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Bactericidal capacity of oral neutrophils as a marker for clinical course of inflammatory respiratory diseases in children

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Abstract

Aim. To study the number of neutrophils in the oral cavity, their bactericidal potential, to assess as an indicator for predicting the course of recurrent bronchitis (J40) and community-acquired focal pneumonia in children.

Methods. 87 children between 5 and 10 years old, including 52 children with recurrent bronchitis and 35 with focal community-acquired pneumonia were observed. The control group consisted of 37 conditionally healthy children of a similar age. Viral antigens were studied by chemiluminescence immunoassay. Oral neutrophil counts and functional activity were determined. Antibacterial antibodies were measured by an enzyme-linked immunosorbent assay (ELISA).

Results. 70.11% of patients had a viral antigen, and 57.47% had immunoglobulins M and G against bacterial pathogens. Oral neutrophil counts increased in the main group compared to the control group: up to 163.8±26.5 cells ($p < 0.001$) in recurrent bronchitis, to 110.9±25.5 ($p < 0.05$) in community-acquired pneumonia. By the recovery period, the number of oral neutrophils counts decreased in recurrent bronchitis (1.7 times higher compared to the control group, $p < 0.01$) and remained practically unchanged in community-acquired pneumonia (115.0±26.9, $p < 0.05$). Myeloperoxidase level had opposite changes for the groups compared to the control group: with recurrent bronchitis, it was 1.61±0.09 to the level in the control group ($p < 0.05$), with community-acquired pneumonia — 0.73±0.09 to the level in the control group ($p < 0.001$). The level of lysosomal cationic proteins decreased to 0.77±0.09 to the level in the control group ($p < 0.05$) in recurrent bronchitis, and to 0.80±0.09 ($p < 0.05$) in pneumonia.

Conclusion. In inflammation of the respiratory tract, neutrophil migration to the oral cavity, as well as myeloperoxidase level, increases, indicators of spontaneous luminol-dependent chemiluminescence are activated, and a deficiency of lysosomal cationic proteins occurs; this prevents the penetration of the pathogen into the lower respiratory tract.

Keywords: oral neutrophils, children, recurrent bronchitis, community-acquired pneumonia, chemiluminescence.

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Background. The problem of bronchopulmonary system lesions in pediatric patients remains an urgent problem in modern medicine [1–4]. It is studied from different positions, including immunological ones [5, 6], and this approach has real grounds. First, the high sensitivity of indicator tests associated with the analysis of functional rearrangements in various parts of the immune system is well known [7]. Second, effector mechanisms of immunity are considered a probable pathogenetic determinant in the development of the inflammatory process in the respiratory tract [8,9].

Recently, there has been an increased interest in the multifunctionality of neutrophilic granulocytes [10–12], which are considered not only factors of exclusively antimicrobial protection but also effectors of structural hemostasis [13, 14].

It has been proven that in the macroorganism, the process of neutrophil migration from the vascular bed to the oral cavity occurs continuously. It is the neutrophils from oral secretion that serve as a starting barrier for penetrating an extensive flow of foreign agents into the body and implementing their pathogenic properties [15].

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To assess the balance of the microbicidal protection components of neutrophilic granulocytes, registration of oxygen-dependent and oxygen-independent mechanisms of pathogen destruction is proposed. The oxygen-independent function of microorganism destruction is implemented by cytotoxic enzymes (such as myeloperoxidase, lysosomal cationic proteins [LCP], and alkaline phosphatase) contained in neutrophil granulocytes [16].

Another informative and accessible way of probing the bactericidal function of oral neutrophils is luminol-dependent chemiluminescence (LDCL), which characterizes the generation of reactive oxygen species.

Thus, the protection of the child's body from the effects of infectious pathogens, the development of inflammatory lesion of the bronchopulmonary tract, and the nature of its course depend on the reserves of the homeostatic resources of neutrophils that migrated into the oral cavity and on their bactericidal activity.

The study aimed to analyze the oral neutrophil count and their bactericidal potential to assess the indicator role in predicting the course of recurrent bronchitis (RB) and community-acquired tabular pneumonia (CAP) in pediatric patients.

Materials and methods. The work was conducted in the pulmonology department of the Children's Hospital of the Central City Clinical Hospital No. 18 of Kazan and the Central Research Laboratory of the Kazan State Medical University. The analytical study, conducted in accordance with the ethical principles of good clinical practice, included 87 children aged 5 to 10 years, including 52 RB and 35 CAP pediatric patients (main groups). Exclusion criteria were exacerbated concomitant chronic diseases and inflammatory changes in the teeth and oral mucosa. The control group consisted of 37 conditionally healthy children of the same gender and age who had no signs of diseases in the aggregate of anamnestic and clinical and laboratory data.

The diagnosis was established based on a thorough analysis of anamnestic data and clinical results as well as paraclinical data and own studies. The clinical examination was performed over time, and it included the monitoring of children during the entire period of hospital stay. The children were examined by highly specialized doctors according to indications.

The antenatal and postnatal factors influence the formation of RB and pneumonia. In our study, we analyzed the course of pregnancy of mothers, the morbidity of parents, the nature of feeding, and the presence of concomitant diseases and medical and social factors.

Paraclinical examination included general blood tests, urinary tests, feces tests, and, according to indications, biochemical blood tests. Instrumental research methods were used, namely, external respiration function assessment with subsequent computer processing of the obtained results, chest X-ray, hepatobiliary system ultrasound, and electrocardiography.

The disease etiology was established in the laboratories of the Smorodintsev Research Institute of Influenza by detecting viral antigens in the epithelium of the nasal passages using standard fluorescent antibodies obtained at the Enterprise for the production of diagnostic preparations, as well as serologically in the complement binding reaction, hemagglutination inhibition, indirect hemagglutination, and enzyme immunoassay.

We used fluorescent immunoglobulins (Ig) to viruses of influenza A2 and B, parainfluenza, respiratory syncytial virus, rhinovirus and adenovirus, and *Mycoplasma pneumoniae* to verify pathogens in RB. The presence of viral antigen was confirmed in 70.11% of cases, including adenovirus in 28.74%, respiratory syncytial virus in 32.18%, parainfluenza virus in 9.2%, rhinovirus in 15.1%, and combined viral antigens in 14.78% of pediatric patients.

Specific antibodies of classes M and G were determined by enzyme immunoassay to verify etiologically significant bacterial pathogens in CAP. An IgM titer of 1:100 and higher and IgG titer of 1:80 were considered an active infectious process. Of pediatric patients, 57.47% had positive examination results, including Staphylococcus, *Haemophilus influenzae*, and atypical microbiota (*M. pneumoniae* and *Chlamydia pneumoniae*).

In our work, special research methods include the study of the count and functional activity of neutrophils that migrated to the oral cavity and their bactericidal potential. Obtaining informed consent, history taking, and examining the patients were performed with the approval of the Ethics Committee of Kazan State Medical University. Written informed consent was obtained from all participants before enrollment.

The methodology for studying the neutrophil count that migrated to the oral cavity included the following (Ashkenazi, 1998): Oral neutrophils were obtained in the morning on an empty stomach by rinsing the mouth with 10 mL of isotonic sodium chloride solution for 2 min. Then, the washes were placed in test tubes and centrifuged two times at 1500 rpm. After which, they were washed for 5 min with an isotonic sodium chloride solution, and thin uniform smears were prepared from the sediment. They were dried, fixed with 10% formalin alcohol

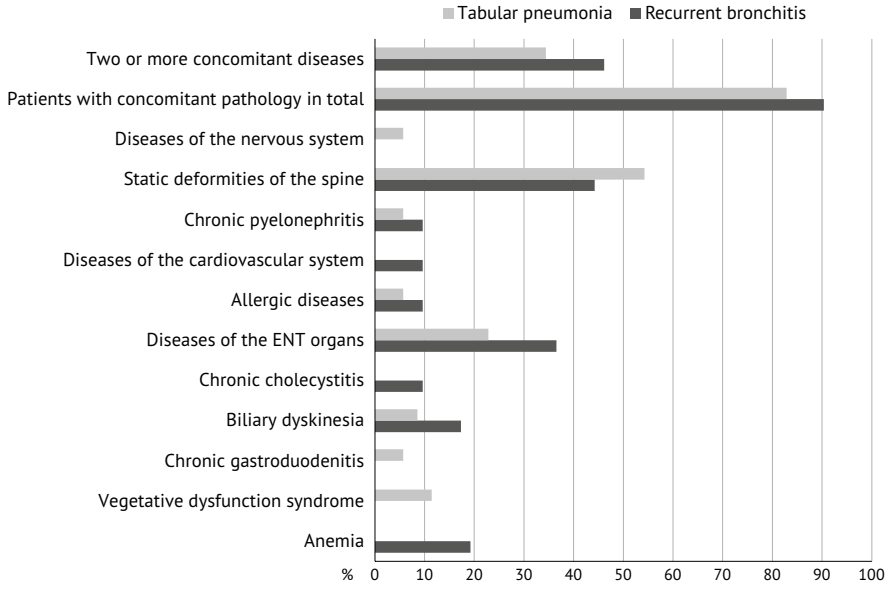

Fig. 1. Structure of concomitant chronic diseases in patients of the groups examined.

for 30–40 s, and stained by Romanowsky-Giemsa (15–20 min). In the finished smears, the absolute neutrophil count was counted through the microscope (at 900× magnification with oil immersion).

Myeloperoxidase in neutrophils was determined by the Graham–Knoll method, which is based on the oxidation of benzidine by the peroxide–peroxidase system into a brown product [17].

Cationic proteins in oral neutrophils were determined using the Slavinsky method [18].

The average cytochemical coefficient was calculated (conditional units) according to Kaplow [19].

Evaluation of the microbicidal function state of oral neutrophils was also investigated by the LDCL method on the CL 3403 apparatus. Indicators were calculated based on the number of pulses per minute. The results were registered using a conventional method by counting the number of pulses on the isoline without a stimulator (spontaneous test) and the number of pulses at the peak height with a stimulator (induced test). Recalculation was performed per 1000 polymorphonuclear neutrophils to unify the indices of induced LDCL.

Microsoft Excel 2010 program was used for statistical processing of the obtained results. The variation series was processed, and the calculation of arithmetic mean values (M), mean error (m), significance indicators (95%), and degree of relationship between the indicators calculated using the Pearson correlation coefficient was performed.

Results. The obstetric history of the parents showed that 23.39% ± 1.4% and 21.9% ± 1.1% of CAP and RB patients ($p = 0.043$ and 0.039 in relation to the control), respectively, had foci of chronic infection (chronic tonsillitis, sinusitis, etc.). The physiological course of pregnancy was noted in 33.06% ± 2.1% of mothers of CAP pediatric patients and 34.02% ± 1.9% of RB pediatric patients, and the rest of the women had a threat of miscarriage in 8.06% ± 0.8% ($p = 0.026$) and 7.85% ± 0.8% ($p = 0.022$) of cases, respectively, and gestosis in the first two trimesters of pregnancy in 45.97% ± 3.1% ($p = 0.034$) and 41.42% ± 2.9% of cases ($p = 0.027$), respectively. Pyelonephritis, anemia, and arterial hypertension of pregnant women were registered in 12.91% of cases in the main group.

Most of the children (70.97%) were born at term with a normal body weight. Of pediatric patients, 34.68% had asphyxia during labor, and 50.81% were breastfed. A burdened allergic anamnesis was recorded in 23.39% of patients. RB episodes were repeated two to four times a year, whereas 73.39% of the patients had an increased incidence of acute respiratory infections (more than eight times a year).

The premorbid body state is significant in the formation of the infectious and inflammatory process. In this regard, during the clinical and instrumental examination, we paid close attention to the presence and severity of concomitant diseases in the patients examined (Fig. 1).

It should be noted that at the time of examination of RB pediatric patients, all chronic diseases were in remission.

The changes in the general blood count were registered in 66.67% of the patients examined (moderate leukocytosis in 14.94% of cases,

Table 1. Oral neutrophil count in the children examined (conditional units)

Nosology	Oral neutrophil count			
	Acute period		Convalescence period	
	M ± m	p	M ± m	p
Recurrent bronchitis	163.8±26.5	<0,001	104,6±25,5	<0,05
Tabular pneumonia	110.9±25.5	<0,05	115,0±26,9	<0,05
Control	60.77±7.14			

Note: *p* is given in relation to the control.

decreased hemoglobin concentration in 10.34%, and increased erythrocyte sedimentation rate in 36.78%). Notably, the erythrocyte sedimentation rate increased in half of the cases with an unchanged total leukocyte count, which was regarded as a hyporeactive state. The leukogram was characterized by changes in the form of neutrophilia in 32.18% of cases, aneosinophilia in 16.09%, and lymphopenia in 27.59% (Fig. 2).

Generally, deviations from normal blood parameters in CAP patients were detected almost two times more than in RB patients.

Radiographically, bronchopulmonary pattern deformation and nonstructural lung root nature were revealed in RB patients and focal lung tissue infiltration in CAP patients. Summarizing the described clinical presentation of the patients monitored, a tendency to protracted course should be noted, as well as torpidity and repeated episodes of bronchopulmonary infection exacerbations with minor changes in general blood tests in more than half of the children examined, which we regarded as a reflection of the child's hyporesistance.

The oral neutrophil count was studied in all children examined. Thus, in the control group, the indices varied from 52 to 72 cells per smear, which averaged 60.77 ± 7.14 cells (Table 1).

Table 1 shows that there was a significant increase in the flow of neutrophils into the oral cavity in RB, and the mean values increased to 163.8 ± 26.5 cells ($p < 0.001$). A more severe course of the process when comparing paraclinical and clinical manifestations of the disease with different intensities of neutrophil migration into the oral cavity was registered when the number of oral neutrophils was less than 110 cells in a smear. At the same time, RB was protracted in nature with a long-lasting cough and persistent physical data. During conventional therapy, the quantitative indicators of oral neutrophils decreased by the recovery period, although they were 1.7 times higher than the control values ($p < 0.01$). By the time of discharge from the hospital, we noted a complete recovery of oral neutrophil count to the borderline values of the norm in only 22.99% of cases.

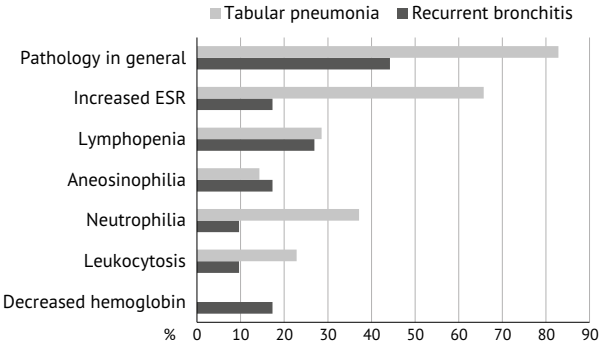

Fig. 2. Deviations from normal blood counts in the children examined.

In patients with tabular CAP, the migration of neutrophilic granulocytes did not increase, similar to that in patients with RB. The average number of oral neutrophils in the oral cavity was 110.9 ± 25.5 cells in a smear ($p < 0.05$), which also differed significantly from the control. By the period of clinical recovery, the indicators of granulocyte count remained practically unchanged (on average, 115.0 ± 26.9, $p < 0.05$). An inverse relationship between the initial values of the oral neutrophil count and the resolution duration of the inflammatory process in the lungs ($r = 0.69$, $p < 0.01$) was noted. The lower the indices of granulocyte count in the oral cavity were, the longer the physical changes in the lungs were clinically recorded.

Consequently, the analysis showed that the dynamics of the oral neutrophil count depended on the inflammatory process level in the respiratory tract. Thus, in RB, the migration of neutrophilic granulocytes into the oral cavity increased, which reflects mucosal immunity. At the same time, the slow migration of neutrophils from the systemic circulation, which was recorded in 36.54% of RB patients, may be a factor contributing to the penetration of an infectious agent into the respiratory tract, as evidenced by the low values of quantitative indicators in pediatric patients with tabular CAP.

Along with the assessment of oral neutrophil count, we studied the parameters of their microbicidal activity tested by cytochemical methods. Thus, in the control group, myeloperoxidase indi-

Table 2. Indicators of myeloperoxidase in oral neutrophils and lysosomal cationic proteins in the children monitored (conditional units)

Nosology	Myeloperoxidase level			
	Acute period		Convalescence period	
	$M \pm m$	p	$M \pm m$	p
Recurrent bronchitis	1.41±0.09	<0.05	1.45±0.03	<0.05
Tabular pneumonia	0.73±0.09	<0.001	0.69±0.09	<0.001
Control	1.26±0.09			
Nosology	Cationic protein level			
	Acute period		Convalescence period	
	$M \pm m$	p	$M \pm m$	p
Recurrent bronchitis	0.77±0.09	<0.05	0.72±0.09	<0.001
Tabular pneumonia	0.80±0.09	<0.05	0.68±0.08	<0.001
Control	1.21±0.08			

Note: p is given in relation to the control.

ces in oral neutrophils varied from 1.08 to 1.36 conditional units (1.26 ± 0.09; Table 2).

The study of myeloperoxidase levels in RB patients revealed its significant increase compared with that in the control (1.27 times). Myeloperoxidase levels correlated directly with oral neutrophil levels ($r = 0.63$, $p < 0.05$), and by the time of clinical recovery, myeloperoxidase levels in oral neutrophils has decreased. Thus, the average value in RB pediatric patients was 1.45 ± 0.03 conditional units, which still significantly exceeded the control values ($p < 0.05$), and only 32.69% of cases reached the standard values.

In contrast to RB patients, myeloperoxidase indices decreased to 0.73 ± 0.09 conditional units ($p < 0.001$) in tabular CAP, and during clinical recovery, the enzyme level in oral neutrophils almost did not change and amounted to 0.69 ± 0.09 conditional units ($p < 0.001$). Only 22.86% of patients had a tendency to increase myeloperoxidase levels.

LCP levels were also studied along with myeloperoxidase levels (Table 2).

The study of LCP level in RB patients revealed its decrease during the exacerbation period (0.77 ± 0.09 conditional units; $p < 0.05$). The presence of inverse correlations between the LCP level and the oral neutrophil number was registered ($r = -0.43$). During exacerbation in CAP, the average values of the enzyme were 0.80 ± 0.09 conditional units ($p < 0.05$). We registered low LCP indices in CAP pediatric patients 1.4 times more than in RB. During clinical recovery, there was also no normalization of LCP level despite the ongoing pneumonia therapy. A weak correlation between LCP level and oral neutrophil count in comparison with previous patients should be noted.

One of the aspects of our study was determining the biocidal activity of oral neutrophils in LDCL reaction (Table 3).

In the acute period of the pulmonary inflammatory process, the reactivity of neutrophilic granulocytes is activated, which is confirmed by a significant increase in spontaneous LDCL indicators. As a result, their metabolism is rearranged, and reactive oxygen species are formed.

The study of the antimicrobial potential of neutrophilic granulocytes in induced LDCL revealed a low response of neutrophilic granulocytes to stimulation with zymosan. In patients with tabular CAP, the index was 124.07 ± 19.05 pulses per minute ($p < 0.05$).

The indices of spontaneous LDCL in RB patients upon admission to the hospital were significantly increased compared with that in healthy children; however, with their high variability, they did not exceed the control values. Taking into account the results obtained, the etiological role of viral and bacterial associations in RB in pediatric patients cannot be ruled out.

In induced LDCL, the initial synthesis of reactive oxygen species was less pronounced in RB patients (167.26 ± 27.48 pulses/min). However, after hospital discharge, a significant decrease in induced LDCL was noted, which was up to 66.59 ± 9.6 pulses/min. The low response of oxygen-dependent metabolism of oral neutrophils to zymosan stimulation indicates a decrease in potential resources of granulocyte biocidal activity, which is more pronounced in RB patients.

Thus, despite the multifactorial nature of the processes involved in the development and course of diseases in pediatric patients, a decrease in the

Table 3. Indicators of luminol-dependent chemiluminescence in children examined over time

Indicators	Control (n = 37)	Tabular pneumonia (n = 35)	Recurrent bronchitis (n = 52)
Acute period			
SLDCL (pulses/min)	733.47±45.16	1612.63±58.97**	1496.58±72.3**
ILDCL (pulses/min/1000 PMNL)	137.72±22.28	209.03±7.8*	167.26±27.48
Upon discharge from the hospital			
SLDCL (pulses/min)	733.47±45.16	658.22±59.7	611.35±71.8
ILDCL (pulses/min/1000 PMNL)	137.72±22.28	133.1±21.3	66.59±9.6**

Note: Statistical significance of differences with the control group.

neutrophil functional activity is a common trigger for the disorder of the integrated activity of the organism in the external environment.

Discussion. The work presents a comparative analysis of the neutrophilic granulocyte count that migrated from the vascular bed, as well as indicators of their microbicidal status, depending on the lesion level of the respiratory tract.

There is probably a certain cascade of implementation of the bactericidal resources of neutrophilic granulocytes in the process of protecting the body against an infectious pathogen that enters the child's body through an aerogenic route. In RB, as the migration of neutrophilic granulocytes into the oral cavity increases, the myeloperoxidase level also increases, and indicators of spontaneous LDCL are activated. At the same time, a significant deficiency of LCPs acting as a modifier of enzymatic processes in the cell occurs. The resulting imbalance of enzymes is compensated by an increase in the oral neutrophil count, increase in myeloperoxidase levels, and spontaneous LDCL activation, which prevents pathogen penetration into the lower respiratory tract.

The oxygen-dependent mechanism of the destruction of microorganisms, provided by the substances contained in neutrophil granules, plays a special role in maintaining the homeostatic resources of oral neutrophils. When activated, the generation of reactive oxygen species and the mobilization of the bactericidal reserve increase rapidly. In particular, we have established an inverse correlation between spontaneous LDCL and LCP levels (with RB, $r = -0.65$, $p < 0.05$; with CAP, $r = -0.76$, $p < 0.05$). On the one hand, inhibition of the entry of oral neutrophils into the oral cavity and deficiency of the most important bactericidal enzymes in the cell (myeloperoxidase) affected negatively the nature of the course of RB with clinical manifestations of torpidity, a tendency to a protracted variant, and occurrence of repeated episodes of recurrent respiratory tract infection with a risk of disease chronicity. On the other hand, in a similar situation, pathogens enter the lower respiratory tract, followed by the development of an inflammatory process in the pulmonary parenchyma.

CONCLUSIONS

1. In inflammatory lesions of the respiratory tract, as the migration of neutrophils into the oral cavity increases, the level of myeloperoxidase level also increases, the indices of spontaneous luminol-dependent chemiluminescence are activated, and a deficiency of LCP occurs; this prevents the pathogen penetration into the lower respiratory tract.

2. Considering the data presented, it can be assumed that the homeostatic mechanisms of bactericidal activity of oral neutrophils are balanced, and when one factor is inhibited, another one is increased. Therefore, by testing the amount of neutrophils that migrated into the oral cavity from the vascular bed and their cytotoxic potential, the course of the inflammatory process in the bronchopulmonary system can be predicted, and the metabolic disorders revealed in granulocytes can be timely corrected.

3. Evaluation of the cytopathogenicity of neutrophils migrated into the oral cavity has a number of advantages, since, with high information content, it is one of the atraumatic methods of examining children and can provide practical assistance to a doctor with a differentiated approach to the choice of rational therapy management.

Author contributions. O.I.P. was responsible for the analysis and interpretation of the results; R.A.F. was the work supervisor; A.M.Z. and Z.Ya.S. conducted research and were responsible for collecting results; E.L.R. and E.V.V. worked with medical documents, collected the material, conducted the literature review, and translated the article abstract into English.

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