АНТИГЕНПРЕЗЕНТИРУЮЩАЯ СУБПОПУЛЯЦИЯ CD66b$^+$CD16$^+$CD33$^+$HLA-DR$^+$ НЕЙТРОФИЛЬНЫХ ГРАНУЛОЦИТОВ ПРИ ОСТРОМ ОСТЕОМИЕЛИТЕ У ДЕТЕЙ: ИММУНОМОДУЛИРУЮЩИЕ ЭФФЕКТЫ ВЛИЯНИЙ ИММУНОТРОПНОГО ГЕКСАПЕПТИДА В ЭКСПЕРИМЕНТАЛЬНОЙ СИСТЕМЕ *IN VITRO*

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Резюме. Включение нейтрофильных гранулоцитов (НГ) в воспаление зависит от экспрессии рецепторов, обеспечивающих функции НГ. Острый остеомиелит (ООМ) занимает центральное место среди гнойно-воспалительных заболеваний у детей. ООМ – гнойно-некротический процесс, протекает в кости, костном мозге, ответственном за кроветворение. Представляет интерес определение субпопуляций НГ, их фенотипа при ООМ и оценка влияния иммунотропных субстанций для коррекции дисфункций. Цель – уточнить варианты изменений количественных и фенотипических характеристик субпопуляций CD66b$^+$CD16$^+$CD33$^+$HLA-DR$^-$, CD66b$^+$CD16$^+$CD33$^+$HLA-DR$^+$ НГ при остром остеомиелите у детей и оценить возможность их иммуномодулирования под влиянием гексапептида (ГП) – Arginyl-alpha-Aspartyl-Lysyl-Valyl-Tyrosyl-Arginine в эксперименте *in vitro*.

Исследована периферическая кровь (ПК) 24 детей ООМ 8-15 лет – группа исследования (ГИ). Группа сравнения – 13 здоровых детей. Для оценки влияния ГП (10^{-6} г/л) ПК детей с ООМ инкубировали 60 мин (37 °С) – группа исследования 1. Определяли количество НГ субпопуляций CD66b$^+$CD16$^+$CD33$^+$HLA-DR$^+$, CD66b$^+$CD16$^+$CD33$^+$HLA-DR$^-$ (FC 500, Beckman Coulter, США), плотность экспрессии рецепторов по MFI, фагоцитарную активность НГ до и после инкубации с ГП.

При ООМ регистрируется субпопуляция НГ, экспрессирующая HLA-DR – 29,9 (18,4-43,6) % CD66b$^+$CD16$^+$CD33$^+$HLA-DR$^+$, отсутствующая в ПК здоровых детей. Под влиянием ГП выявле-

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но снижение количества $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ в 1,5 раза ($p > 0,05$), повышение в 1,2 раза $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$ НГ ($p > 0,05$) относительно ГИ. Перераспределение субпопуляций, очевидно, происходит за счет связывания ГП с HLA-DR на мембране НГ. Также MFI HLA-DR была низкой -1,7 (1,6-2,2) ($p > 0,05$). При этом выявлено усиление MFI CD66b в 1,3 раза и снижение в 1,4 раза MFI CD16 ($p < 0,05$).

В исследовании впервые показано наличие в ПК у детей с ООМ субпопуляции НГ $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ на фоне уменьшения количества $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$НГ. Субпопуляция $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$НГ при ООМ, свидетельствует о появлении активированной субпопуляции НГ в ПК со свойствами АПК. В системе *in vitro* продемонстрировано позитивное влияние ГП на фенотип субпопуляций $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$, $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ НГ. Восстановление фагоцитарной функции под действием ГП связано с повышением экспрессии CD66b, влияющих на эффекторную функцию НГ, и уменьшением гиперэкспрессии молекулы CD16, что обуславливает снижение повреждающей цитотоксической активности НГ.

Ключевые слова: нейтрофильные гранулоциты, острый остеомиелит, дети, антигенпрезентирующая субпопуляция, гексапептид, система in vitro

ANTIGEN PRESENTING SUBSET OF $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ NEUTROPHILIC GRANULOCYTES IN ACUTE OSTEOMYELITIS IN CHILDREN: IMMUNOMODULATING EFFECTS OF IMMUNOTROPIC HEXAPEPTIDE IN AN *IN VITRO* EXPERIMENTAL SYSTEM

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Abstract. Inclusion of neutrophilic granulocytes (NG) in inflammation depends on the expression of receptors providing the functions of NG. Acute osteomyelitis (AOM) occupies a central place among purulent-inflammatory diseases in children. AOM purulent-necrotic process proceeds in the bone, bone marrow – the site of hematopoiesis. It is interesting to determine the functionally significant NG subsets, their phenotype in OM and evaluate the effect of immunotropic substances for the correction of dysfunctions. Aim: to specify the variants of changes in quantitative and phenotypic characteristics of $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$, $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ NG subsets at AOM in children and evaluate the possibility of their immunomodulation under the influence of hexapeptide (HP) – Arginyl-alpha-Aspartyl-Lysyl-Valyl-Tyrosyl-Arginine *in vitro*.

Peripheral blood (PB) of 24 children 8-15 years old AOM were the study group (SG). The comparison group (CG) – 13 healthy children. HP (10^{-6} g/L) were incubated with PB SG (60 min, 37 °C) to evaluate the effects (SG1). The number of NG subsets $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$, $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$ (FC500, Beckman Coulter, USA), receptor expression density (MFI), phagocytic activity before and after incubation with HP were determined.

The NG subset expressing HLA-DR – 29.9 (18.4-43.6) % $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ was registered in children with AOM. The number of $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ was 1.5 times lower ($p > 0.05$), of $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ was 1.2 times higher ($p > 0.05$) than before incubation with of HP. The redistribution of subsets apparently occurs due to the binding of HPs to HLA-DR on the NG membrane. Also MFI HLA-DR was low ($p > 0.05$); the 1.3-fold increase in MFI CD66b, 1.4-fold decrease in MFI CD16 were revealed ($p < 0.05$).

The study was the first to demonstrate the presence of NG subset of $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ in the PB of children with AOM. Subset of $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$NG in AOM indicates the appearance of an activated subset of NG in PB with the properties of APC. The positive influence of HP on the phenotypic characteristics of subsets $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$, $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$. Restoration of phagocytic function of NGs under the influence of HP is connected with the increase of CD66b expression, which influences the effector function of NGs and decrease of CD16 molecule hyperexpression that stipulates decrease of damaging cytotoxic activity of NGs.

Keywords: neutrophil granulocytes, acute osteomyelitis, children, antigen-presenting subset, hexapeptide, in vitro system

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Introduction

Currently, active scientific research is underway, the purpose of which is to study the causes and mechanisms of the occurrence of atypically occurring purulent-inflammatory diseases (PID) and the role of various dysfunctions of neutrophilic granulocytes (NG) in the immunopathogenesis of these diseases [3].

NGs are known for their contribution to antimicrobial defense, effectively destroying pathogens through phagocytosis, production of antimicrobial peptides, reactive oxygen species, secretion of proinflammatory cytokines and chemokines, and formation of neutrophil extracellular networks (NETs) [11]. NGs showed to be phenotypically and functionally heterogeneous cells that participate in cross-interactions with other leukocyte populations and provide a link between innate and adaptive immunity [11]. In addition, they can influence the adaptive immune response by modulating $CD4^+$T cell responses. It was found that in response to the action of GM-CSF, as well as IFNγ and TNFα, IL-3 NGs express HLA class II molecules on their surface and become antigen-presenting cells [7, 13]

The multifunctionality of the NG is in direct dependence on the expression of various receptors on the surface membrane of the NG. Under the conditions of impaired functioning of the NG receptor complex, dysfunction associated with the absence or defects in signals is observed. There is a lack of quantitative growth of NG in the focus of inflammation, reduced number of actively phagocytosing cells, defective capture and digestion of bacterial antigens, inadequate response of microbicidal systems associated with defects in the activity of NADPH-oxidases, myeloperoxidase, neutrophil elastase, cathepsin G, defensins, etc. [11].

Osteomyelitis (OM) is a purulent-necrotic process developing in bone and bone marrow (BM) as well as in the surrounding soft tissues, is central to the structure of PID of the musculoskeletal system and accounts for 0.3-0.75% per 1000 children [1]. A highly specific pathogen in OM is *S. aureus*. *S. aureus* is able to invade, colonize and reproduce in bone tissue, produces virulence factors: degradation of host tissues, adhesion to components of the extracellular matrix, biofilm formation, evasion of destruction by phagocytes. [3]. In response, the chemokines CXCL8, IL-1β, CXCL2, and CCL3 are produced, attracting and activating even more NGs, creating an inflammatory microenvironment that promotes the formation of bone-resorbing osteoclasts [3].

The peculiarity of OM is the fact that the inflammatory process occurs in the BM, the site of hematopoiesis. The red BM contains the entire spectrum of differentiation of myeloid, erythroid cells, early and mature [14]. The first response to most pathogens includes myeloid cells, especially macrophages and NGs. However, their immature precursors and the pool of MDSCs (a subset of myeloid suppressor cells from mature and immature NGs) in CM are unable to exhibit antimicrobial activity [14]. MDSCs have immunosuppressive functions and contribute to the spread of infection [15]. Moreover, cytokines, bacterial products and reactive oxygen species interact with hematopoietic stem cells to induce emergency myelopoiesis, in which both mature NG and MDSC production are enhanced [15]. NGs constitutively express CD16, CD66b, CD33 receptors on the surface membrane.

CD66b (CEACAM8) is a single-stranded GPI-anchored glycoprotein of the Ig superfamily that is of interest to determine the functionally important subsets of NGs and their expressed only on the NG from the promyelocyte stage, a marker of NG activation [5]. HLA-DR is a receptor that is expressed on myeloblasts. HLA-DR is not present on circulating NGs, but is expressed on the surface of tissue NGs in specific inflammatory conditions such as rheumatoid arthritis, Wegener's granulomatosis [4]. CD33 (Siglec-3), belonging to the immunoglobulin superfamily contains two domains (IgV and IgC2) and is a marker of myeloid cell differentiation. The density of CD33 expression gradually decreases from the myeloblast stage to the segmentonuclear NG. The intracellular part of CD33 contains tyrosine-based inhibitory motifs (ITIM), which are involved in the inhibition of cellular activity [6].

The CD16 (Fc RIII) receptor is a marker for banded NG and segmented NG. Upon contact with a bacterial or viral antigen, this receptor trans-

locates from the cytoplasmic depot of the NG to its surface. Increased expression of membrane CD16 on the NG indicates cell overactivation, while decreased expression or complete absence of CD16 characterizes the immaturity of the NG and/or "reverse differentiation" of the cell, which is observed in severe bacterial infections or tissue necrosis [2].

The immune response to pathogens is significantly modified by local factors in specific tissues. The response to bacteria in bone has unique features compared to other infected tissues. It is phenotype when the cell is included in the inflammatory process, in OM and assess the possibility of influencing the expression level of surface receptors of immunotropic substance molecules to correct the NG functions.

Literature data indicate direct binding of the thymopoietin hormone pentapeptide, Timopentin (TP5), a synthetic analog of the thymopoietin active center, to HLA-DR molecules [8]. Given the molecular similarity of Hexapeptide (HP) – Arginylyl-alpha-Aspartyl-Lysyl-Valyl-Tyrosyl-Arginine and Timopentin – Arginyl-Lysyl-Asptyl-Valyl-Tyrosil, it is interesting to specify the effects of HP on the phenotype of NG subsets expressing on their surface markers CD16, CD66b, CD33 and CD HLA-DR in children with AOM in the experiment *in vitro*.

Purpose of the study: to specify the variants of quantitative and phenotypic characteristics of $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$, $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ neutrophil granulocytes subsets at acute osteomyelitis in children and evaluate their immunomodulation possibility under the influence of HP in experiment *in vitro*.

Materials and methods

Peripheral blood (PB) samples of 24 children with acute hematogenic (AHO) and acute posttraumatic (APTO) of 8-15 years old (2 girls, 22 boys), the Study Group, were studied. The Comparison Group consisted of PB samples of 13 healthy children aged 8-15 years.

To assess the effect of HP, the PB samples of children with AOM of the study group were incubated with HP (10-6 g/L) for 60 min, 37 °C – study group 1.

Flow cytometry (FC 500, Beckman Coulter, USA) tested the number of NG subsets of $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$, $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$ simultaneously expressing CD66b, CD16, CD33, HLA-DR and receptor expression density by fluorescence intensity (MFI) before and after incubation with HP (Mab, Beckman Coulter International S. A., France). Phagocytic activity of NGs before and after incubation with HP was determined in parallel. We evaluated the content of active-phagocytic NGs (%PhAH), the volume of captured bacterial pathogen *S. aureus* (strain 209) by indicators – phagocytic number (PhN), phagocytic index (PhI); to evaluate the killing activity – percentage of digestion (%D), digestion index (DI).

All legal representatives of patients obtained informed consent to participate in the study and blood sampling according to the WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects, 2013). The study was approved by the local ethical committee of the Federal State Educational Institution of Higher Professional Education "KubSMU" of the Russian Ministry of Health.

Statistical data processing performed using Microsoft Excel 2016 and StatPlus 2020. Wilcoxon–Mann–Whitney parameters used after assessing normality of distribution of laboratory indices. Presentation of the results in the form of median (upper and lower quartile) – Me ($Q_{0.25}$-$Q_{0.75}$). Determination of statistically significant differences at $p < 0.05$.

Results and discussion

The NG phenotyping in the comparison group of healthy children identified one $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$ subset in 98.8 (98.0-100.0) %. It was characterized by a low MFI CD66b molecule expression density of 4.6 (4.2-5.0), CD33 of 3.7 (3.3-4.6) and a mean MFI CD16 value of 81.5 (69.3-99.2).

Two subsets of $CD16^+CD16^+CD33^+HLA\text{-}DR^-$ and $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$, whose content in PB varied depending on the localization of the infectious process, were identified in the study group of children with AOM. Both subsets showed elevated expression levels of activating CD66b and CD16 receptors.

We revealed a 1.4-fold decrease in the relative amount of NGs in the $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$ subset to 71.2 (52.5-80.5) % relative to 98.8 (98.0-100.0) % in the comparison group ($p < 0.05$). There was an increase in MFI CD16 receptor expression density to 114.5 (100.3-139.0) *versus* 81.5 (69.3-99.2) and CD66b to 6.23 (5.7-7.3) *versus* 4.6 (4.2-5.0) in the comparison group ($p_{1,2} < 0.05$) and unchanged MFI CD33 – to 2.9 (2.5-3.1) *versus* comparison group ($p > 0.05$) (Figure 1).

At the same time, a subset expressing the HLA-DR receptor – $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$NG – was detected in AOM, which was absent in the PB of the healthy control children. The proportion of this subset was 29.9 (18.4-43.6) %. MFI HLA-DR – 2.2 (1.8-4.0), MFI CD33 – 3.5 (3.3-4.2), the density of expression of CD66b and CD16 molecules was comparable with that of $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$NG subset (Figure 2)

It showed that NGs BM exposed to GM-CSF, IL-3, IFNγ can differentiate into neutrophil-DC hybrids, exhibiting a DC-like phenotype and antigen-

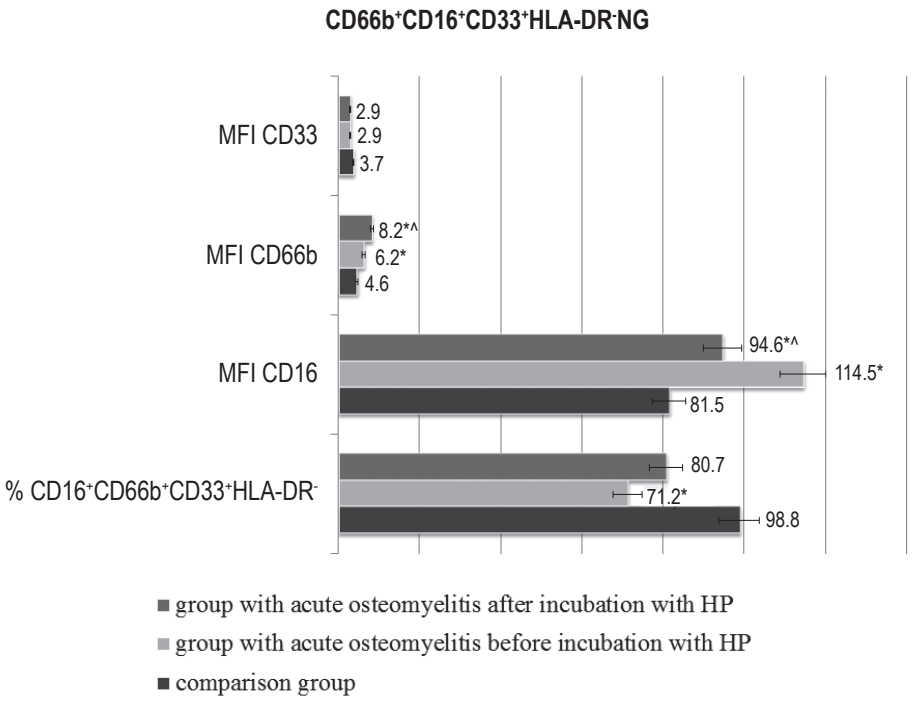

Figure 1. Number of CD66b$^+$CD16$^+$CD33$^+$HLA-DR$^-$NG subset and density of receptor expression (MFI) receptor expression density in acute osteomyelitis before and after *in vitro* incubation of the system with hexapeptide

Note. *, significant differences between the parameters of the comparison group and the study group (AOM), $p < 0.05$; ^, significant differences between the parameters of the study group and the study group after *in vitro* incubation with hexapeptide, $p < 0.05$.

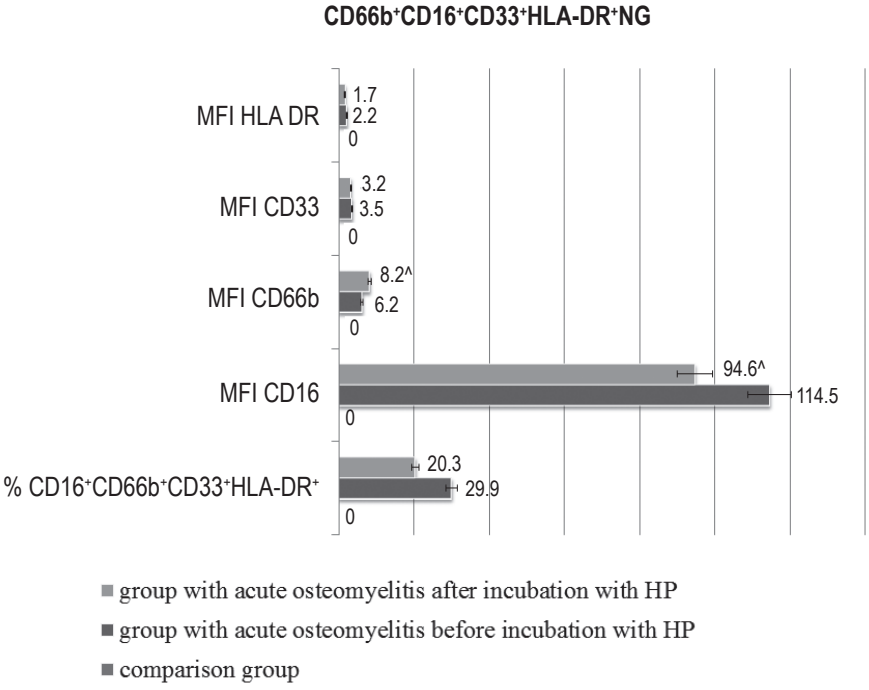

Figure 2. Number of CD66b$^+$CD16$^+$CD33$^+$HLA-DR$^+$ subset and density of receptor expression (MFI) in osseous osteomyelitis before and after incubation of the system *in vitro* with hexapeptide

Note. ^, significant differences between the parameters of the study group (AOM), and the study group after *in vitro* incubation with hexapeptide, $p < 0.05$.

presenting function, while retaining the properties of NGs [10]. In addition, it demonstrated that cytokine-exposed NGs acquire the ability to stimulate T cells via histocompatibility complex (MHC-II) molecules. In addition, the CD66b molecule expressed exclusively on NG can function as a receptor for galectin-3, which is expressed by $CD4^+$ memory T cells, at a low level by naive T cells [7]. Receptor-ligand interactions between memory T cells and NGs can provide the necessary signals to initiate MHC-II expression on the NG membrane. When MHC-II is expressed on the NG membrane, further amplification of MHC-TCR ligation occurs. As a result, more cytokine-secreting T cells are activated, causing an increase in MHC-II expression on the NG membrane. This positive feedback loop may play a central role in the induction and maintenance of HGH antigen presentation [7].

In addition, it was found that during the incorporation of NG into the immune response, there is an additional translocation of intracellular reserve pools of CD16, CD66b receptors to the membrane of NG, which manifests itself in a multiple increase in the number of highly equipped activated NGs. CD16 molecules are able to activate degranulation, phagocytosis, and oxidative burst, allowing NGs to destroy opsonized pathogens [2].

In addition, activation of CD66 on the NG surface induces functional responses such as cell aggregation and protein kinase activity. Interestingly, activation of CD66b on the NG membrane promotes the secretion of presynthesized IL-8, which forms a chemotactic pathway to attract other NGs to the inflammation zone [5].

Analysis of the effect of HP on NG subsets of $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$, $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ children from study group 1 showed different effects.

Thus, a lower NG level of the $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ subset was detected at 20.3 (18.7-39.9) *% versus* 29.9 (18.4-43.6) *%*. ($p > 0.05$) in the study group before incubation with HP. At the same time, there was a higher number of NG of the $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$ subset – 80.7 (72.5-84.5) *% versus* 71.2 (52.5-80.5) *%* before incubation ($p > 0.05$). This redistribution of subsets was apparently due to the binding of HPs to HLA-DRs on the NG membrane. HLA-DR expression density was also quite low MFI HLA-DR – 1.7 (1.6-2.2) *versus* MFI HLA-DR 2.2 (1.8-4.0) in the study group before incubation ($p > 0.05$). We found a 1.3-fold increase in CD66b expression density, 8.2 (8.0-11.1) *versus* 6.23 (5.7-7.3) in the study group ($p < 0.05$) and a 1.4-fold decrease in MFI CD16 to 94.6 (72.7-97.3) *versus* 114.5 (100.3-139.0) before incubation ($p < 0.05$). No effects of HP were detected in relation to MFI of CD33 receptors in both subsets (Figure 2).

Thus, the data obtained from the experiment *in vitro* demonstrated the possibility of a positive immunomodulatory effect of HP on both subsets. The increase of CD66b molecule under the influence of HPs apparently improves chemotactic, adhesive properties of cells necessary for realization of the NG effector functions. In addition, HP statistically significantly reduces the density of CD16 expression on the NG membrane, and therefore reduces the hyperactivation of cells with cytotoxic activity.

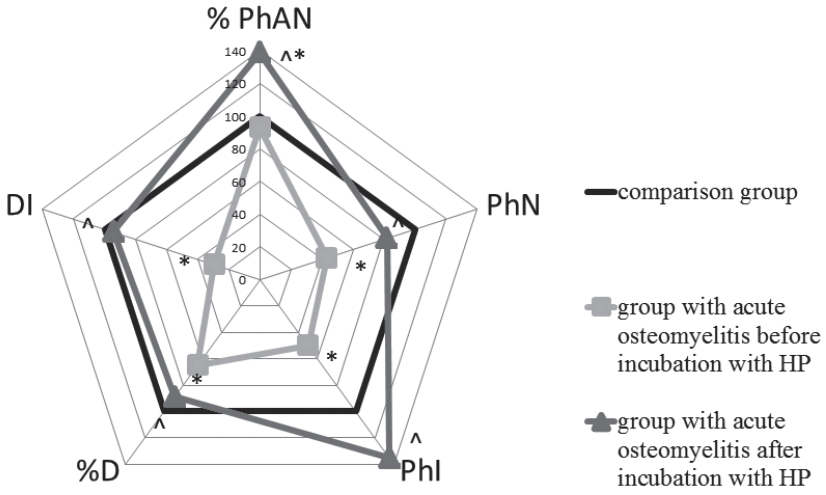

Figure 3. Phagocytic activity of neutrophil granulocytes in acute osteomyelitis before and after incubation of the system *in vitro* with hexapeptide

Note. As for Figure 1.

An *in vitro* study of the effect of HP on the phagocytic function of NG in children with AOM revealed an increase in %PhAN to 76.0 (70.0-77.0) % *versus* 51.0 (42.8-58.3) % ($p < 0.05$) in the study group and 54.7 (51.0-57.0) % in the comparison group ($p < 0.05$). We also found an increase in the killing ability of NG from 41.9 (37.8-44.8) % in the study group with AOM to 57.4 (53.6-61.1) % ($p < 0.05$), i.e. almost to the comparison group values of 64.5 (62.6-66.9) ($p < 0.05$) (Figure.3).

Thus, the positive effects of HP on phagocytic function were established, which was associated with the previously identified modulating effects of HP on subsets of $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$ and $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ subsets, as previously revealed

Conclusion

The present study was the first to demonstrate the appearance of $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ subset against the background of statistically significant decrease of $CD66b^+CD16^+CD33^+HLA\text{-}DR^-NG$ in children with AOM NG. Appearance of $CD66b^+CD16^+CD33^+HLA\text{-}DR^+NG$ subset in children with APO indicates appearance of activated subset in PB with APC properties presenting AG to T lymphocytes [12].

The analysis of individual indicators revealed a significant dispersion interval of the number of NG of this subset (18.4-43.6), which seems to depend on the inflammatory focus of the lesion and its localization, but this issue requires further study.

In an experimental system *in vitro*, a positive effect of HP on the phenotypic characteristics of NG subsets $CD66b^+CD16^+CD33^+HLA\text{-}DR^-$, $CD66b^+CD16^+CD33^+HLA\text{-}DR^+$ was demonstrated. From our point of view, the restoration of phagocytic function of NGs under the influence of HP is associated with an increase in the expression of CD66b, which influences the effector function of NGs and a decrease in the hyperexpression of CD16 molecule, which determines a decrease in the damaging cytotoxic activity of NGs.

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