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PROPERTIES OF Ca^{2+} -DEPENDENT AND Ca^{2+} -INDEPENDENT ISOFORMS OF NO-SYNTASE IN BLOOD LYMPHOCYTES OF OVARIAN CANCER WOMEN

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The purpose of the present work was to study the enzyme activity and kinetic properties of NO-synthase in blood lymphocytes of women with ovarian cancer. Studies were conducted on the blood lymphocytes of women with ovarian cancer of the II-IV degrees (n=26) and healthy women (n=24, control group). It has been found that constitutive isoform of NO-synthase of blood lymphocytes of women with ovarian cancer was 4.0 fold lower compared to control group. The activity of inducible NO-synthase in blood lymphocytes of practically healthy women was (1.52 ± 0.46) nmol NADPH(H^+)/min·mg of protein and in women with ovarian cancer it increased in 144 times. The maximum rate of constitutive isoform of NO-synthase reaction in patients with ovarian cancer was reduced 5 fold and for inducible NO-synthase reaction was increased in tens of times compared to control group. For oncopathology the imaginary affinity constant to arginine for inducible NO-synthase was in 5.4 times lower compared to inducible NO-synthase of control group. The inhibition of constitutive isoform of NO-synthase occurs by competitive type – by reducing the rate of enzyme reaction.

Keywords: ovarian cancer, lymphocytes, Nitrogen (II) oxide, NO-synthase, arginase.

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ВЛАСТИВОСТІ Ca^{2+} -ЗАЛЕЖНИХ І Ca^{2+} -НЕЗАЛЕЖНИХ ІЗОФОРМ NO-СИНТАЗИ В ЛІМФОЦИТАХ КРОВІ ЖІНОК ХВОРИХ НА РАК ЯЄЧНИКІВ

Метою даної роботи було вивчити активність та кінетичні властивості NO-синтази в лімфоцитах крові жінок з раком яєчників. Дослідження проводили на лімфоцитах крові жінок з раком яєчників II-IV стадії (n=26) та здорових жінок (n=24, група контролю). Встановлено, що активність конститутивної ізоформи NO-синтази лімфоцитів крові жінок з раком яєчників була в 4,0 рази нижчою порівняно з контрольною групою. Активність індукційної NO-синтази в лімфоцитах крові практично здорових жінок становила $(1,52 \pm 0,46)$ нмоль NADPH(H^+)/хв·мг протеїну, а у жінок з раком яєчників активність ензиму була вищою у 144 рази. Максимальна швидкість реакції каталізованої конститутивною ізоформою NO-синтази у пацієнтів з раком яєчників була нижчою у 5 разів, а для іNO-синтази максимальна швидкість реакції зростала у десятки разів порівняно з контрольною групою. У пацієнтів з онкопатологією уявна константа спорідненості iNOS до аргініну була в 5,4 рази нижчою порівняно з індукційною NO-синтазою в осіб контрольної групи. Інгібування активності конститутивної ізоформи NO-синтази відбувається за конкурентним типом – шляхом зниження швидкості ензиматичної реакції.

Ключові слова: рак яєчників, лімфоцити, Нітроген (II) оксид, NO-синтаза, аргіназа.

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Ovarian cancer is considered to be one of the most severe diseases of the female reproductive tract. The study of the pathogenesis of ovarian cancer is being directed to improve methods of diagnosis and detection of tumor formation process at early stages [3, 11].

It is known that metabolism of *L*-arginine and synthesis of nitric oxide (NO) plays an important role in carcinogenesis and tumor growth [1, 3, 12]. It is assumed that NO modulates cancer-dependent events, including angiogenesis, apoptosis, cell cycle, invasion and metastasis. The *L*-arginine is converted into NO by means of nitric oxide synthase (NOS) or ornithine with the participation of arginase, is carried out by separate metabolic pathways [4]. According to physiological conditions, the NO production from *L*-arginine occurs by constitutive isoforms of NO-synthase (cNOS), which are Ca^{2+} -dependent enzyme. The increase in NO production occurs as level of calcium ions increases. The stimulating factors of this receipts and, as a result, increase in calcium-dependent cNOS activity are acetylcholine, serotonin, glutamate, ADP and other biologically active substances [2, 4].

In pathophysiological conditions inflammatory stimuli induce the expression of the inducible isoform NO-synthase (iNOS), which is Ca^{2+} -dependent isoform [11]. One or more NOS isoforms exist in different human cells. These isoenzymes are expressed as expression products of different genes localized in separate chromosomes. Post-translational modification of these isoenzymes may affect their intracellular localization and activity.

Although all known isoforms of NO-synthase (eNOS, nNOS, iNOS) have a similar molecular structure [4, 11], their regulation and functional role in different biological processes is the subject of

research, as well as creation of selective and specific NOS inhibitors. Particular interest in the regulation mechanisms of each NOS isoforms is due to the fact that NO causes pleiotropic biochemical effects.

Separation of constitutive and inducible NO synthesis is conditional, since they form one product – NO molecule, which readily diffuses across cell membranes and does not require receptors for implementations of its effects. The balance between physiological, regulatory and/or cytotoxic properties is largely due to the local level of NO, as well as the oxidant status of tissues in which NO is synthesized and realizes its effects [10].

It is known that NO also easily reacts with a superoxide anion radical. The reaction of NO with superoxide anion (O_2^-) with formation of peroxyxynitrite ($OONO^-$) radical and hydroxyl radical (OH^-) is a second NO pathway. These compounds are highly reactive free radicals, have prooxidant properties and cause destructive effects on proteins and lipids [5, 10]. Peroxyxynitrite is able to mediate the NO cytotoxic effects, such as damage to DNA, oxidation of low density lipoprotein, isoprostane formation, nitration of tyrosine, inhibition of aconitase and mitochondrial respiration [2, 5].

As for nitric oxide, according to various literary data, it plays a double role in processes of malignant growth. In high concentrations, it inhibits, and at low levels – stimulates tumor growth and metastasis [3, 11]. At the same time study the characteristics of changes in activity of individual isoforms of NOS during neoplastic transformation can be used as a basis for improving the quality of diagnosing and efficiency of this pathology correction.

In order to recognize the pathological states of organism, peripheral blood lymphocytes can serve as one of the most appropriate model, which can objectively reflect changes in the genetic and metabolic homeostasis of organism and maladaptive states [7]. This allows the use of metabolic parameters of lymphocytes as indicators of the functional state of organism.

The purpose of the study was to determine both the activity and kinetic properties of different isoforms of NO-synthase in blood lymphocytes of women with ovarian cancer.

Material and methods. Study was carried out on peripheral blood lymphocytes of practically healthy women and women with neoplastic changes in the ovary. Total number of practically (clinically) healthy women, representative by age (mean age 53.8 ± 5.4 years), was 24. This group was formed from volunteers among the employees of the Danylo Halytsky Lviv National Medical University, as well as employees of the Lviv Regional Clinical Hospital. Group of women with neoplastic ovary changes (ovarian cancer degree II–IV) consisted of 26 persons, aged 24–75 years (mean age 55.4 ± 5.3 years) who were hospitalized in the Lviv State Oncological Regional Treatment and Diagnostic Center during the years 2015–2018 and underwent a complete clinical and laboratory examination. Only women with confirmed diagnosis of ovarian cancer without the presence of concomitant diseases at the start of the study were enrolled in this study.

For allocation of lymphocytes, the peripheral blood collection in women of experimental groups was performed after the preliminary completion of their clinical examination before an appointment of a course of treatment for them. Blood sampling was conducted by elbow venipuncture in the morning hours under conditions of physiological tranquility, before meals in the amount of 20 ml in test tubes which were stabilized with heparin (final dilution 1:100). Isolation of peripheral blood lymphocytes was carried out in accordance with method of Boyum A. [7] with some modifications [12]. Counting of the number of live and dead cells was carried out in the Goryaev grid. The integrity and viability of blood lymphocytes in all experiments was not less than 95 %. For permeabilization of blood lymphocyte membranes and disclosure of latent enzymatic activity 0.2 % saponin was added to the suspension.

Determination of NOS enzyme activity on saponin-perforated lymphocytes was performed in accordance with the method [12]. The activity of Ca^{2+} -independent isoforms (iNOS) was evaluated by adding inducible isoform selective inhibitor aminoguanidine instead $CaCl_2$ to the incubation medium. The activity of the Ca^{2+} -dependent isoform corresponding to cNOS was calculated as the difference between the total activity of NOS activity and activity of Ca^{2+} -dependent isoform of NOS. The NOS activity was expressed in nmoles of oxidized NADPH(H^+)/min-mg of protein. The imaginary kinetic parameters of the isoforms NOS were determined in the Lineweaver-Burk plot by linearizing the curves and calculating the constants.

Variational and statistical processing of data was carried out using the software package for personal computers Microsoft Excel. The following statistical parameters, such as the arithmetic mean (M) and standard deviation (m), were determined. The significance of the changes was established according to the Student's t-criterion.

Results of the study and their discussion. Endothelial and inducible isoforms of NO-synthase were found that in blood lymphocytes cNOS activity in practically healthy women is (71.4 ± 6.9) nmol NADPH(H^+)/min-mg of protein (fig. 1).

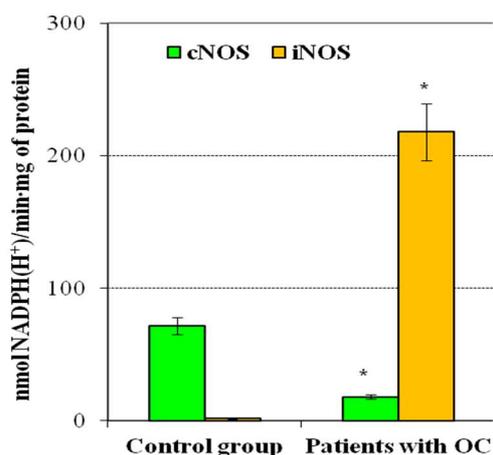


Fig. 1. cNOS and iNOS activity in blood lymphocytes of practically healthy women and patients with ovarian cancer (OC), $M \pm m$.

Note: changes are significant in terms of parameters in lymphocytes of the control group individuals, * $p < 0.001$.

activity up to (218.2 ± 21.5) nmol NADPH (H⁺)/min·mg of protein, in 144 fold ($p < 0.001$) was observed.

Obtained results are consistent with the data obtained earlier by the researchers, which also showed an increase in iNOS activity and expression in women with ovarian cancer [12]. It also was shown that in women with ovarian cancer, the iNOS expression correlated with the stage of the tumor differentiation, and intracellular NO level – with the ovarian cancer stage. Besides, iNOS inhibition by specific inhibitors may be a potential therapeutic target in the ovarian cancer treatment [11]. The hyperexpression of iNOS was detected in different types of malignant tumors. Selective inhibitors of iNOS suppress the tumors growth, the development of which is induced by different carcinogens [6].

Mostly, NOS-dependent production of physiologically necessary NO ("basal NO") occurs by eNOS and nNOS, and the NOS-dependent production of additional amounts of NO in the cell for the development of various pathological states is realized by iNOS. Therefore activation of iNOS is an integral part of numerous adaptive-protective reactions of a cell and an organism [6, 11]. The results obtained by us indicate the violation of the blood lymphocytes arginase/NO-synthase system, which leads to an imbalance of regulatory systems of lymphocytes, in particular, the NO regulatory function. Multiple times growth of iNOS activity is associated with a decrease in cNOS activity and indicates the NO hyperproduction in blood lymphocytes under conditions of oncopathology. It has been shown that NO formed in excessive amounts in pathological states of the organism has a marked cytotoxic effect as a result of the formation of peroxynitrite is a product of the interaction of NO and superoxide anion radical which

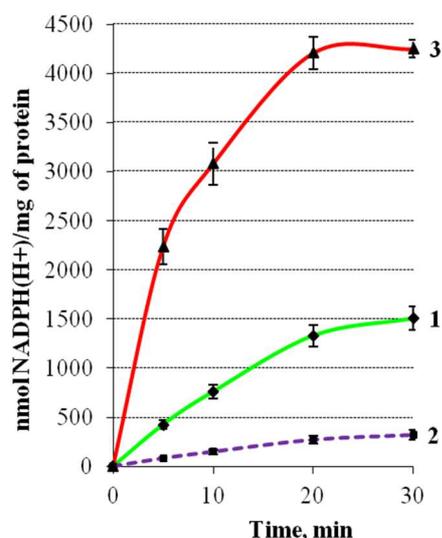


Fig. 2. Dynamics of NADPH(H⁺) consumption in NOS reaction of blood lymphocytes of practically healthy women (1 – cNOS) and patients with ovarian cancer (2 – cNOS, 3 – iNOS)

In literature there is a significant variability in the absolute values of NOS enzymatic activity in blood lymphocytes, which is probably due to various methodological approaches to studying the enzyme activity. In the blood lymphocytes obtained from women with ovarian cancer, the cNOS activity is reduced by 4.0 times comparing to the control group and is (18.0 ± 1.6) nmol NADPH(H⁺)/min·mg of protein ($p < 0.001$).

It is known that cNOS produces low concentrations of NO, while iNOS produces a high levels of NO (>300 nM) [11]. Inducible NOS isoform is calcium-independent and, unlike the constitutive isoform NOS, not always expressed (constitutively).

It has been found that iNOS activity in blood lymphocytes in practically healthy women is identified insignificantly, practically at the margin of error, and is (1.5 ± 0.46) nmol of NADPH(H⁺)/min·mg of protein. Contrary to cNOS inhibition in blood lymphocytes in patients with ovarian cancer, a sharp increase in iNOS

destroys almost all of the cell components. Cells in which an uprising of NO concentrations was marked have an increased growth rate [2, 9].

The change in the activity of the NOS enzymatic system only indicates the direction of dysmetabolic disturbances in the NO-homeostasis system. However, the biochemical mechanisms which caused changes in the NOS activity in the development of ovarian cancer remain unclear. Therefore, the next stage of our research was devoted to the study of kinetic properties of cNOS and iNOS isoforms of lymphocytes in the blood. In order to study the features and mechanism of NOS functioning, the maximum instantaneous reaction rate (V_0), the maximum of the formation of the product of the reaction (P_{max}) and the characteristic reaction time (t) were determined. To establish these kinetic parameters of NOS, the dynamics of NADPH(H⁺) reduction which indicates the NO production was studied. For this, the suspension of lymphocytes was incubated in a standard incubation medium for various intervals of time (0-30 min.). The obtained results shown that the kinetic curves of NO production in NOS reaction in blood lymphocytes tend to saturate (fig. 2).

From this figure it can be seen that the kinetics of the NO production with the participation of cNOS, corresponds to the laws of zero order reaction in the time range of 0-20 min: in this time interval the graph of the dependence of the NO generation on the incubation period is practically linear. Therefore, in subsequent experiments, the duration of incubation of lymphocytes and, accordingly, NO-synthase reaction was 20 minutes. The dynamics and the amount of NO generation by cNOS in lymphocytes in women with ovarian cancer are significantly lower than in practically healthy women.

At the same time, throughout the range of the time factor, the NO generation NO by iNOS in blood lymphocytes of women with ovarian cancer significantly exceeds these values for cNOS. By linearization of the data in the coordinates {P/t; P} the main kinetic characteristics of NOS reaction in blood lymphocytes were calculated (table 1).

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Kinetic parameters of NOS reaction in blood lymphocytes of practically healthy women and patients with ovarian cancer (M±m, n=8)

Kinetic parameters	Practically healthy women	Patients with ovarian cancer
cNOS		
V_0 , nmol NADPH(H ⁺)/min·mg of protein	94.0±7.4	18.5±1.5 *
P_{max} , nmol NADPH(H ⁺)/mg of protein	3413±315	908.6±186.2 *
τ , min	36.7±4.6	50.6±12.9
iNOS		
V_0 , nmol NADPH(H ⁺)/min·mg of protein		739.0±59.6
P_{max} , nmol NADPH(H ⁺)/mg of protein		5536.4±57.7
τ , min		7.6±0.7

Note: changes are significant in relation to the values of blood lymphocytes in individuals in the control group (physiological norm), *p<0.001.

The values of kinetic parameters for cNOS and iNOS lymphocytes in practically healthy women and patients with ovarian cancer are significantly different. Thus, V_0 in the control group was (94.0±7.4) nmol NADRH (H⁺)/min·mg of protein. In women with ovarian cancer, V_0 significantly decreased to (18.5±1.5) nM NADRH(H⁺)/min·mg of protein (p<0.001).

The NO production by cNOS in women with oncopathology is slower and less active than at norm. Regarding the maximum amount of the produced product of the reaction produced in the cNOS reaction, in the case of ovarian cancer it was 3.8 fold less than in women of control group (p<0.001). In the iNO-synthase reaction, Pmax in ovarian cancer women was 6 times higher than in the cNO-synthase reaction (p<0.001). Analysing the characteristic time of the reaction, it was found that in women with ovarian cancer, its value was increased in 1.4 times (p<0.05).

The obtained kinetic parameters confirm the data that in blood lymphocytes of women with ovarian cancer hyperinsynthesis of NO occurs by iNOS, and the “basal” NO generation in normal physiological conditions occurs by cNOS. Changes in L-arginine concentration in the incubation medium are likely to affect the rate of NOS reaction. In this regard, an important characteristic of NOS is its dependence of the NOS activity on the substrate concentration in the incubation medium, which is determined by imaginary constants of affinity to the substrate K_{L-Arg} . It was calculated by determining the specific NOS activity in the incubation medium containing L-arginine in the concentration range from 0.1 to 30 mM (with a constant concentration of CaCl₂ – 10 mM and NADPH – 0.12 mM).

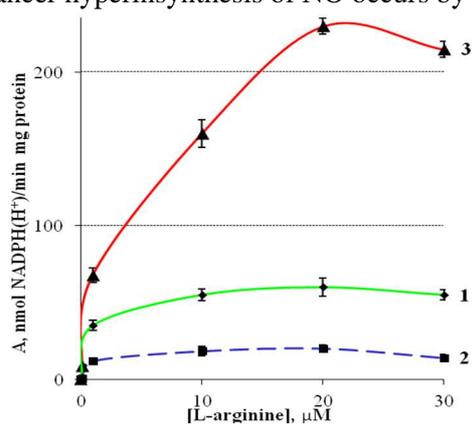


Fig. 3. Concentration dependence of L-arginine level on NOS activity in blood lymphocytes of practically healthy women (1 – cNOS) and patients with ovarian cancer (2 – cNOS, 3 – iNOS), M±m, n=8.

It was found that increasing the L-arginine concentration in the incubation medium in the concentration range from 0.1 to 30 mM leads to a gradual increase in the rate of NOS reaction by both isoforms NOS with plateau (fig. 3).

The maximum activity of cNOS lymphocytes in practically healthy women and iNOS blood lymphocytes in women with ovarian cancer is tested in the presence of 20 mM L-arginine in an incubation medium. The cNOS activity in lymphocytes of women with ovarian cancer reaches the plateau at significantly lower substrate concentrations. By the linearization of the obtained concentration dependences in the Lineweaver-Burk plot, the basic kinetic parameters of NOS in blood lymphocytes were calculated (table 2).

Kinetic parameters of certain isoforms of NOS in blood lymphocytes of practically healthy women and patients with ovarian cancer, determined by *L*-arginine ($M \pm m$, $n=8$)

Kinetic parameters	Practically healthy women	Women with ovarian cancer
	cNOS	
V_{max} , nmol NADPH(H^+)/ min per mg of protein	235.6 \pm 44.0	131.2 \pm 30.7*
K_{L-Arg} , mM	14.6 \pm 2.7	21.2 \pm 4.1
iNOS		
V_{max} , nmol NADPH(H^+)/ min per mg of protein	–	244.3 \pm 6.9
K_{L-Arg} , mM	–	2.7 \pm 0.2 **

Note: changes are significant for the values of cNOS in blood lymphocytes of the control group individuals (physiological norm), * $p < 0.05$, ** $p < 0.001$.

As follows from the table data, the V_{max} value for nNOS of the control group is by 1.8 times higher than that value for cNOS lymphocytes in patients with ovarian cancer ($p < 0.05$). At the same time, the value of K_{L-Arg} for all studied groups does not significantly differ from each other, which suggests that while of oncopathology, the affinity of cNOS to *L*-arginine practically does not change. The V_{max} value for an iNOS activated in the case of cancer pathology is practically no different from this value for cNOS of blood lymphocytes in both groups ($p > 0.05$). However, blood lymphocytes iNOS of patients with ovarian cancer has a much higher affinity to *L*-arginine: K_{L-Arg} value for iNOS is lower in 5.4 times comparing to blood lymphocytes cNOS of the control group individuals ($p < 0.001$).

Consequently, in interpreting the obtained kinetic parameters determined by *L*-arginine, it was shown that in the oncopathology the imaginary constant of iNOS affinity to *L*-arginine is by 5.4 times lower ($p < 0.001$) than for cNOS in healthy women of the control group and inhibition of cNOS activity occurs in a competitive type – by reducing the enzyme reaction rate. Consequently, under the conditions of oncopathology, the ratio of NO-synthase and arginase metabolism of *L*-arginine is disturbed, indicating dismetabolic changes in the NO synthesis system, namely its hyperproduction.

From the literary data it is known that NO promotes a carcinogenesis as a modifier of xenobiotics metabolism and as an agent that violates pro- and anticarcinogenic genetic balance, causing one- and double-stranded DNA breaks [8, 10]. Nitric oxide metabolite peroxynitrite ($ONOO^-$) is a potent mutagen [2, 5]. The NO effects on the cell depends on its concentration, which in turn depends on the various NOS isoforms, however, is usually associated with iNOS. The main function of NO generated by iNOS is participation in immune processes, including antipathogenic reactions, nonspecific cytotoxicity, antitumor protection, transplant rejection, etc. [2, 4]. According to the literature, data in lymphocytes endothelial and inducible isoforms of NO-synthase were identified [4, 10]. High activity of this enzyme leads to NO accumulation and initiation of cell pathological processes (inhibition of mitochondrial enzymes, DNA damage, etc.). Excessive NO generation occurs during transplant rejection, arthritis, septic shock, inflammatory processes. It also leads to the destruction of nerve cells and may lead to Alzheimer's disease, inhibits cell proliferation and increased lymphocytes apoptosis and macrophages and causing secondary immune deficiencies. This mechanism is related to the reaction of nitric oxide of DNA or RNA of cells directly by NO. Therefore, the selective inhibition of each of NOS isoforms is of particular importance relevance, and, first of all, of iNOS inhibition. In this regard, the considerable efforts of researchers and are aimed at studying the mechanisms of regulating of the of NOS isoforms activity. Since high NO levels are toxic to cells, iNOS is considered to be pathological in contrast to the constitutive isoform.

Conclusions

1. In women with ovarian cancer, cNOS activity in the blood lymphocytes decreases and iNOS activity increases several folds.
2. The affinity of cNOS to *L*-arginine in blood lymphocytes of two studied groups is practically identical.
3. A decrease in cNOS activity in ovarian cancer women is due to a decrease in the reaction rate.
4. Furthermore, in women with ovarian cancer, the iNOS affinity to *L*-arginine is lower than that for cNOS.

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CYTOKINES AS MEDIATORS OF THE IMMUNE SYSTEM AND THEIR ROLE IN THE PATHOGENESIS OF COMMUNITY-ACQUIRED PNEUMONIA

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The clinical and immunological examination of patients with community-acquired pneumonia has been carried out that included the study of the IL-2, IL-4, IL-6, IL-8 and TNF α cytokines' system. Immune system disorders that determine the severity of pneumonia, the amount of inflammatory damage to the lung tissue, disrupted elimination of the immune complexes have been found. These tendencies to a decrease in the immunoglobulins M and G result in a moderate course of community-acquired pneumonia and lobar lesions of the lungs, and the immune system disorders are reliably associated with imbalance of cytokines, with a predominance of their pro-inflammatory activity and a decrease in regulatory functions. The findings of the immunological monitoring indicate that standard antibiotic therapy in patients with community-acquired pneumonia leads to its clinical and radiological resolution, though it is not accompanied by the normalization of immunity parameters. The imbalance of the cytokine component of immunity justifies the need for further development of pathogenetic, as well as immunocorrective therapy, in patients with community-acquired pneumonia.

Keywords: community-acquired pneumonia, pathogenesis, immunity, cytokines.

В.І. Березняков, О.М. Корж, С.Б. Павлов, Г.А. Єрошенко, К.В. Шевченко, Н.М. Пивовар ЦИТОКІНИ ЯК ПОСЕРЕДНИКИ ІММУННОЇ СИСТЕМИ ТА ЇХНЯ РОЛЬ В ПАТОГЕНЕЗІ НЕГОСПІТАЛЬНОЇ ПНЕВМОНІЇ

Проведено клініко-іммунологічне дослідження хворих з негоспітальною пневмонією, що включає вивчення систем цитокінів-IL-2, IL-4, IL-6, IL-8 та TNF α . Виявлені порушення в системі імунітету, визначаючи ступінь важкості пневмонії, об'єм запального ураження легеневої тканини, порушення елімінації імунних комплексів. Ці тенденції до зменшення імуноглобулінів М і G обумовлюють середньо-тяжкий перебіг протягом негоспітальної пневмонії і долевої поразки легень, порушення в системі імунітету, достовірно пов'язані з дисбалансом цитокінів, з переважанням їх прововоючої активності та зменшенням регуляторних функцій. Результати імунологічного моніторингу свідчать про те, що стандартна антибактеріальна терапія хворих з негоспітальною пневмонією призводить до її клініко-рентгенологічного врегулювання, але не спровокована нормалізація показників імунітету. Дисбаланс цитокінового зрівогого імунітету визначає необхідність подальшої розробки патогенетичної та, у тому числі, імунокоригуючої терапії, у хворих із негоспітальною пневмонією.

Ключові слова: не госпітальна пневмонія, патогенез, імунітет, цитокіни.

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Currently, pneumonia is ranked 4th-5th in the structure of causes of death worldwide after cardiovascular and oncological diseases, cerebrovascular pathology, injuries and poisoning, and is ranked first among infectious diseases [7]. Mortality in hospital patients with severe form of the disease ranges from 14 to 40 % and increases among patients over 60 years of age [3].