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Neutrophil extracellular traps in the anti-infectious defense of human organism

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ABSTRACT

Background. Neutrophil extracellular traps (NETs) are net-like structures that have been investigated in inflammatory diseases. However, the presence of NETs in infected persons without clinical symptoms has not been yet studied.

Aim. To reveal NETs in healthy persons during and after the H1N1 influenza pandemic as well as to study the functional activity of NETs.

Materials and methods. The study included two groups of volunteers (n = 10 in each group) aged 20–25 years. The first group of volunteers was examined in the absence of acute diseases during one month before the study and in the absence of chronic diseases in the medical history. Volunteers of the second group were in contact with patients with influenza, but did not get sick. The comparative study also included patients with acute inflammation in the abdominal cavity (appendicitis, cholecystitis, abscess; 12 patients) and 9 patients with non-specific ulcerative colitis. Neutrophils were isolated from the blood by the traditional method of Ficoll density centrifugation. The number, morphology, and functional activity of NETs were determined (by capture of *Klebsiella pneumoniae*). SYBR Green I-based fluorescence microscopy was used to visualize and quantify NETs.

Results. In healthy volunteers who were not in contact with infected patients, spontaneous NETs formation did not occur. Neutrophils of persons who were in contact with infected patients spontaneously formed NETs. In this case the number of NETs reached 8.58 ± 0.51%, and the size of NETs amounted to 39.68 ± 3.52 μm. NETs effectively captured cells of the tested microorganism, which was accompanied by retraction of network fibers and transformation of the network structure into a cloud-like one, which retained 89.38 ± 5.86 microbial cells. For comparison, the NETs in patients with acute inflammation in the abdominal cavity captured and bound 20.2 ± 1 .67 microbial cells and with non-specific ulcerative colitis – 5.53 ± 0.34 cells.

Conclusion. High binding capacity of NETs is a factor contributing to effective defense of the body against the development of an infectious disease with manifested clinical symptoms.

Keywords: functional activity of NETs, neutrophil extracellular traps, cloud-like appearance, healthy volunteers, H1N1 influenza, *Klebsiella pneumoniae*, inflammatory diseases

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at Pirogov Russian National Research Medical University (Protocol No. 203 of 21.12.2021).

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Противоинфекционная защита организма человека с участием нейтрофильных сетей

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РЕЗЮМЕ

Введение. Нейтрофильные экстраклеточные ловушки (НЭЛ) в форме сетей исследованы при воспалительных заболеваниях. Присутствие нейтрофильных сетей у инфицированных людей без клинических симптомов не изучено.

Цель. Выявление НЭЛ у неболеющих людей в период пандемии гриппа H1N1 и вне этого периода, а также исследование функциональной активности НЭЛ.

Пациенты и методы. Две группы волонтеров (по 10 человек) в возрасте 20–25 лет. Первая группа добровольцев обследована в спокойный эпидемиологический период, при отсутствии острых заболеваний в течение 1 мес до исследования и хронических заболеваний в анамнезе. Волонтеры 2-й группы контактировали с больными гриппом, но при этом не заболевали. В исследование также были включены больные (12 человек) с острым воспалением в брюшной полости (аппендицит, холецистит, абсцесс) и 9 – с неспецифическим язвенным колитом. Нейтрофилы выделяли из крови традиционным методом на градиенте фиколла. Определяли количество, морфологию и функциональную активность НЭЛ (по захвату *Klebsiella pneumoniae*). Для визуализации и подсчета НЭЛ использовали флуоресцентную микроскопию с красителем SYBR Green.

Результаты. У здоровых волонтеров, не контактировавших с больными, спонтанного формирования НЭЛ не возникало. Нейтрофилы же добровольцев, контактировавших с больными, спонтанно формировали нейтрофильные сети. Количество НЭЛ у них достигало 8,58 ± 0,51%, а размеры НЭЛ – 39,68 ± 3,52 мкм. НЭЛ эффективно захватывали клетки тестового микроорганизма, что сопровождалось ретракцией волокон сети и преобразованием сетевидной структуры в вуалеобразную, которая удерживает 89,38 ± 5,86 микробных клеток. Для сравнения: нейтрофильная сеть больных с острым воспалением брюшной полости захватывает и связывает 20,2 ± 1,67 микробных клеток, при неспецифическом язвенном колите – 5,53 ± 0,34.

Заключение. Высокая связывающая способность нейтрофильных сетей является фактором эффективной защиты от развития инфекционного заболевания с манифестацией клинических симптомов.

Ключевые слова: функциональная активность нейтрофильных сетей, нейтрофильные экстраклеточные ловушки, вуалеобразная форма, не болеющие волонтеры, грипп H1N1, *Klebsiella pneumoniae*, воспалительные заболевания

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

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. Все участники исследования подписали добровольное информированное согласие. Исследование одобрено этическим комитетом РНИМУ им. Н.И. Пирогова (протокол № 203 от 21.12.2021).

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INTRODUCTION

Neutrophils form neutrophil extracellular traps (NETs) as a body defense mechanism against pathogens. In previous studies on responses of cells of innate immunity (neutrophils) to viral infections (hantaviruses, adenoviruses, human immunodeficiency virus, influenza viruses), data were obtained on the formation of NETs in patients with various acute viral diseases [1–3]. At the same time, researchers found that excessive formation of NETs is dangerous, as it can cause tissue damage and lead to complications of the underlying disease [3–6]. The role of neutrophils in viral infection has its own particular features, and

the threshold at which protective functions are surpassed by damage mechanisms, including immunopathological ones, is still quite unclear.

Recent studies have established that influenza viruses are capable of inducing the formation of NETs in the blood of healthy donors *in vitro* [7, 8]. At the same time, it was found that the formation of NETs in the form of net-like structures, as a response of innate immunity cells contributing to the fight against acute viral infection, is accompanied by a pronounced antiviral effect that helps control the virus at the systemic level [9]. It is generally accepted that NETs are absent in the blood of healthy people and are found only during inflammation. NETs have been described in detail, but their presence in the body of people without clinical manifestations of the disease is unknown. There is no exact data on the presence of NETs in the peripheral blood of people who are in contact with infected individuals but do not become ill. In addition, the mechanisms underlying the formation of NETs are still unclear.

Most researchers are not aware of various morphological forms of NETs; their functional activity has not been studied. One approach to elucidating these issues is to determine the content of NETs, their morphological structure, and functional activity in people who do not become ill during and after an influenza pandemic.

Th aim of the study was to reveal NETs in healthy persons during and after the H1N1 influenza pandemic as well as to study the functional activity of NETs.

MATERIALS AND METHODS

The study included two groups of volunteers (n = 10 in each group) aged 20–25 years. The inclusion criterion was the absence of acute diseases at the time of the study and chronic diseases in the medical history. The exclusion criterion was acute infectious diseases within a month before the day of the study.

Volunteers of the first group were examined in May – June 2022. During this period, the lowest morbidity rates for influenza and COVID-19 were recorded. Members of this group had no contact with people suffering from acute respiratory diseases.

Volunteers of the second group were examined in December 2022. During this period, the epidemic threshold for H1N1 influenza was exceeded in children and adults. Moreover, the volunteers of the second group during the month preceding the study were in constant contact with H1N1 influenza patients but did not get sick themselves.

The study also included patients treated in Moscow clinical hospital No. 51. Patients of the first group (n = 12) were hospitalized with acute inflammation in the abdominal cavity (acute appendicitis, acute cholecystitis, abscess). Patients of the second group (n = 9) were diagnosed with non-specific ulcerative colitis.

The blood test was carried out at the Department of Pathophysiology and Clinical Pathophysiology of Pirogov Russian National Research Medical University. All procedures were performed in accordance with the ethical standards of the WMA Declaration of Helsinki (as amended in 2004) and the patient's written informed consent. The study was approved by the Ethics Committee at Pirogov Russian National Research Medical University (Protocol No. 203 of 21.12.2021).

Determining NETs content. Obtaining cell fractions of neutrophils. The Vacutainer EDTA blood collection tubes were used to sample blood from volunteers to prevent clotting. Isolation of neutrophils from the EDTA-treated venous blood was performed by Ficoll density gradient centrifugation. To do this, the blood was diluted 4 times with sodium phosphate buffer solution, pH 7.4, and layered on the Ficoll – Hypaque density gradient. The top layer density was 1.077 g / cm^3, and the density of the bottom layer was 1.190 g / cm^3. After centrifugation (1,600 rpm, 30 min), neutrophils accumulated at the interface between the gradients (98−100% purity). Neutrophils were twice washed with sodium phosphate buffer (50 mM, pH 7.4) to remove Ficoll impurities. Sedimentation of blood cells was performed by centrifugation (1.200 rpm, 15 min). The isolated neutrophils in the RPMI-1640 medium were used for cell culture experiments. The viability of the isolated neutrophils was 95 % (test with 0.1% trypan blue solution).

Immunofluorescence detection of NETs. Fluorescence microscopy was used for detection and quantification of NETs [10]. The results were expressed as a percentage, as the ratio of the number of NETs to the total number of cells in the field of view. NETs were detected using the SYBR Green I fluorescent dye (Evrogen, Russia), which is able to bind specifically to double-stranded DNA. Microscopy, quantification, and photo registration of cells and extracellular structures were performed at ×1,000 magnification.

Capture of the test microorganism. The functional activity of NETs was determined using the test for capturing *Klebsiella pneumoniae* (ATCC 700603). To do this, the microbial culture of *Klebsiella pneumoniae* in the RPMI-1640 medium at a concentration of

10^3 / µl was added to neutrophils immobilized on the poly-L-lysine-coated glass slides. Net-like structures captured the test microorganism in accordance with the potential functional activity of NETs. After staining (SYBR Green, 15 min) and removing excess dye during microscopy, the number of *Klebsiella pneumoniae* cells associated with each NET was determined.

The STATISTICA 12.0 software package (StatSoft Ink., USA) was used for statistical data processing. The results were reported as the mean and the standard error of the mean ($M \pm m$). Comparison of quantitative variables was performed using the Mann – Whitney U-test and the Kruskal – Wallis test. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Quantitative and qualitative characteristics of NETs were determined in *in vitro* research. We determined variants of their morphological structure, quantity, size, and functional activity (in the test with the capture of *Klebsiella pneumoniae*).

The results of the study in two groups of volunteers (those who had no contact with infected people (Group 1) and those who had contact with infected people, but did not have clinical signs of the disease (Group 2)), are shown in Table.

Table

Results of the study of NETs in healthy volunteers, $M \pm m$

Characteristics of the groups	Parameters of NETs		
	Morphological structure of NETs	Number of NETs, %	Size of NETs, µm
Volunteers who had no contact with infected patients, $n = 10$	–	0.00	0.00
Volunteers who contacted with infected patients and did not get sick, $n = 10$	net-like structure	8.58 ± 0.51*	39.68 ± 3.52*

* $p < 0.05$ compared to the controls

In healthy volunteers who had no contacts with infected people and were examined not during the pandemic (Group 1), spontaneous formation of net-like NETs did not occur. The neutrophils of the volunteers from Group 1 were presented as classic mature segmented granulocytes (Fig. 1).

In healthy volunteers who contacted with infected people (Group 2), the results were different. In this case, neutrophils spontaneously formed net-like traps (Fig. 2, 3). The number of neutrophil extracellular traps was 8.58 ± 0.51%, and the size of NETs amounted to 39.68 ± 3.52 µm. Neutrophils in this group spontaneously formed net-like structures without any additional stimulation. Consequently, volunteers who had contacts with infected patients were infected themselves, and their neutrophils were previously activated.

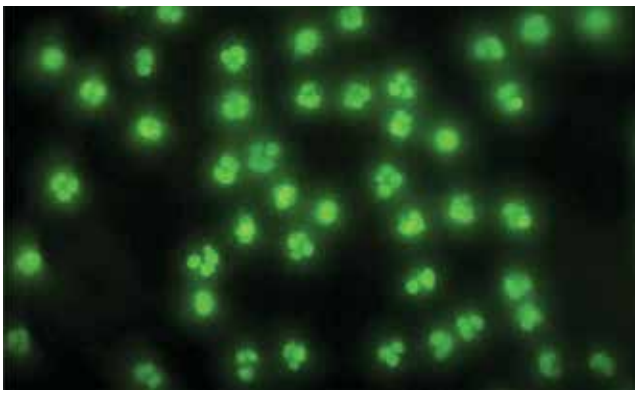

Fig. 1. Neutrophils of healthy people who had no contact with infected patients. Staining with CYBR Green I, x1,000.

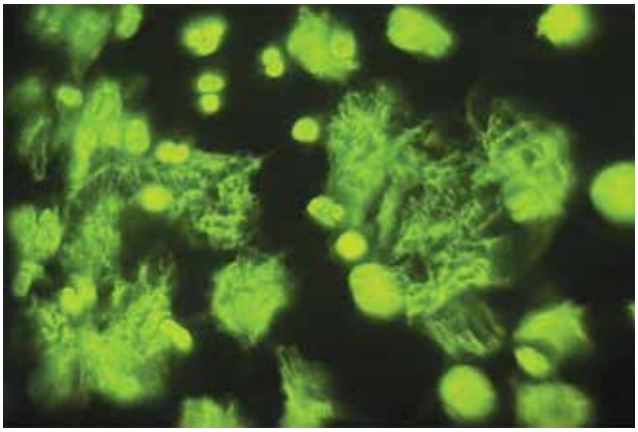

Fig. 2. NETs of people who had been in contact with infected patients but did not get sick: incubation time 1 hour. Staining with CYBR Green I, x1,000.

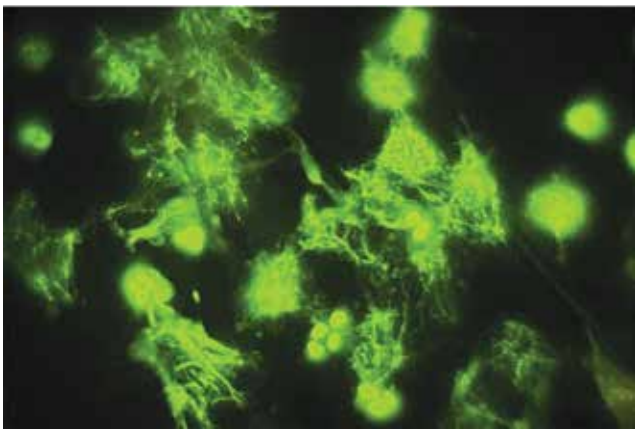

Fig. 3. NETs of people who had been in contact with infected patients but did not get sick: incubation time 1 hour. Staining with CYBR Green I, x1,000.

In the next series of experiments, the functional activity of the NETs found in the healthy volunteers who had contacts with infected patients (Group 2) was determined in the test with the capture of *Klebsiella pneumoniae* (ATCC 700603).

Neutrophils of the volunteers from Group 2 were added to the cells of the test microorganism *Klebsiella pneumoniae* placed on the glass slides (Fig. 4). The neutrophils of the disease-free volunteers who were examined during an unfavorable epidemiological period formed NETs (Fig. 2, 3) and captured the cells of the test microorganism. It was accompanied by retraction of network fibers and transformation of the net-like structure into a cloud-like one. Moreover, the size of the newly formed cloud-like traps, together with the captured cells of *Klebsiella pneumoniae*, became 2–3 times smaller than the size of the original net-like traps (Fig. 6). Our observations show that the capture of the test microorganism cells was very effective. An extensive array of *Klebsiella pneumoniae* cells was almost completely cleared of this pathogen, and all microorganisms were trapped inside the cloud-like structures (Fig. 4). Each cloud-like structure that originated from the net-like NET of the volunteers who were not ill but had contacts with infected people captured and retained 89.38 ± 5.86 cells of *Klebsiella pneumoniae* (Fig. 5).

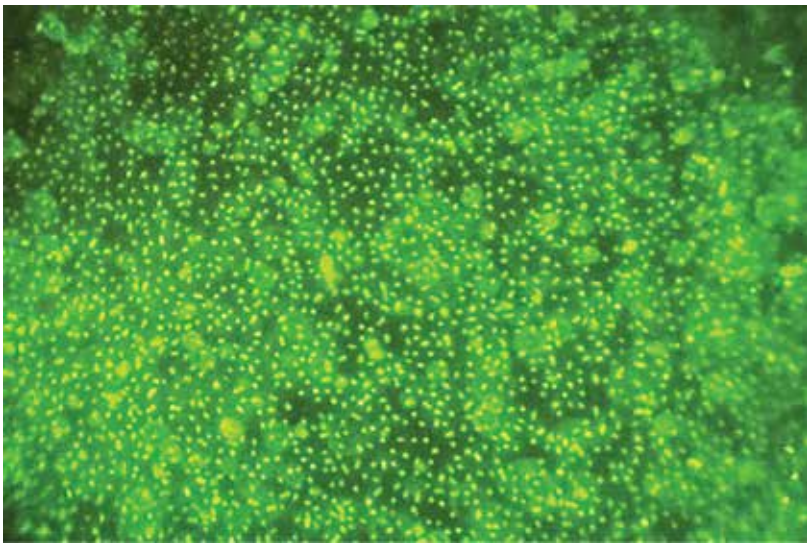

Fig. 4. Cells of *Klebsiella pneumoniae* (ATCC 700603) placed on glass slides. Staining with CYBR Green I, x1,000

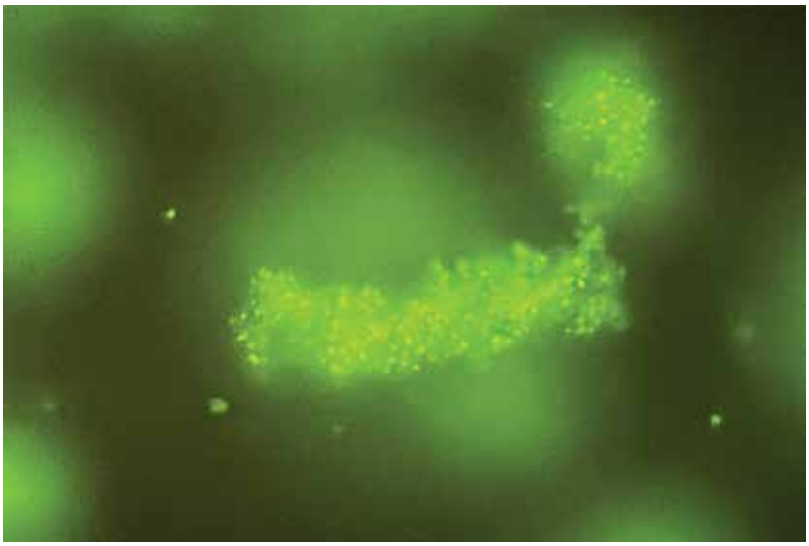

Fig. 5. Net-like NETs effectively capture and bind *Klebsiella pneumoniae* cells and turn into cloud-like structures. Incubation time of NETs with *Klebsiella pneumoniae* cells is 2 hours. Staining with CYBR Green I, x1,000

For comparison, the functional activity of neutrophils in the disease-free volunteers was compared with the results obtained in the test with the capture of *Klebsiella pneumoniae* in two other groups of patients. We studied the capture and binding of test microorganism cells by NETs in patients with acute infection and inflammation in the abdominal cavity (acute appendicitis, acute cholecystitis, abscess) and in patients with non-specific ulcerative colitis.

The functional activity of NETs in patients with acute infection and inflammation in the abdominal cavity was reduced. Each NET in such patients captured and bound 20.2 ± 1.67 microbial cells. The binding capacity decreased by more than 4 times compared to clinically healthy, disease-free volunteers (Fig. 6).

The study of the capturing and binding capacity of NETs the in patients with ulcerative colitis revealed a very weak functional activity of NETs in this group of patients. The number of Klebsiella pneumoniae microorganisms captured by NETs in patients with ulcerative colitis was only 5.53 ± 0.34 microbial bodies. Thus, the results of the comparative study of cell binding capacity in relation to the test microorganism (Fig. 7) showed that NETs in the healthy volunteers who had contacts with infected patients had the highest binding capacity. High binding capacity of NETs in such patients, apparently, was a factor in their effective protection from the development of the infection with manifestation of clinical symptoms.

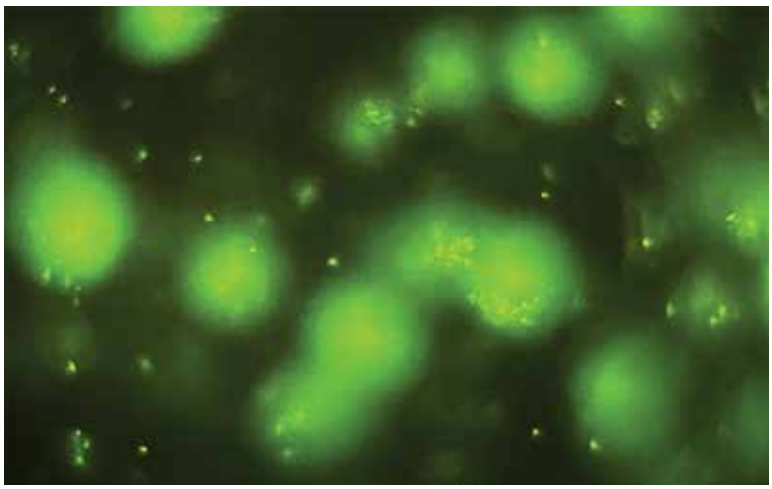

Fig. 6. Weakening of pathogen binding in patients with acute inflammation in the abdominal cavity. Net-like NETs of patients with inflammation in the abdominal cavity capture and bind *Klebsiella pneumoniae* cells and turn into cloud-like structures. Incubation time of NETs with *Klebsiella pneumoniae* cells is 2 hours. Staining with CYBR Green I, x1,000

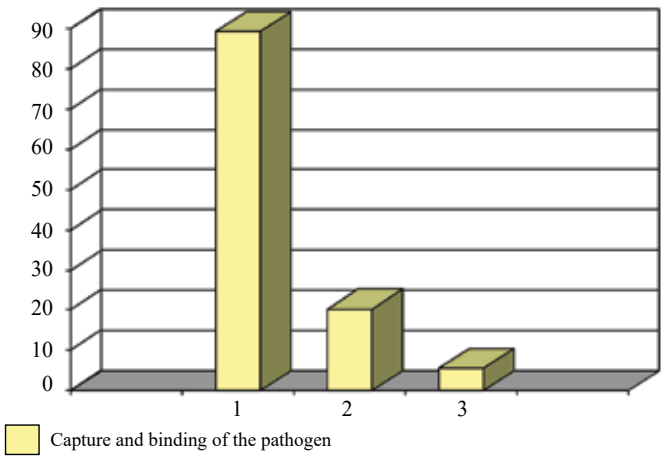

Fig. 7. Capture and binding of the *Klebsiella pneumoniae* pathogen by NETs in healthy people and in patients with various inflammatory diseases: on the vertical axis – the number of microbial bodies captured by one neutrophil structure, on the horizontal axis – healthy volunteers who had contact with infected patients (1); patients with acute inflammation in the abdominal cavity (2); patients with non-specific ulcerative colitis (3)

NETs in the patients with acute infection and inflammation in the abdominal cavity (appendicitis, cholecystitis, abscess) had a reduced capturing and binding capacity; however, it still remained at a fairly high level. The transformation of the net-like NETs into cloud-like structures in such patients was accompanied by weakening of their binding capacity in relation to the cells of the test microorganism.

NETs in the patients with ulcerative colitis had the lowest ability to bind pathogen cells. The weakening of the capturing and binding capacity of NETs in patients with this chronic autoimmune disease indicates reduced functional activity of NETs and, apparently, underlies comorbidity in patients with ulcerative colitis.

CONCLUSION

Obtaining detailed information about the morphological variants of NETs is based on the adequate method for their visualization, which we described earlier [10]. The results of the *in vitro* study demonstratев the structure of NETs and changes in their morphology during the response of neutrophils to the pathogen, which indicates the crucial role of these innate immunity cells in the body defense against infection.

The results obtained showed that the group of disease-free volunteers who were in contact with infected people were protected from infection due to the effective work of the neutrophil link of innate immunity. Their neutrophils were already pre-activated, as they were able to spontaneously form NETs. Net-like traps very effectively captured pathogen cells, developed fiber retraction, and turned into compact cloud-like structures. Then these structures naturally underwent phagocytosis; intracellular hydrolysis developed, and antigen presentation occurred. From this moment, the adaptive immunity response began.

In the patients with acute infection and inflammation in the abdominal cavity (appendicitis, cholecystitis, abscess), the experiment confirmed weakening of the capturing and binding capacity of NETs, which in the clinical setting can increase the risk of developing complications of the underlying disease.

The functional activity of NETs was drastically weakened in the patients with ulcerative colitis, which explains increased sensitivity to infection and high comorbidity in these patients.

The results of the study show that body defense against infection with the participation of innate immunity cells consists in the effective capture and binding of the pathogen. Weakening of the binding capacity of NETs carries a potential risk of complications.

The authors draw attention to the fact that this is the first study devoted to the study of the functional activity of NETs in the human body.

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Authors' contribution

Kazimirskii A.N. – carrying out the experiment, drafting of figures and diagrams, drafting of the article. Salmasi J.M. – editing of the article. Poryadin G.V., Stupin V.A. – conception and design. Panina M.I. – editing of the article, statistical processing of the material. Kim A.E. – carrying out the experiment. Titova E.G. – carrying out the experiment, collection and processing of the material. Rogozhina L.S. – collection and processing of the material.

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