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Transformation of NETs under the effect of pathogens and IgG

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ABSTRACT

Background. Many studies have shown that neutrophil extracellular traps (NETs) in the form of web-like structures are present in the peripheral blood of patients with inflammatory diseases. In our research, in addition to traditional web-like NET structures, several anomalous forms were identified, including NETs with cloud-like appearance.

Aim. To investigate morphological and functional transformation of NETs under the influence of *Klebsiella pneumoniae* and immunoglobulin G (IgG).

Materials and methods. The study included 42 patients of Moscow City Clinical Hospital No. 51: 28 patients with acute inflammation in the abdominal cavity (appendicitis, cholecystitis, pancreatitis, peritonitis), 6 patients diagnosed with ulcerative colitis, and 8 patients with hernias. Neutrophils were isolated using gradient-density centrifugation. To calculate NETs, we used SYBR Green I-induced fluorescence microscopy (Evrogen, Russia), with the dye specifically interacting with double-stranded DNA. The functional activity of NETs was determined in the *Klebsiella pneumoniae* (ATCC 700603) capture test.

Results. In patients with inflammatory diseases of the abdominal cavity in the postoperative period, the functional activity of NETs was several times lower than in healthy individuals. NETs in these patients capture and bind no more than 20 cells of the microorganism. Under the effect of IgG, neutrophil networks transform into loose cloud-like structures, which can hardly capture and bind the pathogen, binding only 8.46 ± 0.44 cells of the microorganism. Spontaneous enzymatic degradation of cloud like NETs may be accompanied by the production of secondary alteration factors.

Conclusion. The results of the study provide the grounds for the development of new approaches to elaborating vaccination regimens and using immunobiologics that require preliminary monitoring of the state of innate immunity, in particular, neutrophil networks in the patient's body.

Keywords: neutrophil extracellular traps, neutrophil web-like structure, neutrophil cloud-like structures, inflammation, NET functional activity, vaccination, immunobiological therapy

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Conformity with the principles of ethics. All study participants 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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Трансформация нейтрофильных сетей под влиянием патогенов и иммуноглобулинов класса G

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РЕЗЮМЕ

Введение. Исследования многих авторов показали, что в периферической крови пациентов с воспалительными заболеваниями присутствуют нейтрофильные экстраклеточные ловушки (НЭЛ, NETs) в морфологической форме нейтрофильных сетей. В наших исследованиях помимо традиционной структуры НЭЛ в виде нейтрофильных сетей были выявлены некоторые аномальные формы, в том числе и вуалеобразные формы НЭЛ.

Цель. Исследование морфофункциональной трансформации НЭЛ под влиянием *Klebsiella pneumoniae* и иммуноглобулинов класса G (IgG).

Материалы и методы. В исследование включены 42 больных 51-й ГКБ г. Москвы: 28 – с острыми воспалительными процессами в брюшной полости (аппендицит, холецистит, панкреатит, перитонит), шесть – с диагнозом «язвенный колит», восемь – с грыжами. Нейтрофилы выделяли, используя градиентное центрифугирование. Для подсчета НЭЛ использовали флуоресцентную микроскопию с красителем SYBR Green (ЗАО «Евроген», Россия), специфично взаимодействующего с двухцепочечной ДНК. Функциональную активность НЭЛ определяли в тесте с захватом *Klebsiella pneumoniae* (ATCC 700603).

Результаты. У больных с воспалительными заболеваниями брюшной полости в послеоперационном периоде функциональная активность НЭЛ ослаблена по сравнению со здоровыми в несколько раз. Нейтрофильные экстраклеточные ловушки у этих больных захватывают и связывают не более 20 клеток микроорганизма. Под влиянием IgG нейтрофильные сети превращаются в рыхлые вуалеобразные структуры. Эти нейтрофильные структуры обладают очень слабой способностью к захвату и связыванию патогена, соединяя 8,46 ± 0,44 клеток микроорганизма. Спонтанная ферментативная деградация нейтрофильных «вуалей» может сопровождаться продукцией факторов вторичной альтерации.

Заключение. Результаты исследования создают предпосылки для формирования новых подходов к разработке режимов вакцинации и применения иммунобиологических препаратов, требующих предварительного контроля состояния врожденного иммунитета, в частности, нейтрофильных сетей в организме пациентов.

Ключевые слова: нейтрофильные экстраклеточные ловушки, нейтрофильные сети, нейтрофильные вуали, воспаление, функциональная активность НЭЛ, вакцинация, лечение иммунобиологическими препаратами

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 информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом РНИМУ им. Н.И. Пирогова (протокол № 203 от 21.12.2021).

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INTRODUCTION

Components of innate immunity and, in particular, neutrophils form the first line of defense against various foreign agents (viral, bacterial, etc.) invading the human body. Numerous studies have shown that the protective function of neutrophils is implemented through the formation of neutrophil extracellular traps (NETs). The NET formation is an effective mechanism for combating invading microorganisms, and the lack of NET formation or hydrolysis of the main NET nucleotide chain by

bacterial DNases makes the human body susceptible to infections [1].

NETs are a form of reaction of pre-activated neutrophils to interactions with host cells in a state of apoptosis, as well as various microorganisms, including viruses [2]. Neutrophils receive signals to release NETs through various innate immunity receptors (TLRs).

Many authors in their studies have shown that NETs are present in the peripheral blood of patients with inflammatory diseases in the form of neutrophil web-like structures. However, in our research, in addition to traditional web-like NET structures, some anomalous forms were identified, including NETs with cloud-like appearance [3].

These cloud-like NETs can be formed under the effect of immunoglobulin G (IgG) [4]. Some reports also mention cloud-like NETs that were found in the blood of patients with inflammatory diseases and in some healthy persons [5, 6]. However, the causes underlying the formation of various forms of NETs are still unclear. The functional role of web-like and cloud-like NETs is also unclear.

The aim of the study was to investigate morphological and functional transformation of NETs under the influence of *Klebsiella pneumoniae* and IgG.

MATERIALS AND METHODS

The study included 42 patients treated at Moscow City Clinical Hospital No. 51: 28 patients who underwent surgery for acute inflammatory processes in the abdominal cavity (acute appendicitis, acute cholecystitis, acute pancreatitis / necrotizing pancreatitis, peritonitis), 6 patients with ulcerative colitis undergoing non-surgical treatment, and 8 patients with umbilical and inguinal hernias (5 patients did not undergo surgery, while 3 patients underwent surgery).

The study of blood samples was carried out in the laboratory at the Department of Pathological Physiology and Clinical Pathological Physiology of the Institute of Human Biology and Pathology (Pirogov Russian National Research Medical University). All procedures were performed in accordance with the ethical principles of the WMA Declaration of Helsinki. 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).

Determining the composition of NETs

Obtaining neutrophil cell fractions. Vacutainer EDTA blood collection tubes were used for blood sampling. Neutrophils were isolated from venous blood treated with EDTA by the traditional method using gradient density centrifugation. The purity of the isolated neutrophil fraction was 98–100%. Neutrophils were washed twice from Ficoll impurities with a sodium phosphate buffer solution (50 mM, pH 7.4). Blood cells were precipitated by centrifugation (600 g, 15 min). Isolated neutrophils were resuspended in the RPMI-1640 medium and used in short-term culture experiments. The viability of isolated neutrophils was at least 95% (test with 0.1% trypan blue solution).

Immunofluorescence detection of NETs. We developed a method using fluorescence microscopy, the main stages of which were described earlier, and used it to register NETs [7]. NETs were detected using a fluorescent SYBR Green-I dye (Evrogen; Russia), which specifically interacts with double-stranded DNA. Microscopy, counting, and photo registration of cells and extracellular structures were performed at x 1,000 magnification. The results were expressed as a percentage, the ratio of the number of extracellular traps to the total number of cells in the field of view.

Culture of neutrophils with IgG. Human IgG preparation (Sorbent, Russia) was added to the sterile isolated cells and incubated with the cells in an atmosphere of 5% CO_2 at 37 °C for 1 hour. A 100 µl sample prepared in the RPMI-1640 medium contained neutrophils and IgG preparation (5 µg / ml). The final concentration of cells in the culture medium was 2×10^5 / ml.

Capturing a test microorganism. The functional activity of web-like NETs was determined using the *Klebsiella pneumoniae* capture test (ATCC 700603). To do this, a microbial culture of *Klebsiella pneumoniae* in the RPMI-1640 medium at a concentration of 10^3 / µl was added to neutrophils immobilized on poly-L-lysine coated glass slides. Web-like NETs capture the test microorganism in accordance with their potential functional activity. After staining (SYBR Green-I, 15 min) and washing off the excess dye, the morphological structure of NETs and the number of *Klebsiella pneumoniae* cells associated with each extracellular structure were determined by microscopy.

Statistical processing

The results obtained were processed using the Statistica 12.0 (StatSoft Inc., USA). NET parameters obtained in the study of blood samples obtained from

patients were processed using nonparametric statistics and presented as the median and the interquartile range *Me* [Q_{25}–Q_{75}]. Quantitative variables were compared using the Mann – Whitney *U* test and the Kruskal – Wallis analysis of variance. The results of experiments on short-term neutrophil culture, characterized by normal data distribution, were presented as the mean and the standard error of the mean ($M \pm m$). Quantitative variables were compared using the Student's *t*-test. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Morphological characteristics of NETs in patients with inflammatory diseases of the abdominal cavity. Web-like NETs were present in the peripheral blood in all patients with inflammatory diseases of the abdominal cavity in the postoperative period. The number of neutrophil web-like structures was registered in the range from 14.2 % [7.7–17.9%] in acute appendicitis to 17.6% [10.3–26.6%] in the development of peritonitis. Uncomplicated abdominal inflammation in the postoperative period was characterized by a similar number of NETs in the peripheral blood. Thus, in local acute inflammation, the relative count of NETs corresponded to the range of *Me* 14.2–15.1%. Diffuse peritonitis was accompanied by an increase in the number of NETs up to the level of *Me* 17.6% (Fig.1). Thus, when inflammation spread to other organs and serous membranes of the abdominal cavity (in the case of peritonitis), the number of web-like NETs increased ($p = 0.668$).

Determination of the size of NETs in microns showed a statistically significant ($p = 0.0189$) increase in the size of NETs in patients with acute cholecystitis and pancreatitis. The size of NETs increased up to 53.65 [43.9–88.45] microns in acute cholecystitis and up to 91.9 [62.0–120.0] microns in acute pancreatitis compared to the group of patients with acute appendicitis, where it was 44.65 [35.6–57.6] microns (Fig. 2). In addition to web-like NETs, neutrophilic structures in the form of single strands, fibers, and clouds were found in patients. The increase in the size of NETs in patients with cholecystitis and pancreatitis was associated with the presence of these unusual extracellular structures in the blood that previously were not thoroughly described and studied. The smallest size of NETs was found in patients with ulcerative colitis (comparison group) who received non-surgical treatment (32.2 (6.3–57.4) microns).

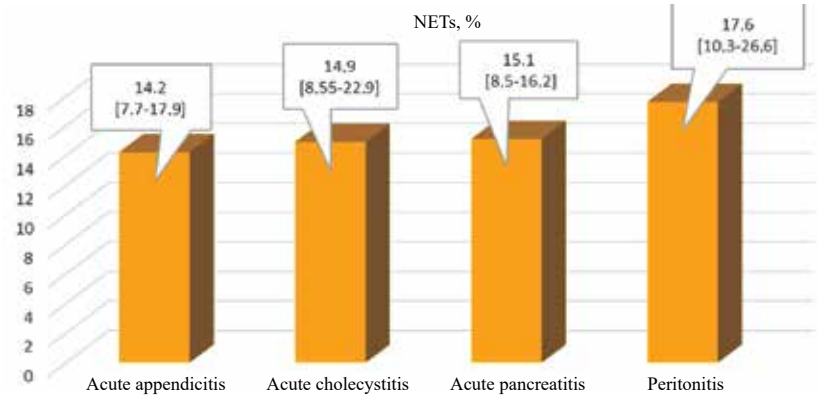

Fig. 1. The number of NETs in inflammatory diseases of the abdominal cavity (postoperative period), *Me* [Q_{25}–Q_{75}], %

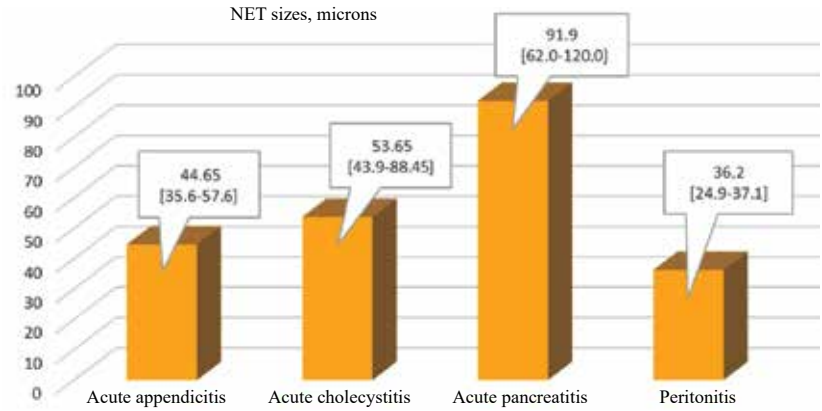

Fig. 2. Sizes of NETs in various inflammatory diseases of the abdominal cavity, *Me* [Q_{25}–Q_{75}], microns

The study of the morphological characteristics of NETs in patients with inflammatory diseases (Fig. 3, 4) raised a number of important questions related to understanding the process of functional transformation of neutrophil extracellular structures. Solving the issues of morphological and functional transformation of web-like NETs might be the key to understanding the role of various forms of NETs in the defense against infections and will allow to draw conclusions regarding their functional activity.

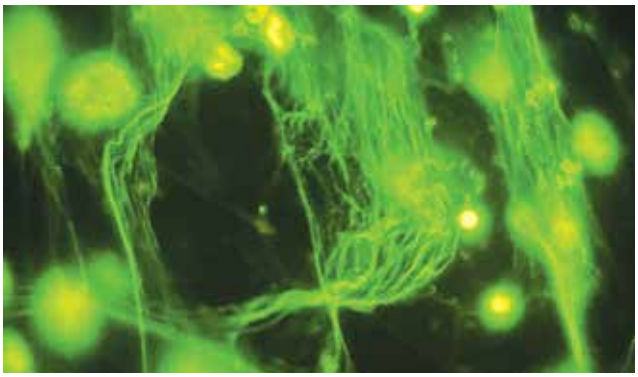

Fig. 3. Web-like NETs. Large structures. Uncomplicated appendicitis after surgery. An example of an uncomplicated inflammatory process with a favorable course. Here and in Fig. 4, 5, 7, incubation time is 1 h. Staining with CYBR Green I. x1,000

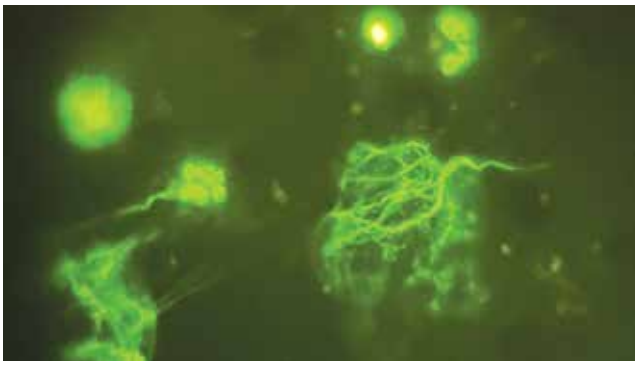

Fig. 4. Web-like NETs. Ulcerative colitis (non-surgical treatment). Small structures

Functional characteristics of NETs in healthy persons and patients with inflammatory diseases of the abdominal cavity. To study the role of NETs in the immune defense of the body and their functional activity, we developed a methodological approach in which NETs obtained from patients interacted *in vitro* with cells of the test microorganism *Klebsiella pneumoniae* (ATCC 700603). During the interaction of neutrophils with pathogen cells, the protective potential of innate immunity cells was realized.

Healthy donors. Neutrophils obtained from healthy donors have high functional activity [3]. Contact interactions with *Klebsiella pneumoniae* cells cause the formation of web-like NETs, as well as capture and binding of a large number of cells of the test microorganism (Fig. 5). During this process, web-like NET fibers are retracted and transformed into a cloud-like structure. The range of binding pathogen cells by NETs obtained from healthy donors is 70–90 cells per cloud. Each NET under our experimental conditions captured and retained an average of 78.05 ± 10.58 *Klebsiella pneumoniae* cells. Moreover, almost all cells of the test microorganism were localized inside NETs.

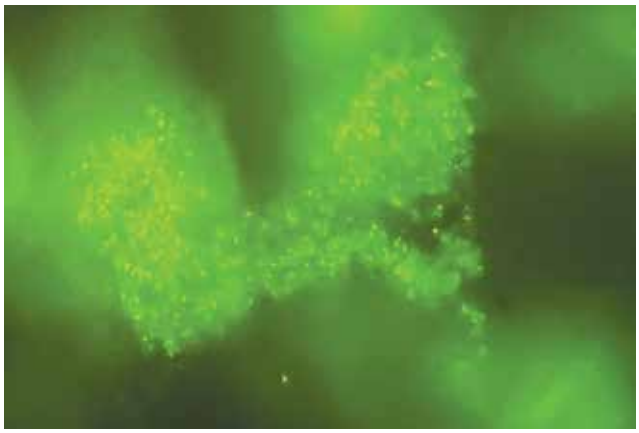

Fig. 5. Web-like NETs of healthy donors capture and bind a large number of *Klebsiella pneumoniae* (ATCC 700603) cells and acquire a cloud-like appearance

Patients with inflammatory diseases of the abdominal cavity. In patients with inflammatory diseases of the abdominal cavity (appendicitis, cholecystitis, pancreatitis) in the postoperative period, the functional activity of NETs is much more weakened compared to healthy donors. NETs in these patients capture and bind on average no more than 20 cells of the test microorganism. Some of the cells of the test microorganism remain unbound by NETs, which may contribute to the development of a postoperative infectious complication in these patients [8].

Dynamics of changes in the functional activity of web-like NETs. The dynamics of changes in the functional activity of NETs (pathogen capture and binding) in patients during web-like NET formation was studied in a group of patients with umbilical and inguinal hernias. In patients with non-strangulated

hernias who did not undergo surgery, the number of pathogen cells captured *in vitro* by a single NET varied from 4.62 ± 0.36 to 26.56 ± 3.45 after 1 and 2 hours of culture, respectively, which means it increased during culture by 5.8 times.

In patients who underwent surgery for strangulated hernias, pathogen capture reached 38.17 ± 3.74 *Klebsiella pneumoniae* cells per NET after 1 hour of culture and 25.85 ± 3.20 pathogen cells per one NET after 2 hours of culture. The sharp increase in the capture and binding of the pathogen by NETs in operated patients after 1 hour of culture may be explained by the involvement of *in vivo* pre-activated neutrophils in *in vitro* interaction between neutrophils in the operated patients and pathogen cells.

The subsequent decrease in the functional activity of NETs in operated patients during the second hour of culture may be due to the fact that part of the initially formed web-like NETs together with pathogen cells after 1 hour of culture turn into cloud-like structures that are absorbed by neutrophils capable of developing phagocytic activity.

A decrease in the number of NETs after their formation was investigated *in vivo* and *in vitro* and described in detail in the works of other researchers [9]. The conducted studies revealed the dependence of the elimination of formed NETs on both the phagocytic activity of neutrophils and macrophages [10, 11] and the enzymatic activity of pancreatic DNase I [12, 13]. Proinflammatory cytokines have been shown to stimulate phagocytosis and accelerate the destruction of NETs by macrophages and dendritic cells [14]. In patients with severe bacterial infections, vascular occlusions were caused by impaired elimination of NETs *ex vivo*, which was accompanied by the formation of intravascular blood clots containing NETs [15].

Functional characteristics of NETs in patients with inflammatory diseases of the abdominal cavity under the influence of IgG. Web-like NETs are very sensitive to IgG. Under the influence of IgG they turn into loose cloud-like structures (Fig. 6), while their size increases [4], and the ability to capture and bind the pathogen sharply deteriorates (Fig. 8).

In addition to the fact that cloud-like NETs formed under the IgG influence have a very weak ability to capture and bind the pathogen, they also bind cells of the test microorganism only on the periphery of the cloud-like structure (Fig. 7). This type of binding, apparently, makes it possible for the pathogen to avoid the influence of damaging factors produced in activated neutrophils.

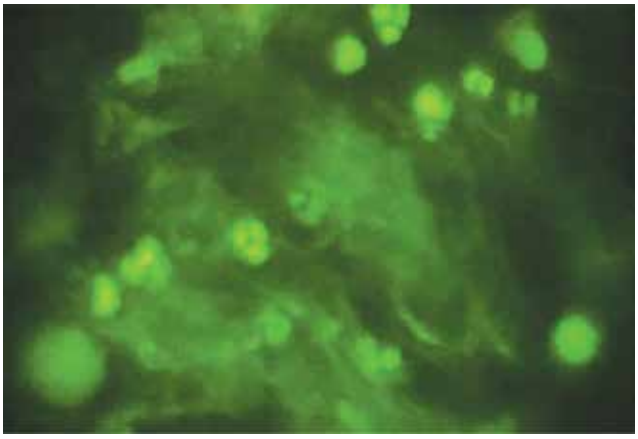

Fig. 6. Cloud-like NETs. Cloud-like forms of NETs were obtained from web-like neutrophil structures during their incubation with IgG (5 mcg / ml). Incubation time was 30 min. Coloring with CYBR Green-I. x1,000

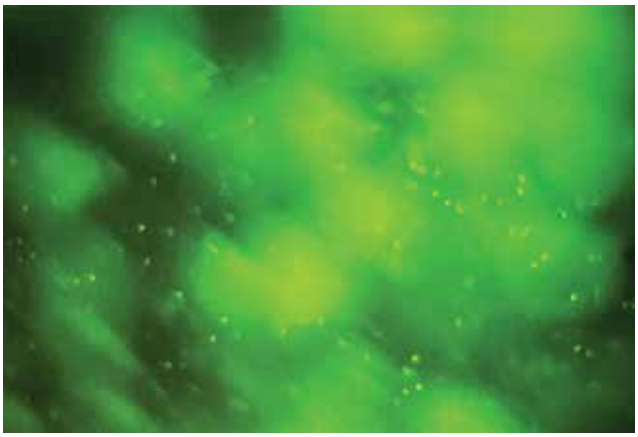

Fig. 7. Attenuation of pathogen capture and binding (*Klebsiella pneumoniae*) by cloud-like NETs. Peripheral binding of the pathogen by cloud-like NETs

It should be noted that similar cloud-like extracellular structures were also found in some patients. They also had a low binding capacity in relation to *Klebsiella pneumoniae* and also bound them only on the periphery of the cloud-like structure.

The results of the study of the functional activity of cloud-like NETs in comparison with web-like structures are shown in Table.

Table

Effect of IgG on neutrophil trapping of *Klebsiella pneumoniae* in patients with acute inflammatory processes in the abdominal cavity

Morphological structure of NETs and incubation conditions	Number of microorganisms captured by neutrophil traps, $M \pm m$
Neutrophil web-like structures without IgG influence	20. ± 1.67

End of table

Morphological structure of NETs and incubation conditions	Number of microorganisms captured by neutrophil traps, $M \pm m$
Cloud-like structures formed under IgG influence	8.46 ± 0.44*

* $p < 0.001$ compared to parameters without IgG influence.

In addition to the weakening of functional activity, cloud-like NETs are more susceptible to spontaneous enzymatic degradation of DNA fibers that form the basis of this morphological structure of NETs in comparison with web-like NETs, which carries an obvious risk of developing subsequent long-term complications in these patients. As a result of DNA fiber degradation in the extracellular space, the content of extracellular purine bases, which are factors of secondary alteration, can significantly increase. This effect was previously found and described in patients with post-COVID syndrome. NETs in the form of single DNA strands of considerable size were found in patients with post-COVID syndrome. Spontaneous enzymatic degradation of these strands causes an increase in the concentration of extracellular purine nitrogenous bases and is an additional factor in tissue damage and inhibition of T lymphocyte activity.

RESULTS AND DISCUSSION

The results of the study of NETs in patients with acute inflammation in the abdominal cavity revealed the dependence of the quantitative parameters of NETs (their number and size) on the type of inflammation, the size and degree of delimitation of the inflammatory lesion.

A comprehensive analysis of NETs, including not only the quantitative determination of NET parameters, but also the assessment of the morphological and functional transformation of neutrophil structures, will allow to fully identify the pathophysiological patterns of the inflammatory process, will become the key to understanding the role of certain NET forms in the fight against infections, and will allow to determine the functional activity of neutrophil extracellular structures.

The functional activity of NETs, understood as pathogen capture and binding, in healthy donors and in patients with acute infectious inflammation in the abdominal cavity has some similarities, but there are also differences.

In both healthy donors and patients, the interactions of the pathogen and neutrophils cause the formation of web-like NETs, and then the formed web-like structures capture and bind the pathogen. During this process, the DNA fibers are shortened (retracted), the size of this neutrophil structure becomes more compact, and the web-like NET is transformed into a cloud-like one. The pathogen cells are localized in the central part of this cloud-like structure.

The differences relate to the number of pathogen cells captured by cloud-like NETs. Cloud-like neutrophil structures originating from web-like ones in healthy donors capture and bind a significant number of cells of the test pathogen, several times more than similar cloud-like structures in patients. Some pathogen cells remain unbound in the study of the functional activity of NETs in patients with inflammatory diseases.

A morphological and functional restructuring of the NETs is observed under the influence of IgG. The results of the study demonstrate changes in the structure of NETs under the influence of IgG. Neutrophil web-like structures turn into loose cloud-like ones that have a weakened ability to bind the pathogen, which means they acquire low functional activity. A feature of the functional activity of IgG-induced NETs is peripheral binding of the pathogen.

The study demonstrates two types of cloud-like structures: functionally active clouds, which are formed from neutrophil web-like structures after pathogen capture, and ineffective clouds, which are formed from neutrophil web-like structures under the influence of IgG. In our opinion, these ineffective cloud-like structures pose a certain danger to the human body due to the possible generation of secondary alteration factors in the form of extracellular purine bases. The content of these extracellular purine nitrogenous bases can significantly increase as a result of spontaneous enzymatic hydrolysis of DNA in neutrophil cloud-like structures and induce a decrease in immune resistance as a result of inhibition of the activity of T cells in the immunity.

In addition, extracellular purine nitrogenous bases can cause damage to the structures of the nervous system and cells of internal organs. Their long-term effect on the body can cause long-term adverse effects.

CONCLUSION

From a practical perspective, the study and its results create prerequisites for new approaches to the development of vaccination regimens and the use of immunobiologic drugs, in particular, monoclonal antibody drugs, especially considering the fact that at present new medical technologies are sought to improve the effectiveness of patient treatment.

The lack of knowledge about the mechanisms of interaction between innate immunity and adaptive immune responses in the human body can significantly limit the use of vaccines and monoclonal antibody preparations. It also contributes to certain distrust of this type of therapy in patients [16]. Cases of thrombosis in combination with thrombocytopenia after vaccination [17] and progressive multifocal leukoencephalopathy caused by polyomavirus during treatment with monoclonal antibodies [18] have been described.

As the indications for the use of monoclonal antibody drugs expand, there are reports of complications and even deaths after the use of these drugs [19, 20] due to the development of multiple organ dysfunction syndrome. The causes of adverse effects after monoclonal antibody therapy are still insufficiently studied, but it can be assumed with a high degree of probability that the formation of ineffective neutrophil cloud-like structures can become a mechanism for inducing hemocoagulation and producing secondary alteration factors. Therefore, it is important to correlate the vaccination and treatment regimen with immunobiologic drugs with control of the state of innate immunity, in particular, with detection of spontaneous neutrophil web-like structures in the blood of patients.

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Kazimirskii A.N. – experimental research, preparation of illustrative material, drafting of the manuscript. Salmasi J.M. – editing of the article. Poryadin G.V. – conception and design. Panina M.I. – editing of the article, statistical processing of the data. Kim A.E. – experimental research. Rogozhina L.S. – collection and processing of the data.

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