds to the expressiveness of the dysbiotic manifestations and was confirmed by the results of the conducted correlation analysis.

### Table 1. The state of mucosal immunity in frequently and episodically ill children in the period of clinical well-being, M ± m

<table>
<thead>
<tr>
<th>Indicators</th>
<th>Group of the frequently ill children, n = 42</th>
<th>Group of the episodically ill children, n = 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-defensins 1–3, pg/ml</td>
<td>2266.9 ± 93.4*</td>
<td>2702.46 ± 91.90</td>
</tr>
<tr>
<td>Lactoferrin, ng/ml</td>
<td>28.2 ± 2.5</td>
<td>32.1 ± 4.4</td>
</tr>
<tr>
<td>SIgA, mg/l</td>
<td>85.6 ± 9.7*</td>
<td>370–670</td>
</tr>
</tbody>
</table>

Note: * — p < 0.05 compared with a group of episodically ill children.

### Table 2. The content of some biologically active substances in plasma and serum in children with recurrent bronchitis, M ± m

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Patients with recurrent bronchitis, n = 32</th>
<th>Control group, n = 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-defensins, pg/ml</td>
<td>6576.7 ± 602.8**</td>
<td>3583.3 ± 735.4</td>
</tr>
<tr>
<td>Interleukin-6, pg/ml</td>
<td>10.1 ± 2.0</td>
<td>9.1 ± 1.1</td>
</tr>
<tr>
<td>Interleukin-10, pg/ml</td>
<td>4.42 ± 1.00*</td>
<td>1.41 ± 0.59</td>
</tr>
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Notes: * — p < 0.05 compared with the control group; ** — p < 0.01 compared with the control group.
The next stage of the work was to study the content of α-defensins 1–3 in the blood plasma of children with recurrent bronchitis (table 2).

According to the results of the study, the content of this antimicrobial peptide in the blood plasma of children of the control group was 3583.3 ± 735.4 pg/ml, while in the group of children with recurrent bronchitis the α-defensin level was 1–3 times higher than the results obtained almost twice and was 6576.7 ± 602.8 pg/ml, p < 0.01.

In the study of the serum cytokine profile, it was found that in children with recurrent bronchitis compared with the control group, the content of proinflammatory interleukin-6-cytokine, which regulates the inflammatory process and provides mobilization of the inflammatory response, as well as an antigen-specific immune response, had only a tendency to increase (10.1 ± 2.0 pg/ml vs 9.1 ± 1.1 pg/ml in the control group, p > 0.05). Against this background, we observed an increase of more than 3 times the level of antiinflammatory interleukin-10, which is a product of type 2 helper cells, responsible for the formation of a humoral antigenspecific immune answer — 4.42 ± 1.0 pg/ml vs 1.41 ± 0.59 pg/ml, respectively (p < 0.05). As is known, excess interleukin-10 leads to a decrease in antiinfective protection and may contribute to chronic inflammation in the respiratory tract [12]. Thus, there was an imbalance between pro- and antiinflammatory cytokines in children with recurrent bronchitis.

Discussion

Among the many molecular effectors of the non-specific defense system of the respiratory tract, the metal-binding protein lactoferrin occupies a special place and is evolutionarily the youngest member of the transferrin family, cationically active iron-binding glycoproteins [13, 14]. Lactoferrin is a natural antibacterial, antifungal and antiviral protein, has antioxidant and immunomodulating properties. Antibacterial properties of the protein are due to the ability of lactoferrin to bind iron and thereby deprive the bacterial microflora necessary for its growth and vital activity microelement [13]. The bactericidal properties of LF are also due to the presence of specific lactoferrin receptors on the cell surface of microorganisms, and the bacteriostatic effect is achieved due to intracellular changes in bacteria, without violating membrane permeability [15]. The bacteriostatic effect of apo-LF against Strep. mutans, S. salivarius, S. muttor, S. pneumonia, Vibro cholerae 5698, Pseudomonas aeruginosa has been proved [16]. LF also causes changes in the permeability of the outer membrane of some gramnegative bacteria [17], and the LF of cow’s milk shows bacteriostatic activity against of E. coli [18]. Lactoferrin has antiviral activity against a broad spectrum of human and animal viruses with DNA and RNA genomes [19]. At this point shown the effect of LF against herpes simplex virus 1 and 2, cytomegalovirus, HIV, hepatitis C virus, hantavirus, rotavirus, poliovirus first type, adenoviruses, respiratory syncytial virus [20–22], murine leukemia virus Freud [23]. The most studied mechanism of the antiviral activity of lactoferrin is the prevention of the entry of viral particles into target cells [24]. In addition to interaction with cellular receptors, lactoferrin directly binds to viral particles and prevents their penetration into cells, which is confirmed by the antiviral action of the LF protein against rotaviruses, for which the cellular receptors are hydrocarbon residues differing in composition from glycosaminoglycans.

Defensins are cationic peptides of the immune system that are active against bacteria, fungi and many enveloped and non-enveloped viruses [25]. Immune cells use defensins to kill bacteria, absorbed in phagocytosis. Typically, defensins attach to the cell membrane of the microbe and deepen into it, forming porous-like discontinuities [26, 27]. Among human antimicrobial proteins, α-defensins 1–3 are unique, neutrophils contain up to 99 % of all defensins, and therefore only neutrophils are practically the only source of HNP 1–3 in blood plasma and other body fluids [28, 29]. According to the published data, the increase of α-defensins is registered against a background of significant activation of acute or chronic inflammatory process, reaching a maximum at sepsis. Furthermore, HNP 1–3 have a bactericidal effect, chemotactic, immunomodulatory and cytotoxic activity. It has been proved that HNP 1–3 have antiviral effect, contributing to anti-HIV-1 activity of CD8 antiviral factor [30].

Taking into account the above data on the known mechanisms of action of antimicrobial proteins of lactoferrin and defensins and taking into account the results of the study, it can be stated that the frequently ill children have changes in the mucosal protection indexes, and a significantly low content of α-defensins 1–3 in the oropharyngeal secret in comparison with episodically ill children allows to assert about the lack in the FIC in the period of clinical well-being of acute or exacerbation of a chronic infectious process and the absence of activation or enhancement neutrophilic inflammatory process in the bodies of the examined children.

At the same time, at the present stage it is proved that high levels of α-defensins induce the release of interleukin-8 and neutrophil-activating protein 78 from respiratory epithelial cells, which leads to additional migration of polymorphonuclear leukocytes to the inflammatory focus [3, 31]. Excessive accumulation of neutrophils in the lung parenchyma and capillaries, in turn, contributes to a local “protease explosion” to damage the components of surfactant, the basement membrane of alveoli and of the endothelial cells. In addition, α-defensins 1–3 in high concentrations increase the permeability of the microcirculatory network both directly and by stimulation of mast cell degranulation [31, 32]. That is, under such conditions, the compensatory-adaptation reaction, aimed at overcoming the contamination of the pathogen, takes on the character of a pathological and acts as an additional factor in the destruction of the respiratory system. It should be noted that high concentrations of defensins inhibit the phagocytic activity of neutrophils [33]. Thus, in children with recurrent bronchitis, there is an increased production of α-defensins 1–3, in all probability, induced by bacterial agents. However, this superproduction of α-defensins can cause inhibition of phagocytosis by polymorphonuclear leukocytes, which leads to the appearance of recurrent forms of the infection process of the respiratory tract.
Conclusions

1. For frequently ill children in the period of clinical well-being is characteristically a decrease in the level of protective immunity of mucous membranes and strain of local defense mechanisms, which is manifested by a low content of antimicrobial protein α-defensins HNP-1, -3 and SlgA in the oropharyngeal secretion.

2. In children with recurrent bronchitis, there is an imbalance between pro- and anti-inflammatory cytokines, manifested by an increase in interleukin--10 serum levels against a background of insufficient synthesis of interleukin-6.

Conflicts of interests. Authors declare the absence of any conflicts of interests that might be construed to influence the results or interpretation of their manuscript.

References


Содержание антимикробных белков у детей с воспалительными заболеваниями респираторного тракта

Резюме. Актуальность. Воспалительные заболевания дыхательных путей часто связаны с нарушением синтеза антимикробных пептидов, представителями которых являются дефензины и лактоферин (ЛФ). Цель исследования заключалась в определении концентраций антимикробных пептидов в орофарингеальном секрете и плазме крови у детей с воспалительными заболеваниями дыхательных путей. Материалы и методы. Исследуемая группа включала 111 детей в возрасте от 4 до 17 лет из детских домов. Содержание антимикробных белков ЛФ, α-дефензинов 1–3 (человеческие нейтрофильные пептиды (ЧНП) 1–3), секреторного иммуноглобулина А в ротоглоточном секрете, ЧНП 1–3 в плазме крови, интерлейкина-6 (ИЛ-6) и -10 в сыворотке крови у детей определяли с использованием метода иммуноферментного анализа. Результаты. Анализ амнистических данных у соматически здоровых детей, больных рецидивирующим бронхитом, на фоне респираторных инфекций в течение года, подтвердил наличие от 4 до 6 эпизодов воспалительных заболеваний дыхательных путей. Установлено, что для запальних захворювань дихальних шляхів часто пов’язано з порушенням синтезу антимікробних пептидів, представниками яких є дефензини та лактоферин (ЛФ). Метою дослідження було встановлення концентрацій антимікробних пептидів в оофарингеальному секреті та плазмі крові у дітей з запального стани дихальних шляхів.

Матеріали та методи. Досліджувана група включала 111 дітей віком від 4 до 17 років із дитячих будинків. Уміст антимікробних білків ЛФ, α-дефензинів 1–3 (человеческие нейтрофильные пептиды (ЧНП) 1–3), секреторного іммуноглобуліну А в ротоглоточному секрєті та плазмі крові у дітей із запальними стани дихальних шляхів. Матеріали та методи. Досліджувана група включала 111 дітей віком від 4 до 17 років із дитячих будинків. Уміст антимікробних білків ЛФ, α-дефензинів 1–3 (человеческие нейтрофильные пептиды (ЧНП) 1–3), секреторного іммуноглобуліну А в ротоглоточному секрєті та плазмі крові у дітей із запальними стани дихальних шляхів.

Результати. Результати дослідження показали, що для запальних захворювань дихальних шляхів часто пов’язано з порушенням синтезу антимікробних пептидів, представниками яких є дефензини та лактоферин (ЛФ). Метою дослідження було встановлення концентрацій антимікробних пептидів у дітей із запальними стани дихальних шляхів.

Висновки. Встановлено, що для запальних захворювань дихальних шляхів часто пов’язано з порушенням синтезу антимікробних пептидів.