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ИЛЛЮСТРАЦИИ К СТАТЬЕ «ЗАКОНОМЕРНОСТИ В РАЗВИТИИ КОЛЛЕКТИВНОГО ИММУНИТЕТА К SARS-CoV-2 В ХОДЕ ПАНДЕМИИ COVID-19» (АВТОРЫ: ПОПОВА А.Ю., СМИРНОВ В.С., ЕГОРОВА С.А., ДРОЗД И.В., МИЛИЧКИНА А.М., ДАШКЕВИЧ А.М., НУРМАТОВ З.Ш., МЕЛИК-АНДРЕАСЯН Г.Г., РУЗИЕВ М.М., ТОТОЛЯН АРЕГ А. [с. 759-766])

ILLUSTRATIONS FOR THE ARTICLE "PATTERNS IN THE DEVELOPMENT OF COLLECTIVE IMMUNITY TO SARS-CoV-2 DURING THE COVID-19 PANDEMIC" (AUTHORS: POPOVA A.YU., SMIRNOV V.S., EGOROVA S.A., DROZD I.V., MILICHKINA A.M., DASHKEVICH A.M., NURMATOV Z.S., MELIK-ANDREASYAN G.G., RUZIEV M.M., TOTOLIAN AREG A. [pp. 759-766])

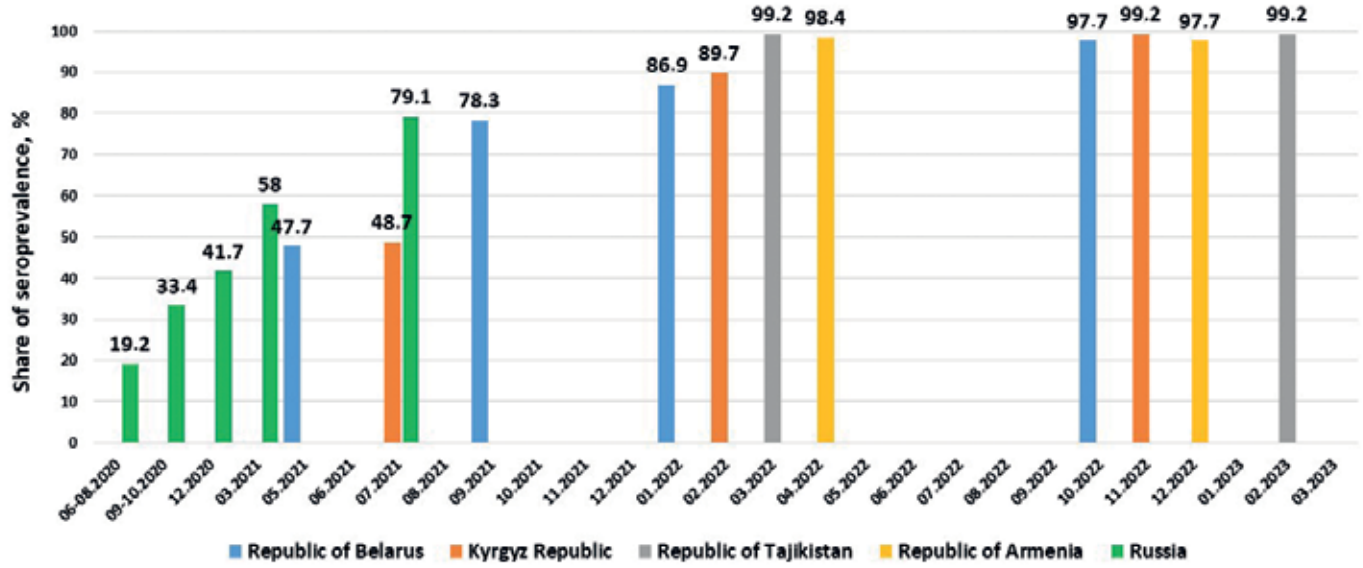

Figure 3. Dynamics of total seroprevalence in the population during the COVID-19 pandemic. Total refers to the presence of antibodies to Nc and/or RBD as one group

ИЛЛЮСТРАЦИИ К СТАТЬЕ «СНИЖЕНИЕ MDC/CCL22 ПРИ COVID-19 И В ПОСТКОВИДНОМ СИНДРОМЕ» (АВТОРЫ: КОРОБОВА З.Р., ТОТОЛЯН АРЕГ А. [с. 773-778])

ILLUSTRATIONS FOR THE ARTICLE "MDC/CCL22 DEPLETION IN COVID-19 AND POST-COVID" (AUTHORS: KOROBOVA Z.R., TOTOLIAN AREG A. [pp. 773-778])

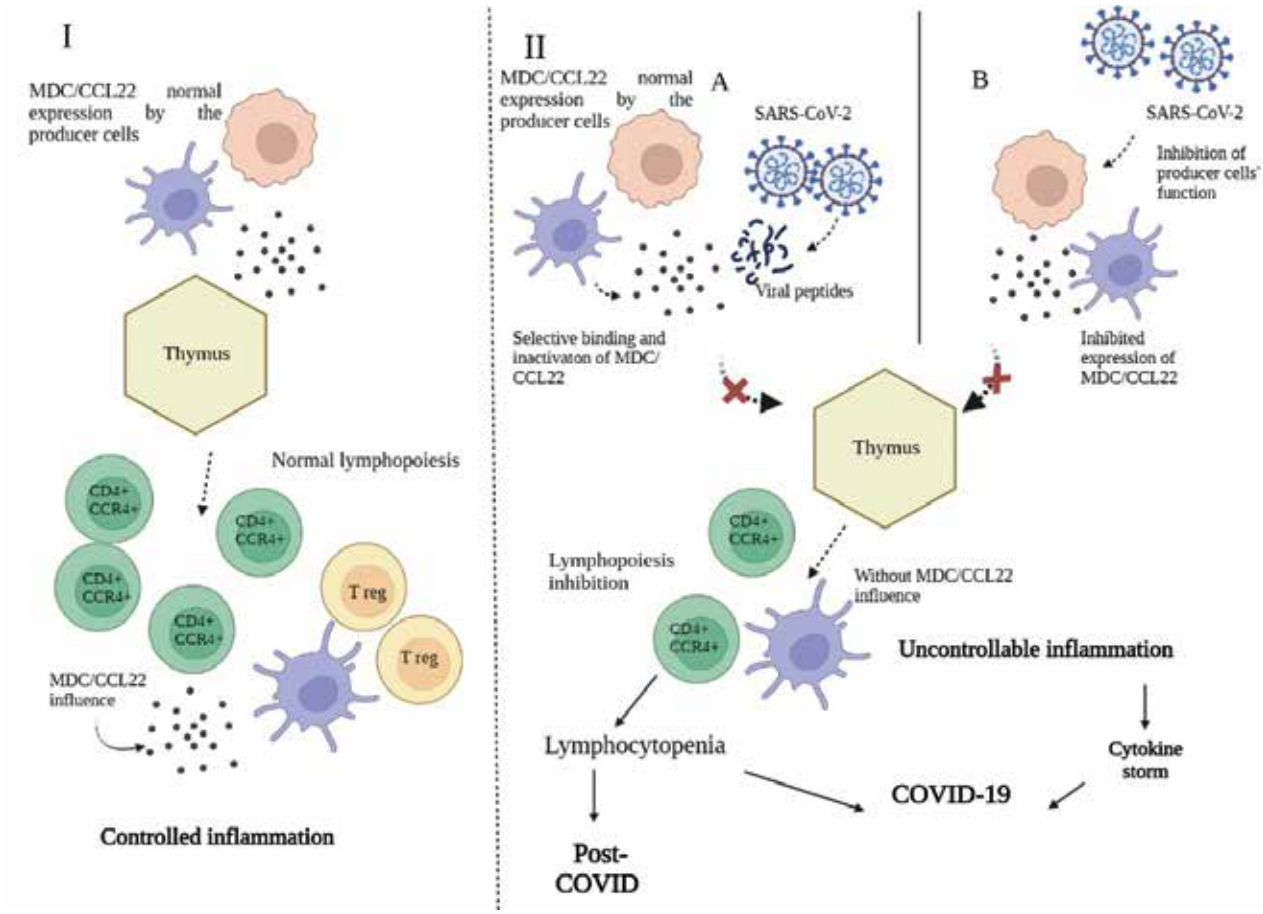

Figure 3. Role of MDC/CCL22 in immunity and the SARS-CoV-2 infectious process

Note. I, MDC/CCL22 influence on T lymphocyte maturation in thymus via the CCR4 receptor. The presence of this chemokine also mediates an adequate balance between regulatory T cells and helper T cells, thus creating restrictions on inflammatory reactions. II, SARS-CoV-2 influence on T cell maturation in thymus via depletion of MDC/CCL22: A, decrease in MDC/CCL22 concentrations associated with selective binding to SARS-CoV-2 viral peptides; B, restriction of MDC/CCL22 secretion by producer cells due to their functional failure.

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ИДЕНТИФИКАЦИЯ Th1-ПОЛЯРИЗОВАННЫХ КЛЕТОК Th17: РЕШЕНИЕ ПРОБЛЕМЫ

Куклина Е.М., Глебездина Н.С.

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Резюме. Т-хелперы, продуцирующие IL-17 (Th17), обладают высокой пластичностью: рестимуляция лимфоцитов в воспалительном окружении способна индуцировать их трансформацию в клетки с другим фенотипом, и наиболее частым является сдвиг в направлении Th1. Результатом такой трансформации является появление клеток, экспрессирующих наряду с классическими маркерами клеток Th17 ключевые Th1-ассоциированные молекулы. В наиболее общей форме такая популяция представлена $CD4^+CD161^+CCR6^+CXCR3^+IL\text{-}17^+IFN\gamma^+$Т-клетками, и в современной литературе она чаще всего обозначается как Th17.1. Часть клеток Th17.1 может полностью утрачивать продукцию IL-17, сохраняя при этом экспрессию других Th17-ассоциированных молекул, – это так называемые клетки ex-Th17 ($CD4^+CD161^+CCR6^+CXCR3^+IL\text{-}17^-IFN\gamma^+$Т-клетки). Как следствие, популяция Th1-поляризованных Th17 включает клетки Th17.1, ex-Th17 и ряд дополнительных переходных форм. Она имеет уникальные функциональные свойства – повышенный провоспалительный потенциал и способность преодолевать гистогематические барьеры. Именно этим клеткам в настоящее время отводится ключевая роль в патогенезе многих аутоиммунных заболеваний, а процесс редифференцировки Th17 в Th1 рассматривается как перспективная терапевтическая мишень. Однако развитие этого направления осложняет слабая сопоставимость данных о размерах такой популяции. Проведенный в рамках настоящей работы анализ методов определения Th1-поляризованных Th17 *in vivo* и *in vitro* позволил разрешить эти противоречия и разработать оптимальные подходы к идентификации данной популяции. В большинстве работ, особенно клинических, ее идентифицируют по коэкспрессии ключевых цитокинов (IL-17/IFNγ) или хемокиновых рецепторов (CCR6/CXCR3), редко – по их комбинации. При таком подходе коэкспрессия CCR6/CXCR3 маркирует общую популяцию Th1-подобных Th17, включающую и Th17.1, и ex-Th17, тогда как коэкспрессия цитокинов IL-17/IFNγ идентифицирует клетки Th17.1, а субпопуляцию ex-Th17 в этом случае ошибочно классифицируют как классические Th1. Такая «недооценка» субпопуляции ex-Th17 существенно занижает результаты, поскольку именно на долю ex-Th17 приходится основная часть Th1-подобных Th17. И только одновременная оценка коэкспрессии цитокинов и Th17-ассоциированных мембранных молекул позволяет идентифицировать клетки Th17.1 и ex-Th17 отдельно, что важно учитывать при интерпретации данных по проблеме и при планировании клинических исследований.

Ключевые слова: Th17, редифференцировка, Th1-поляризованные Th17, IFNγ-продуцирующие Th17, Th17.1, ex-Th17

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IDENTIFICATION OF Th1-POLARIZED Th17 CELLS: SOLVING THE PROBLEM

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Abstract. Helper T cells producing IL-17 (Th17) have high plasticity: restimulation of lymphocytes in an inflammatory environment can induce their transformation into cells with another phenotype, and a shift towards Th1 is the most common. The result of this transformation is the appearance of cells expressing along with the classical markers of Th17 cells key Th1-associated molecules. In its most general form, this population is represented by $CD4^+CD161^+CCR6^+CXCR3^+IL\text{-}17^+IFN\gamma^+$T cells, and in the current literature it is most often referred to as Th17.1. Some Th17.1 cells can completely lose the production of IL-17, while maintaining the expression of other Th17-associated molecules; these are the so-called ex-Th17 cells ($CD4^+CD161^+CCR6^+CXCR3^+IL\text{-}17^-IFN\gamma^+$T cells). Consequently, the population of Th1-polarized Th17 includes Th17.1, ex-Th17 cells and a number of additional transitional forms. It has unique functional properties – an increased pro-inflammatory potential and the ability to overcome histohematic barriers. It is these cells that are currently assigned a key role in the pathogenesis of many autoimmune diseases, and the process of Th17 redifferentiation into Th1 is considered as a promising therapeutic target. However, the development of this direction is complicated by the weak comparability of data on the size of such a population. The analysis of methods for determining Th1-polarized Th17 *in vivo* and *in vitro*, carried out in this work, made it possible to resolve these contradictions and develop optimal approaches to identifying this population. In most studies, especially clinical ones, it is identified by co-expression of key cytokines (IL-17/IFNγ) or chemokine receptors (CCR6/CXCR3), rarely by their combination. In this approach, co-expression of CCR6/CXCR3 marks the total population of Th1-like Th17, including both Th17.1 and ex-Th17, while co-expression of IL-17/IFNγ cytokines identifies only Th17.1 cells, and the subpopulation of ex-Th17 is misclassified as classic Th1 in this case. Such "underestimation" of the ex-Th17 subpopulation significantly marks down the results, since it is ex-Th17 that accounts for the bulk of Th1-like Th17. And only a simultaneous assessment of the co-expression of cytokines and Th17-associated membrane molecules allows identification Th17.1 and ex-Th17 cells separately, which is important to consider when interpreting data on the problem and when planning clinical trials.

Keywords: Th17, redifferentiation, Th1-polarized Th17, IFNγ-producing Th17, Th17.1, ex-Th17

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Introduction

Differentiation of T helper cells producing IL-17 (Th17) is usually initiated upon activation of naive $CD4^+$ T cells in the presence of cytokine combinations IL-6/TGF-β or IL-6/IL-1β, and later IL-23 enters the process, providing cell expansion and supporting their functioning, in particular, the synthesis of IL-17 [11]. Classical Th17 cells express the key transcription factor RORC, carry specific membrane markers such as the lectin-like killer cell receptor CD161 and the chemokine receptor CCR6, and are capable of producing their characteristic cytokines IL-17A, IL-17F, and IL-22 [9]. However, differentiated Th17 lymphocytes are unstable and can transform into cells of a different phenotype upon restimulation in a local cytokine environment. The most common variant of this transformation is a shift towards Th1, which is accompanied by the formation of cells that co-express along with traditional Th17-associated molecules Th1 cell markers – transcription factors STAT4/T-bet, the chemokine receptor CXCR3, and the key Th1 cytokine IFNγ [8, 10, 12].

Normally, the population of IFNγ-producing Th17 is present in trace amounts in peripheral blood, but its content in infiltrates of inflamed tissues reaches 60% [15], and it is these cells that are currently assigned a key role in the pathogenesis of many diseases, including autoimmune ones. Thus, the presence of IFNγ-producing Th17 was found in the sites of inflammation in sarcoidosis [15], in the CNS in multiple sclerosis [3, 6, 13], in the synovial tissue of patients with rheumatoid arthritis [2, 12], in inflamed tissues of the gastrointestinal tract of patients with Crohn's disease [1, 7], and for many pathologies a direct association with this non-classical T helper population has been convincingly shown. As a result, the number and activity of Th1-polarized Th17 cells are currently considered as promising diagnostic and/or prognostic markers. However, this is hampered

by inconsistencies in how this population is defined and by incomparability of data between studies.

The purpose of this work is to analyze methods for assessing Th1-polarized Th17 *in vivo* and *in vitro*, and to develop optimal approaches to identify this population.

Materials and methods

A literature search was performed in the PubMed database using appropriate keywords, without language and publication date limitations. The subject of interest was generally not a biological effects, but rather the ways of identification the non-classic T cell population and comparing its size between studies.

Results and discussion

Contradictions in the data on IFNγ-producing Th17 are primarily associated, apparently, with a large number of potential markers of these cells, and the markers are diverse. As noted earlier, this population combines the phenotypic and functional characteristics of both lines, Th17 and Th1, including the expression of membrane molecules, cytokines, and key transcription factors, although the latter are not usually used for identification. In the most general form, this population is presented in the works as $CD^+CD161^+CCR6^+CXCR3^+IL\text{-}17^+IFN\gamma^+$T cells [10, 12]. It is called differently in the current literature; in this paper we will use its most common name, Th17.1 [2, 14, 15]. However, Th17.1 cells are not the only variant of Th1-polarized Th17 lymphocytes: some of these cells lack the production of IL-17, but demonstrate the expression of the Th17-associated transcription factor RORC and CD161/CCR6 membrane molecules ($CD4^+CD161^+CCR6^+CXCR3^+$ $IL\text{-}17^-IFN\gamma^+$T cells) [1, 12]. In the literature, they are usually referred to as ex-Th17. Most of the available evidence suggests that Th17.1 and ex-Th17 cells are not separate subpopulations, but rather different stages of Th17 redifferentiation into Th1, during which classical Th17 first acquire the expression of Th1-associated molecules, and at the next stage lose IL-17 synthesis, producing only IFNγ, but retaining other "attributes" of the original population.

A more detailed analysis of data on the Th17.1 subpopulation shows that in a number of cases, when these cells are identified only with membrane molecules, two more chemokine receptors, CCR4 and CCR10, are added to the line of markers in order to separate the populations of classical Th17 and Th22, which upon identification using these membrane markers have the same phenotype ($CCR6^+CXCR3^{-/low}$), but can be differentiated by the combination of CCR4/CCR10: CCR4 is present in both populations, while CCR10 is highly expressed on Th22 cells but absent in Th17 [14]. As a result, the subpopulation of cells that co-produce IL-17/IFNγ will have the $CD4^+CD161^+CCR6^+CCR4^{-/low}CCR10^-$ $CXCR3^+$ phenotype, in contrast to the classical Th17 with the $CD4^+CD161^+CCR6^+CCR4^+CCR10^-$ $CXCR3^-$ phenotype [14]. In addition, in *ex vivo* studies, Th17.1 and exTh17 cells are usually isolated from pre-fractionated memory T cells, either central ($CCR7^+CD45RA^-$) or effector ($CCR7^-CD45RA^-$/ $CD45R0^+$).

Of course, this is not the limit of detail; there are studies in which, along with membrane molecules, a wide range of intracellular molecules are evaluated, both at the mRNA and protein levels, however, in most studies, especially clinical ones, this subpopulation is identified by key cytokines (IL-17/IFNγ), chemokine receptors (CCR6/CXCR3) or, very rarely, combinations thereof. Obviously, when using only CCR6/CXCR3 chemokine receptors as markers, a general population of Th1-like Th17 is identified, including both Th17.1 and ex-Th17 [2, 3, 13], whereas in the case of detection of Th1-like Th17 cells by co-expression of IL-17/IFNγ cytokines, we are talking about the Th17.1 subpopulation [1, 4, 12]. It is important to emphasize that the ex-Th17 subpopulation ($IL\text{-}17^-IFN\gamma^+$T cells) is in this case improperly classified as classic Th1. And only a simultaneous assessment of the expression of cytokines (IL-17/IFNγ) and Th17-associated membrane molecules (CD161 or CCR6) allows us to identify both the Th17.1 subpopulation ($CD4^+C$ $D161^+IL\text{-}17^+IFN\gamma^+$T lymphocytes) and the second the key subpopulation of Th1-like Th17 – ex-Th17 ($CD4^+CD161^+IL\text{-}17^-IFN\gamma^+$T cells), differentiating it using CD161 from classical Th1 lymphocytes lacking this marker ($CD4^+CD161^-IL\text{-}17^-IFN\gamma^+$T cells) [10].

Conclusion

The use of CCR6/CXCR3 membrane markers makes it possible to identify both major subpopulations of Th1-like Th17 lymphocytes, Th17.1 and ex-Th17, but does not allow them to be differentiated from each other. Evaluation of co-expression of IL-17/IFNγ cytokines makes it possible to detect Th17.1 cells, but not ex-Th17 cells; in this case, they are improperly attributed to the population of classical IFNγ-producing Th1. Moreover, such an "underestimation" of the ex-Th17 subpopulation significantly reduces the informativeness of the results, since it accounts for the bulk of T lymphocytes co-expressing Th17/Th1-associated membrane markers CCR6/CXCR3 [5, 15]. Not surprisingly, the size of the study population varies greatly from study to study. Therefore, when interpreting and comparing data on Th1-like Th17-lymphocytes, it is fundamentally important to take into account the method of their identification, and when planning work, to give preference to a combined approach, with simultaneous assessment of cytokines (IL-17/IFNγ) and Th17-associated membrane molecules (CD161 or CCR6), which allows to determine separately the subpopulations of Th17.1 and ex-Th17.

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ПОКАЗАТЕЛИ СИСТЕМЫ ЦИТОКИНОВ У ПРАКТИЧЕСКИ ЗДОРОВЫХ ЖЕНЩИН РАЗНОГО ВОЗРАСТА И ВЗАИМОСВЯЗЬ С ЭМОЦИОНАЛЬНЫМ СОСТОЯНИЕМ

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Резюме. Многочисленные исследования показывают роль цитокиновой сети в патогенезе тревожности, депрессии. Однако в настоящее время исследования сопряженности уровней провоспалительных и противовоспалительных цитокинов с уровнем эмоциональной нагрузки достаточно немногочисленны. Целью исследования был анализ сывороточных уровней провоспалительных и противовоспалительных цитокинов и эмоционального состояния у практически здоровых женщин в зависимости от возраста. В сыворотке крови были исследованы уровни IL-1β, IL-6, IL-17, IFNγ, IL-10 и IL-4 у 100 практически здоровых женщин, которые были распределены на 3 группы в зависимости от возраста (ВОЗ): 18-44 (молодой возраст) 30 человек, 45-59 (средний возраст) 40 человек, 60-74 (пожилой возраст) 30 человек (методом сендвич-варианта твердофазного иммуноферментного анализа, пг/мл). Для оценки эмоционального компонента здоровья все обследуемые проходили опросник SF-36 «Оценка качества жизни», где из 8 шкал оценивалась только шкала ролевого функционирования (эмоциональное состояние), значения выражали в баллах. Статистическую обработку полученных данных проводили с помощью аналитического программного обеспечения IBM SPSS Statistics, 22.0. У практически здоровых женщин обнаружено повышение значений IL-1β и IL-6 в группе пожилого возраста ($p < 0,05$), при этом между группами молодого и среднего возраста различий выявлено не было. Уровень IFNγ во всех возрастных группах женщин статистически значимо не отличался. При этом в группе пожилого возраста уровни IFNγ у 40% варьировались от 1,04 до 8,76 пг/мл, а у 60% женщин – от 24,85 до 28,5 пг/мл. IL-17 также был высоким ($p < 0,05$-$0,01$) в группе женщин 60-74 лет. В противовоспалительном звене наблюдалась противоположная картина, так женщин молодого и среднего возраста уровни IL-10 и IL-4 были выше показателей группы пожилого возраста. Таким образом, проведенный анализ позволил констатировать, что показатели цитокинового профиля

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у женщин сопряжены с возрастом. При оценке степени, в которой эмоциональное состояние мешает выполнению работы или другой обычной повседневной деятельности в группе женщин среднего возраста прослеживалось снижение ее уровня (50,6 баллов, $p < 0,01$), а в группах молодого и пожилого возраста значения шкалы приближались к 90 баллам (89,6 и 85,9, баллов соответственно).

Ключевые слова: цитокины, возраст, женщины, стресс, интерлейкины, качество жизни

INDICATORS OF THE CYTOKINE SYSTEM IN PRACTICALLY HEALTHY WOMEN OF DIFFERENT AGES AND INTERRELATION WITH THE EMOTIONAL STATE

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Abstract. Numerous studies show the role of the cytokine network in the pathogenesis of anxiety and depression. However, at present, studies of the correlation between the levels of pro-inflammatory and anti-inflammatory cytokines and the level of emotional stress are rather few. The aim of the study was to analyze the serum levels of pro-inflammatory and anti-inflammatory cytokines and the emotional state in apparently healthy women depending on age. Serum levels were tested IL-1β, IL-6, IL-17, IFNγ, IL-10 and IL-4 in 100 apparently healthy women, who were divided into 3 groups depending on age (WHO): 18-44 (young age) 30 people, 45-59 (middle age) 40 people, 60-74 (old age) 30 people (sandwich variant of enzyme-linked immunosorbent assay, pg/mL). To assess the emotional component of health, all the subjects passed the questionnaire SF-36 "Assessment of the quality of life". Statistical processing of the obtained data was carried out using the analytical software IBM SPSS Statistics, 22.0. In practically healthy women, an increase in the values of IL-1β and IL-6 was found in the elderly group ($p < 0.05$), while no differences were found between the groups of young and middle age. The level of IFNγ in all age groups of women did not differ significantly. At the same time, in the elderly group, the levels of IFNγ in 40% ranged from 1.04 to 8.76 pg/mL, and in 60% of women – from 24.85 to 28.5 pg/mL. IL-17 was also high ($p < 0.05$-0.01) in the group of women aged 60-74. In the anti-inflammatory link, the opposite picture was observed, for example, in young and middle-aged women, the levels of IL-10 and IL-4 were higher than in the elderly group. Thus, the analysis made it possible to state that the parameters of the cytokine profile and emotional state in women are associated with age.

Keywords: cytokines, age, women, stress, interleukins, quality of life

Introduction

Currently, an active lifestyle and work make up 80% of our lives. Such employment inevitably leads to an increase in emotional stress. This is also due to the fact that among able-bodied people, especially women, there is high competition and the need to strengthen the stability of their position. At the same time, emerging stress can stimulate the development of cognitive impairment, which leads to depletion of the pituitary-hypothalamic-adrenal axis, manifested by a decrease in cortisol release and pro-inflammatory activity. At the same time, elevated levels of IL-1β, IL-6 and TNFα can be detected not only in the bloodstream, but also in various organs and tissues [1, 2]. An important role is played by stress resistance, which depends on the psychological portrait of the individual; in this regard, the nature of human life is also an important aspect in the relationship between stress and the immune system. The aim of the study was to analyze the serum levels of the cytokine system and the emotional state in practically healthy women depending on age.

Materials and methods

One hundred practically healthy women were examined, who were divided into 3 groups depending

on age (WHO): 18-44 (young age) 30 people, 45-59 (middle age) 40 people, 60-74 (old age) 30 people. The levels of IL-1β, IL-6, IL-17, IFNγ, IL-10 and IL-4 in blood serum were studied by the sandwich variant of enzyme-linked immunosorbent assay, pg/mL. To assess the emotional component of health, all the subjects took the SF-36 questionnaire "Assessment of the quality of life", where out of 8 scales (physical functioning, role-based physical functioning, pain scale, general health, vitality scale, scale of social functioning, role emotional functioning, psychological health), only the scale of role functioning (emotional state) was assessed; the values were expressed in points. For all scales, the maximum value was 100, the higher the score, the better the quality of life on the scale. Before calculating the indicators, the responses were recoded. Statistical processing of the obtained data was carried out using the analytical software IBM SPSS Statistics, 22.0. For comparative analysis, the chi-square test (χ^2) was used. Spearman's rank correlation coefficient was used to identify the relationship between variables. The scope of the studies performed made it possible to evaluate the results with a reliability of 95-99% of statistical methods.

Results and discussion

It should be noted that the examined women had low values of the studied cytokines in all age groups – from 0.1 to 30 pg/mL. When studying the level of pro-inflammatory cytokines in practically healthy women, an increase in the values of IL-1β and IL-6 was found in the elderly group ($p < 0.05$, Table 1), while there were no differences in the level of the above cytokines between the groups of young and middle age. The level of IFNγ in all age groups of women did not differ significantly. At the same time, in the elderly group, IFNγ levels in 40% ranged from 1.04 to 8.76 pg/mL, and in 60% of women – from 24.85 to 28.5 pg/mL (Table 1).

IL-17 was also high ($p < 0.05$-0.01) in the group of women aged 60-74. In the anti-inflammatory link, the opposite picture was observed, for example, in young and middle-aged women, the levels of IL-10 and IL-4 were higher than in the elderly group. Thus, the analysis made it possible to state that the parameters of the cytokine profile in women are associated with age.

Analysis of the results of the cytokine profile of the blood serum of the examined patients in comparison with the data of other scientists showed that the level of IL-1β in the group of young and middle age is comparable with the data obtained by Markelova E.V. et al. (1.09 pg/mL, 2016), Turmovoy E.P. et al. (1.2 pg/mL, 2017) and Slepovoy A.S. et al. (0.65±0.5, 2016, in the older age group), but lower than the values of patients Slepovoy A.S. et al. (2016) at the age of 26.6±5 years. The level of IL-6 was higher than the values established by Turmovoy E.P. et al. (1.6 pg/mL, 2017, $p < 0.05$) and Slepovoy A.S. et al. (2016), exceeding them by an average of 2 times (7.2±2.3, $p < 0.05$. The levels of IFNγ were comparable to the data obtained in the course of studies by Saibel A.V. (12.52 pg/mL, 2013), but turned out to be higher compared to the results of Turmovoy E.P. et al. (5.5 pg/mL, 2017, $p < 0.05$). IL-17 corresponded to the values of all researchers, except for the values obtained by Seibel A.V. et al. (2013), which, on the contrary, were slightly higher (10.96 pg/mL, $p < 0.05$). There were no significant differences in the level of IL-4 compared with the literature data. The values of IL-10 in the groups of this study were 6 times lower than those of Turmova E.P. et al. (36.7 pg/mL, 2017, $p < 0.01$) and 1.5 times lower than those presented by Seibel A.V. et al. (11.77 pg/mL, 2013, $p < 0.05$), but did not differ from the results of Slepovoy A.S. et al. (2.6 pg/mL, 2016).

When assessing the degree to which the emotional state interferes with the performance of work or other normal daily activities, including spending a lot of time on them, reducing the amount of work done, reducing its quality, a decrease in its level was observed in the group of middle-aged women (50.6 points, $p < 0.01$), and in the young and old age groups, the scale values approached 90 points (89.6 and 85.9 points, respectively). When assessing the degree to which the emotional state interferes with the performance of work or other normal daily activities, including spending a lot of time on them, reducing the amount of work done, reducing its quality, a decrease in its level was observed in the group of middle-aged women (50.6 points, $p < 0.01$), and in the young and old age groups, the scale values approached 90 points (89.6 and 85.9 points, respectively).

It is known that acute stress is associated with the intensification of immune responses, while chronic stress is associated with a limitation of their effectiveness. Currently, a third component has been added to this two-component model, which is characterized by simultaneous activation and suppression of the immune response by changing the secretion pattern during chronic stress [2]. At the early stage of prolonged stress, the production of pro-inflammatory (IL-1β, IL-6, TNFα and IFNγ) is inhibited and the synthesis of anti-inflammatory cytokines (IL-4, IL-10 and IL-13, TGF-β) is enhanced. The next stage of prolonged stress is

TABLE 1. CONTENT OF PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINES IN THE BLOOD SERUM OF PRACTICALLY HEALTHY WOMEN, DEPENDING ON AGE, Me ($Q_{0.25}$-$Q_{0.75}$)

No.	Indicator, pg/mL	Practically healthy women, n = 30 (18-44 years old)	Practically healthy women, n = 40 (45-59 years old)	Practically healthy women, n = 30 (60-74 years old)
		1	2	3
1	IL-1β	1.1 (0.7-3.0) $p_{1-3} < 0.05$	1.22 (0.79-4.38) $p_{2-3} < 0.05$	3.29 (0.77-7.25)
2	IL-6	6.8 (0.96-11.4) $p_{1-3} < 0.05$	7.96 (1.38-19.42) $p_{2-3} < 0.05$	13.92 (3.0-22.1)
3	IFNγ	13.36 (4.06-19.80)	15.24 (5.62-19.84)	16.20 (1.46-28.50)
4	IL-17	3.22 (1.3-10.0) $p_{1-3} < 0.01$	3.80 (1.20-19.40) $p_{2-3} < 0.05$	11.30 (1.34-58.60)
5	IL-10	6.3 (1.86-10.83) $p_{1-3} < 0.05$	7.4 (2.02-19.00) $p_{2-3} < 0.001$	2.37 (0.89-4.01)
6	IL-4	11.00 (3.64-14.50) $p_{1-3} < 0.05$	14.85 (4.00-18.86) $p_{2-3} < 0.05$	9.60 (1.80-12.00)

Note. $p_{1, 2, 3}$ compared groups (only statistically significant differences are indicated).

characterized by "saturation" (or tolerance) of catecholamines and glucocorticoids, the effects of which lead to a weakening of influences on further stress reactions due to changes in the content of the nuclear protein "kappa-B" in immunocytes, a key pro-inflammatory factor. Activators of the nuclear protein blocker are IL-1, TNFα, IFNγ, which turn off this inhibitory connection. In turn, being activated, kappa-B stimulates the expression of IL-1, IL-6, IL-8, TNFα, IFNγ, chemokines and other molecular substances involved in the inflammatory response. In the third stage of chronic stress, pro-inflammatory cytokines and inflammatory mediators are activated again, at a certain level of which the inflammatory process is triggered [3].

Under conditions of chronic stress of moderate intensity, the level of pro-inflammatory cytokines, including IL-6, increases both in the blood and in the brain [1]. Chronic psychosocial stress in humans causes a decrease in the production of cortisol, which regulates the immune response, leading to increased synthesis pro-inflammatory cytokines, as well as a decrease in the concentration of serotonin, norepinephrine and dopamine [4, 5]. In addition, stress stimulates the secretion of norepinephrine in the brain tissues, which, acting through β-adrenergic receptors, induces the release of IL-1β from intracellular depots, and also activates the rapid production of this cytokine de novo. It should be noted that in response to stress, anti-inflammatory mechanisms are also activated in the brain, which provide protection against an excessive inflammatory response [1].

Cytokines IL-1 and IL-6 act in both directions, playing the role of modulators, and their receptors are dispersed in many structures of the central nervous system, powerfully stimulating the production of corticosteroids through the effect on corticoliberin. All this testifies to the important role of IL-1 and IL-6 as mediators of neuroimmune interaction in the body's response to stress. The anti-inflammatory effect of interleukins weakens, and the pro-inflammatory effect increases [3, 4], which was shown in the study, namely in the group of older women, apparently in the third phase of chronic stress.

We support the point of view of Lisitsyna T.A. et al. (2019), this study shows that hyperproduction of IL-6 in women aged 60-74 years can lead to a decrease in the level of serotonin and dopamine, which play an important role in the development of anxiety, chronic fatigue, and sleep disturbance. According to researchers, in healthy people who have experienced

stress, low levels of IL-6 in the blood are an indicator of a rapid regression of bad mood [2]. Differences in the production of IL-6 in response to stress factors are explained by genetic polymorphisms, for example, polymorphism of the IL-6 gene (SNPrs1800795) increases the risk of inflammation in individuals exposed to adverse socioeconomic factors, another polymorphism in the IL-6 receptor gene (rs8192284) leads to functional changes in amino acids, disrupting proteolytic reactions [3].

Conclusion

1. In practically healthy women aged 60-74 years, activation of the pro-inflammatory cytokine profile is observed.

2. When assessing the degree of the emotional component that affects the performance of daily duties, it was in the middle-aged group (45-59 years) that the depletion of the hypothalamic-pituitary-adrenal axis was observed, however, normal IL-6 values indicate a rapid regression of bad mood.

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Т-ЛИМФОЦИТЫ 2-ГО ТИПА ИММУННОГО ОТВЕТА И ИХ РОЛЬ В УСИЛЕНИИ ВОСПАЛЕНИЯ ПРИ ВЫПОЛНЕНИИ ПРОФЕССИОНАЛЬНОЙ ДЕЯТЕЛЬНОСТИ ПОЖАРНЫХ

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Резюме. Т-лимфоциты 2-го типа иммунного ответа способствуют развитию и обострению воспаления, главным образом аллергического. Усиление воспаления при длительном воздействии неблагоприятных факторов при работе пожарных может привести к развитию различных заболеваний. Оценка иммунитета пожарных важна для назначения адекватного лечения и профилактики инфекционных и аллергических заболеваний. Целью работы стал анализ показателей иммунитета у сотрудников государственной противопожарной службы МЧС России в зависимости от возраста и интенсивности профессиональной нагрузки. Обследованы мужчины (n = 79), средний возраст 31 год, стаж работы от 1 года до 22 лет с различной интенсивностью нагрузки. В периферической крови методом проточной цитометрии (Navios, FC 500, Beckman Coulter) оценивали субпопуляции моноцитов, относительное количество Т-лимфоцитов 2-го типа иммунного ответа $CD3^+CD294^+$. Определяли концентрацию общего иммуноглобулина Е (Immulite). В секрете носовых ходов оценивали содержание секреторного иммуноглобулина А (Вектор Бест) (n = 30). Статистическую обработку результатов проводили с помощью пакета Statistica 12.0 (StatSoft). Увеличение количества $CD3^+CD294^+$ клеток наблюдали в 16,5%. Выявили прямую корреляционную зависимость количества Т-лимфоцитов 2 и возраста обследованных лиц ($p < 0,05$). В группе пожарных с более интенсивной нагрузкой в 5 раз выше встречали повышение количества $CD3^+CD294^+$ клеток ($p < 0,05$). Среди пациентов, имевших какое-либо заболевание респираторного тракта, увеличение этой популяции наблюдали статистически значимо чаще – в 26% случаев против 11,5%. Выявили сильную прямую корреляционную зависимость количества Т-лимфоцитов 2 и длительности стажа курения ($p < 0,05$). Установили прямую корреляционную зависимость количества Т-лимфоцитов 2 и концентрации общего IgE ($p < 0,05$). Снижение секреторного IgA в секрете из носовых ходов наблюдали у 23% пожарных, у 13% обследо-

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ванных показатель выходил за верхнюю границу референтного интервала. Значимо чаще выявляли отклонение этого показателя от референтных значений у пожарных с высокой нагрузкой. Установили повышение субпопуляции классических моноцитов в группе обследованных с высоким количеством $CD3^+CD294^+$ клеток ($p < 0,05$). Таким образом, с увеличением профессиональной нагрузки пожарных в неблагоприятных условиях несения службы отмечается угнетение противоинфекционной защиты и усугубление повреждения респираторного тракта при усилении 2-го типа иммунного ответа. Оценка количества Т-лимфоцитов 2-го типа в периферической крови позволит выявить предрасположенность к Т2 профилю иммунного воспаления, что будет способствовать персонифицированному подходу к ведению пациентов.

Ключевые слова: Т-лимфоциты 2, $CD3^+CD294^+$ клетки, пожарные, воспаление, секреторный иммуноглобулин А, общий иммуноглобулин Е

T LYMPHOCYTES OF THE 2nd TYPE OF THE IMMUNE RESPONSE AND THEIR ROLE IN ENHANCING INFLAMMATION DURING THE PROFESSIONAL ACTIVITIES OF FIREFIGHTERS

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Abstract. T lymphocytes of the 2nd type of immune response contribute to the development and exacerbation of inflammation, mainly allergic. Increased inflammation with prolonged exposure to adverse factors during the work of firefighters can lead to the development of various diseases. Evaluation of the immunity of firefighters is important for the appointment of adequate treatment and prevention of infectious and allergic diseases. This paper aimed to analyze the indicators of immunity in employees of the state fire service of EMERCOM of Russia, depending on the age and intensity of the professional workload. The surveyed were men ($n = 79$), mean age 31 years, work experience from 1 to 22 years, with different workload intensity. In peripheral blood, flow cytometry (Navios, FC 500, Beckman Coulter) was used to evaluate subpopulations of monocytes, the relative number of T lymphocytes of the 2nd type of immune response $CD3^+CD294^+$. The concentration of total immunoglobulin E (Immulite) was determined. From nasal secretions, the content of secretory immunoglobulin A (Vector Best) was evaluated ($n = 30$). Statistical processing of the results was performed using the Statistica 12.0 package (StatSoft). An increase in the number of $CD3^+CD294^+$ cells was observed in 16.5%. A direct correlation was found between the number of T lymphocytes 2 and the age of the examined persons ($p < 0.05$). In the group of firefighters with a more intense workload, an increase in the number of $CD3^+CD294^+$ cells were 5 times higher ($p < 0.05$). Among patients who had any disease of the respiratory tract, an increase in this population was observed statistically significantly more often – in 26% of cases *versus* 11.5%. A strong direct correlation was found between the number of T lymphocytes 2 and the duration of smoking experience ($p < 0.05$). A direct correlation was established between the number of T lymphocytes 2 and the concentration of total IgE ($p < 0.05$). A decrease in secretory IgA in the secret from the nasal passages was observed in 23% of firefighters, in 13% of the examined, the indicator went beyond the upper limit of the reference interval. Significantly more often revealed the deviation of this indicator from the reference values in firefighters with a high workload. An increase in the subpopulation of classical monocytes was established in the group of those examined with a high number of $CD3^+CD294^+$ cells ($p < 0.05$). Thus, with an increase in the professional workload of firefighters in unfavorable conditions of service, inhibition of protection to infection and aggravation of damage to the respiratory tract with an increase in the 2nd type of immune response are noted. Evaluation of the number of type 2 T lymphocytes in peripheral blood will reveal a predisposition to the T2 profile of immune inflammation, which will contribute to a personalized approach to patient management.

Keywords: T lymphocytes 2, $CD3^+CD294^+$ cells, firefighters, inflammation, secretory immunoglobulin A, total immunoglobulin E

Introduction

In the pathogenesis of autoimmune, oncological, allergic, and infectious diseases, a significant role is given to disorders in the immune system [8] because it is one of the three regulatory systems of the human organism. Inflammation is a fundamental biological process underlying almost all immune responses [2]. T lymphocytes of the 2nd type of immune response contribute to the development, maintenance, and exacerbation of inflammation, mainly allergic, with the participation of several mechanisms. This cell population is involved in prolonging immediate and delayed types of inflammation, as well as eosinophilic and neutrophilic [1]. An increase in type 2 T lymphocytes in the peripheral blood has been described in patients with allergic [9] and autoimmune diseases [13]. Recently, a hypothesis has been proposed [5] that cells of the 2nd type of immune response, in addition to the development of allergic reactions, control chronic inflammatory diseases, and metabolic homeostasis, take part in the repair and fibrosis of tissues, etc.

Strengthening the processes of local and systemic inflammation, which develop as a result of prolonged exposure to adverse factors in the respiratory tract of firefighters, can lead to the development of diseases of the respiratory and cardiovascular systems and can result in the occurrence of oncological diseases [14]. The complex of unfavorable professional factors for firefighters includes exposure to burning products, high temperatures, physical overstrain, stress, disturbed sleep and wakefulness, nutrition, and others not directly related to the fire extinguishing process. The respiratory tract is primarily involved in counteracting adverse factors of various origins, including pathogens and toxic substances from the environment. In the mucous of the respiratory tract, powerful immune defense mechanisms are used, but unfavorable professional factors that contribute to chronic inflammation can lead to the suppression of immunity to infection and the development of allergic pathology in the performance of firefighters.

Evaluation of cellular and humoral immunity in persons exposed to occupational hazards is important not only for prescribing adequate treatment for advanced diseases but also for determining the type of immune response to prevent possible diseases, including infectious and allergic ones.

This paper **aim** to analyze the indicators of cellular and humoral immunity in employees of the state fire service of EMERCOM of Russia, depending on the age and intensity of the professional workload.

Materials and methods

The study concluded 79 employees of the federal fire service of the state fire service of the EMERCOM of Russia, men, with an average age of 31 years (from 21 to 47 years), and work experience in the specialty from 1 to 22 years. The intensity of the load was calculated depending on the time spent by the fire and rescue team in personal respiratory protection equipment.

In the peripheral blood of all examined individuals, monocyte subpopulations were analyzed, the relative number of T lymphocytes of the 2nd type of immune response, the concentration of total immunoglobulin E (IgE), and the amount of secretory immunoglobulin A (sIgA) in the secretion of nasal passages were measured in 30 firefighters (average age and work experience corresponded to the main group).

The evaluation of the relative number of T lymphocytes of the 2nd type in peripheral blood, as well as subpopulations of monocytes, was carried out by flow cytometry. To determine the subpopulation composition of monocytes, whole blood aliquots were stained with a cocktail of anti-CD14, anti-CD16, and anti-CD45 monoclonal antibodies (Beckman Coulter, USA) according to the manufacturer's instructions. VersaLyse (Beckman Coulter, USA) was used for the lysis of erythrocytes. Samples were analyzed in a multicolor protocol on a Navios flow cytometer (Beckman Coulter, USA). The monocyte population was defined as $CD45^+SSCmodCD14^+$ cells. Depending on the CD16 expression density, three subpopulations were distinguished among $CD14^+$ monocytes: $CD14^+CD16^-$ (classical), $CD14^+CD16^+$ (intermediate), and $CD14^{dim}CD16^+$ (non-classical). To assess type 2 T lymphocytes, whole blood aliquots were stained with a cocktail of anti-CD3 and anti-CD294 monoclonal antibodies (Beckman Coulter, USA) according to the manufacturer's instructions. For the lysis of erythrocytes, OptiLyse C (Beckman Coulter, USA) was used. The population of T lymphocytes 2 was determined as $CD3^+CD294^+$ cells and their relative number from the total pool of lymphocytes was estimated. Samples were analyzed on a Cytomics FC 500 flow cytometer (Beckman Coulter, USA).

The chemiluminescent method (Immulite 2000, Siemens, Germany) was used to determine total immunoglobulin E in blood serum. Determination of the concentration of secretory IgA in nasal secretions was carried out by enzyme immunoassay (IgA secretory-ELISA-BEST, Vector Best, Russia). The material for the study was obtained by blotting nasal secretions with a standard circle of filter paper, after which 500 μL of the RPMI 1640 medium was added to the samples, and this material was subsequently used in enzyme immunoassay (INFINITE F50, TECAN, Austria).

Statistical processing of the results was carried out using the Statistica 12.0 (StatSoft) with the determination of descriptive statistics (mean values, standard error). The statistical significance of differences in the groups was assessed using a non-

parametric Mann–Whitney U test for independent variables. Spearman's non-parametric correlation analysis was used to identify and assess the relationship between quantitative traits. Frequency analysis was performed using four-field contingency tables based on Pearson's X2 test. Differences in the compared parameters were considered statistically significant at $p < 0.05$.

Results and discussion

It is known that lymphocytes of the 2nd type of immune response with the $CD3^+CD294^+$ phenotype are effector memory T cells [1]. An increase in the relative number of this cell population above the upper limit of the reference interval (0.5-1.5%) was observed in 13 examined individuals (16.5%). In the group of firefighters with normal values of T lymphocytes of the 2nd type, this parameter was 0.7±0.3%, in the other group – 2.9±1.6%, the age of the examined in these groups differed statistically significantly – 30.3±6.6% and 36.5 ± 8.4%, respectively, $p < 0.05$. In firefighters younger than 35 years old, on average, 0.83±0.6% of cells of this population among lymphocytes were detected in the group compared with persons older than 35 years old, where this figure was twice as high and amounted to 1.6±1.6% ($p < 0.01$), which exceeds the upper limit of the reference interval. Correlation analysis revealed a direct correlation between the relative number of T lymphocytes of the 2nd type of immune response and the age of the examined individuals (Spearman's correlation coefficient 0.3, $p < 0.05$).

In addition, it was shown that in the group of firefighters with a more intense load, the frequency of occurrence of an increased (more than 2%) relative number of T lymphocytes of the 2nd type of immune response was 5 times higher (17.1% *versus* 3.2%, $p < 0.05$, Pearson's contingency coefficient C 0.242, which indicated the average strength of the connection). An increase in this population of cells in several examined individuals, especially among firefighters with an intensive professional workload, indicated an increase in type 2 immune response and indicated the influence of unfavorable working conditions on the deviation of the immune response.

It should be mentioned that the analysis included mainly young and middle-aged people, but at the same time, a quarter of them had diseases of the upper (in 85% of the total number of diseases) and lower (in 15% of cases) respiratory tract diseases at the time of the study. Among the diseases of the upper respiratory tract dominated allergic and chronic rhinitis, tonsillitis, and rhinosinusitis. Diseases of the lower respiratory tract included mainly chronic bronchitis, bronchial asthma, and chronic obstructive pulmonary disease. When conducting a frequency analysis, it was shown that among patients who had any disease, an increase in the population of type 2 T lymphocytes was observed statistically significantly more often – in 26% of cases *versus* 11.5% in persons without diagnosed diseases ($p < 0.05$).

A strong direct correlation was found between the number of T lymphocytes of the 2nd type and the duration of the smoking experience (Spearman correlation coefficient 0.49, $p < 0.05$). Tobacco smoke activates the epithelial cells of the respiratory tract, as a result of which they produce pro-inflammatory cytokines – TNF, IL-1β and IL-8, granulocyte-macrophage colony-stimulating factor, as well as TSLP (thymic stromal lymphopoietin), which directs the immune response in type 2 [16]. A long time of smoking provokes the appearance of sensitization to allergens [4] and increases the incidence of chronic non-allergic rhinosinusitis [3]. Exposure to various toxic substances contained in tobacco smoke enhances the adverse effect of occupational hazards in firefighters and contributes to the persistence of chronic inflammation, mainly type 2.

In 30% of the firefighters, an increase in the concentration of total immunoglobulin E in the blood serum was revealed. A trend towards an increase in this indicator was shown in the group of patients with an increased relative number of type 2 T lymphocytes in peripheral blood – 144 IU/mL *versus* 103 IU/mL (reference interval 0-85 IU/mL). A direct correlation was found between the number of T lymphocytes of the 2nd type and the concentration of total IgE (Spearman correlation coefficient 0.24, $p < 0.05$). The results confirm the participation of this population of T cells in the switch of B lymphocytes to the synthesis of IgE and indicate a more pronounced IgE-mediated allergic inflammation in the group of firefighters with an increase in the number of type 2 T lymphocytes.

It should be noted that the concentration of total immunoglobulin E in both groups, on average, went beyond the upper limit of the reference interval, which confirmed the general trend of increased allergic inflammation in the group of firefighters compared to healthy individuals in the general population. The development of a specific allergic immune response to inhaled combustion products in firefighters has been described, as evidenced by an increase in the number of eosinophils, eosinophilic cationic protein, and pro-inflammatory cytokines IL-4 and IL-13 in sputum and bronchoalveolar lavage [6].

In addition to assessing the indicators of systemic cellular and humoral immune response in 30 firefighters in the upper respiratory tract, the concentration of secretory IgA, the main immunoglobulin contained in mucous secretions (tears, saliva, sweat, colostrum, discharge from the genitourinary tract, etc.) was determined. It was shown that a decrease in its amount in the secret from the nasal passages was observed in 23% of firefighters, and in 13% of the examined, the indicator went beyond the upper limit of the reference interval (5-30 μg/mL). The main function of secretory IgA is the binding of antigens

(both infectious agents and allergens that can cause sensitization) [7]. At the same time, it is not excluded that the reason for the excessively high content of secretory immunoglobulin A may be an increase in its synthesis in damaged organs [11]. It has been shown in animal models that exposure to high temperatures leads to a decrease in this important humoral mucosal protection factor [10], and adaptation to moderately high temperatures increases the concentration of sIgA locally [12]. Therefore, secretory immunoglobulin A can be considered a marker of the body's adaptation to external influences.

There was a pronounced tendency to increase the amount of sIgA in the secret from the nasal passages in the group of firefighters with work experience of 6-14 years compared with the group, which included persons working less than 5 years – 52±59 μg/mL and 19±59 μg/mL, respectively, $p = 0.07$. When conducting a frequency analysis, it was shown that in the group of firefighters with a high load, a deviation of this indicator from the reference values was significantly more often ($p < 0.05$) – in 8 out of 14 examined persons (in 5 people a decrease was detected, in 3 an increase in sIgA in the secret) against 3 out of 16 people with a lower professional load (2 people showed a decrease, 1 had an increase in sIgA in secret). The results obtained prove the influence of unfavorable working conditions on the lesion of the parameters of the immune system of the mucous of the upper respiratory tract. In the group of firefighters, multidirectional trends can be distinguished – both a decrease immunity to infection with a decrease in one of the main humoral factors of mucosal immunity, and excessive synthesis of sIgA that accompanies damage to the epithelial lining of the respiratory tract. In both cases, as a result, there is a high probability of developing inflammation, both infectious with a decrease in the protection of mucous and aseptic with the toxic effects of burning products.

There was a trend towards an increase in the frequency of occurrence of a reduced amount of sIgA in the secret from the nasal passages in the group of firefighters with an increased number of type 2 T lymphocytes – 3 out of 7 people (43%) *versus* 4 out of 23 (17%). Probably, in several examined individuals, an increase in type 2 immune response leads to inhibition of the synthesis of protective immunoglobulin A with an increase in the production of immunoglobulin E in the mucous, which will contribute to the development of allergic inflammation.

An increase in inflammation in firefighters with a deviation of the immune response towards type 2 was also evidenced by a statistically significant increase in the relative number of classical monocytes (92±3.7% *versus* 88±5.2%, $p < 0.05$), which went beyond the upper limit reference interval (81-90%), in the group examined with a high number of $CD3^+CD294^+$ cells. The main function of classical monocytes is to promote homeostasis by eliminating apoptotic bodies, maintaining inflammation, and participating in tissue repair [15].

Conclusion

In our study, it was found that an increase in the peripheral blood of firefighters T lymphocytes with the $CD3^+CD294^+$ phenotype is observed not only with age, but also with an increase in professional workload, and is also associated with smoking experience and an increase in the incidence in the examined group. In firefighters with an increased number of T lymphocytes of the 2nd type, an increase in the concentration of immunoglobulin E and the relative number of classical monocytes with the phenotype $CD14^+CD16^-$ was found, as well as a tendency to decrease in the secretion from the nasal passages of secretory immunoglobulin A. Therefore, an increase in the relative amount of T lymphocytes type 2 in peripheral blood will contribute to a more likely development of allergic reactions, a decrease in protective forces, and possible fibrosis of lung tissue during prolonged contact with burning products and other inhaled allergens during the professional activities of firefighters, especially with an increase in the smoking experience.

The revealed tendencies of inhibition of immunity to infection and aggravation of damage in the respiratory tract with an increase in type 2 immune response with an increase in professional workload in adverse working conditions contribute to the deterioration of the health of firefighters. Regular in-depth dispensary monitoring of this contingent should be recommended, using laboratory and instrumental methods of examination to prevent the development of diseases and timely detection of severe complications. The use of the relative amount of type 2 T lymphocytes in peripheral blood as a laboratory biomarker to characterize the dominant type of immune inflammation will make it possible to identify in firefighters a predisposition to the T2 profile of the immune response, which will contribute to a personalized approach to patient management.

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КЛИНИКО-ИММУНОЛОГИЧЕСКАЯ ХАРАКТЕРИСТИКА СТУДЕНТОВ МЕДИЦИНСКОГО ВУЗА В ЗАВИСИМОСТИ ОТ ДЛИТЕЛЬНОСТИ И ПРОГРАММЫ ОБУЧЕНИЯ

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Резюме. Хронический психоэмоциональный стресс может стать причиной дисфункции нейроиммуноэндокринной дисрегуляции с последствиями в виде нарушения функционального потенциала иммунной системы. Адаптация к новым условиям жизни при старте учебы в медицинском вузе – одно из неизбежных обстоятельств, которое преодолевают первокурсники. Обучение по программе военной подготовки в медицинском вузе несет в этом аспекте дополнительную стрессорную нагрузку. Исследования, посвященные механизмам формирования адаптивных реакций иммунной системы при обучении по программе военной подготовки офицеров медицинской службы, представляют несомненный интерес. Целью проведенного исследования стала сопоставительная характеристика клинической манифестации иммуноопосредованной патологии и параметров адаптивного и врожденного иммунитета студентов медицинского вуза в зависимости от стажа и программы обучения. Под наблюдением находились 104 студента-медика, все – мужчины, из которых 37 первокурсников и 67 – студенты третьего курсов медицинского университета. Обследуемые каждого курса разделены на две подгруппы в зависимости от программы обучения. Группа первокурсников состояла из 18 человек военного учебного центра (ВУЦ) и 19 – лечебно-профилактического факультета (ЛПФ). Среди третьекурсников студентов ВУЦ – 31, ЛПФ – 36. Для клинической характеристики заболеваемости в течение года обучения использовали регистрационные карты анализа иммуноопосредованной патологии, параметры иммунной системы в конце весеннего семестра исследовали с использованием стандартных методологических подходов. Полученные данные указывают, что на первом курсе адаптация к обучению у студентов, имеющих дополнительную нагрузку в виде программы военной подготовки, проходит более тяжело в сравнении с первокурсниками лечебного факультета. Эти различия состоят в более частой и значимой клинической манифестации инфекционной патологии и отражаются на функциональном потенциале клеточных показателей врожденного иммунитета. Констатация признаков угнетения функциональных потенций клеток макрофагального ряда и натуральных киллеров у первокурсников военного учебного центра является настораживающим фактором возможного срыва адаптационных резервов системы иммунного реагирования, что, вероятно предполагает необходимость разработки программ превенции негативного влияния стрессообразующих факторов. К третьем году обучения у студентов военного учебного центра в сравнении со студентами

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стандартной образовательной программы врачей-лечебников клинические и иммунологические показатели функционирования иммунной системы лучшие. Вероятно предположение, что в этот период завершается процесс психологической адаптации военных студентов-медиков.

Ключевые слова: психоэмоциональный стресс, адаптация, студенты, натуральные киллеры, иммунная дисрегуляция, стрессообразующие факторы

CLINICAL AND IMMUNOLOGICAL CHARACTERISTICS OF MEDICAL STUDENTS DEPENDING ON THE DURATION AND PROGRAM OF STUDY

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Abstract. Chronic psycho-emotional stress can cause dysfunction of neuroimmunoendocrine dysregulation with consequences in the form of a violation of the functional potential of the immune system. Adaptation to new living conditions at the start of studies at a medical university is one of the inevitable circumstances that first-year students overcome. Education under the military training program at a medical university carries an additional stress load in this aspect. Research on the mechanisms of formation of adaptive reactions of the immune system during training under the military training program for officers of the medical service is of undoubted interest. The purpose of the study was to compare the clinical manifestations of immune-mediated pathology and the parameters of adaptive and innate immunity of medical students depending on the length of service and training program. Under observation were 104 medical students, all men, of which 37 were first-year students and 67 were third-year students of a medical university. The subjects of each course were divided into two subgroups depending on the training program. The group of first-year students consisted of 18 people from the military training center (VTC) and 19 people from the medical and preventive faculty (LPF). Among the third-year students of the VUC – 31, LPF – 36. For the clinical characterization of the incidence during the year of study, registration cards for the analysis of immune-mediated pathology were used, the parameters of the immune system at the end of the spring semester were studied using standard methodological approaches. The data obtained indicate that in the first year students with an additional load in the form of a military training program have a more difficult time adapting to learning in comparison with first-year students of the medical faculty. These differences consist in a more frequent and significant clinical manifestation of infectious pathology and are reflected in the functional potential of cellular parameters of innate immunity. The statement of signs of inhibition of the functional potencies of macrophage cells and natural killers in first-year students of a military training center is an alarming factor in the possible disruption of the adaptive reserves of the immune response system, which probably suggests the need to develop programs to prevent the negative impact of stress-forming factors. By the third year of study, the students of the military training center have the best clinical and immunological indicators of the functioning of the immune system in comparison with the students of the standard educational program of general practitioners. It is likely that during this period the process of psychological adaptation of military medical students is completed.

Keywords: psycho-emotional stress, adaptation, students, natural killers, immune dysregulation, stress-forming factors

Introduction

The educational process in a medical university is characterized by high intensity, which affects the psycho-emotional status of students and can lead to the formation of chronic stress [8]. In turn, stress causes a change in the functional activity of the homeostatic systems of the body, including the immune system, contributing to a decrease in immunocompetence and the development of clinical manifestations of immune dysfunction [2, 3, 4, 7]. Education under the military training program carries an additional stress load to the standard educational process in a medical school [9]. On the other hand, military medical students initially have higher health requirements than civilians. Is the combination of the above factors reflected in the change in the functional resources of the immune system of medical students? Studies on the aspects of the formation of adaptive reactions of the immune system during training under the military training program for officers of the medical service are fragmentary and are extremely poorly covered.

The purpose of this work was a comparative assessment of the clinical manifestation of immune-mediated pathology and the parameters of adaptive and innate immunity of medical students depending on the length of service and training program.

Materials and methods

The object of the study were 37 first-year and 67 third-year students of the medical and preventive faculty and the military training center of the Federal State Budgetary Educational Institution of Higher Education of the Rostov State Medical University of the Ministry of Health of Russia. The subjects of each course were divided into two subgroups depending on the training program. The group of first-year students consisted of 18 people from the military training center (MTC) and 19 people from the medical and preventive faculty (MPF). Among the third-year students of the MTC – 31, MPF – 36. The study participants were comparable in age (respectively, years: 19±1 and 19±2 in the first year group; 23±3 and 22±2 in the third year), gender (all men), physical condition (corresponded to the I group of health).

All study participants signed an informed consent in accordance with the protocol approved by the Local Independent Ethical Committee of the Federal State Budgetary Educational Institution of Higher Education Rostov State Medical University (No. 15/19 dated October 08, 2019).

The study was conducted at the end of the second and sixth semesters of study.

The work used registration cards for the analysis of immune-mediated pathology (Sizyakina L.P., 2013), reflecting the incidence during the year from the standpoint of assessing the work of the immune system according to the main syndromes: infectious, allergological, autoimmune, lymphoproliferative. The parameters of the immune system were studied using standard methodological approaches [5].

The inclusion criterion for all participants at this stage was the absence of acute or chronic diseases in the acute stage. Statistical calculations were performed using the StatTech v. 1.2.0 and Statistica SPSS v. 26. The description of the results obtained was carried out by calculating the median (Me) and the interquantile range of values in the form of 25 and 75 percentiles, which is presented in the text as Me ($Q_{0.25}$-$Q_{0.75}$). Comparison of two groups on a non-normally distributed score was performed using the Mann–Whitney U test, and groups with a normal distribution were compared using a parametric Student's t-test. Differences were considered significant at $p < 0.05$.

Results and discussion

Processing of data from registration cards of immune-mediated pathology indicates that during the first two semesters of study at the Medical University, there were no lymphoproliferative disorders, as well as manifestations of autoimmune pathology, in both subgroups of first-year students. Allergic syndrome was absent in MTC students and was detected (seasonal allergic rhinitis) in 10% of MPF students. Infectious manifestations were noted in all students of the MTC (100% of cases) and only in 36% of students of the MPF. Clinically, the infectious syndrome was manifested by acute infections of the respiratory tract. Complications that required the use of antibiotic therapy developed in 36% of cases in military students and in 20% of cases in MPF students.

Comparative characteristics of the parameters of the immune system of the two subgroups did not reveal fundamentally significant changes in the parameters of the subpopulation composition and functional characteristics of T and B-lymphocytes, as well as the level of production of serum immunoglobulins. At the same time, a statistically significant difference was noted in the content of a functionally active subpopulation of natural killers in the peripheral circulation. Thus, in MTC students, the proportion of granzyme-containing $CD3^-CD16^+Gr^+$ lymphocytes is 3 (1.9-4.8) %, while in MPF students this figure is 10 (7-13) % ($p = 0, 0001$). Also, in military medical students, in comparison with the MPF group, a smaller number of peripheral blood monocytes express the pattern-recognizing receptor TLR4: $CD14^+CD284^+$ 13.5 (10-18) % and 19 (13-24) %, respectively ($p = 0.03$).

The data obtained indicate that in the first year students with an additional load in the form of a military training program have a more difficult time adapting to learning in comparison with first-year students of the medical faculty. These differences are also reflected in more frequent and significant clinical manifestations and, most importantly, affect the functional potential of cellular parameters of innate immunity. The results obtained are of particular interest because they are in line with the data of studies emphasizing that among all subpopulations of immunocytes, natural killer cells have the highest sensitivity to chronic stress [1, 6].

The analysis of third-year students' survey data revealed that during the 4th and 5th semesters of study, the clinical manifestation of immune-mediated processes was registered in 58% of the students of the MTC and 61% of the students of the LPF. At the same time, no lymphoproliferative disorders and manifestations of autoimmune pathology were noted. Allergosyndrome (seasonal allergic rhinitis) was recorded in 3% of cases of military doctors and in the same ratio (3%) in the MPF group. Infectious manifestation in the form of acute infections of the upper respiratory tract was manifested in 45% of military doctors and 50% of students of the medical faculty. Complications that required the use of antibiotic therapy developed in 28% of cases in military students and in 45% of students of the MPF.

A comparative analysis of the parameters of the immune status of two subgroups of third-year students

showed that statistically significant differences affected only the functional parameters of monocytes. Thus, students of the MTC have a higher relative amount of $CD14^+HLADR^+$ (77.5 (70-79) % and 71 (64-74) %, $p = 0.02$), as well as $CD14^+CD282^+$ (78 (72.25-81.1) and 72 (66-77), $p = 0.006$), respectively. As follows from a generalized analysis of the data obtained from the survey of third-year students, military medical students in this period of study, in comparison with students without an additional training program, have better indicators of the functioning of the immune system. This postulate is confirmed by a fundamentally lower number of complications after ARVI and a more pronounced activation of cells that provide the processes of primary antigenic recognition and regulation of the adaptive immune response.

Conclusion

Thus, the data obtained indicate that at the start of training at a medical university, military medical students, in comparison with students of the medical and preventive faculty, have a more significant infectious manifestation of immune dysfunction against the background of a decrease in the functional parameters of natural killers and monocytes. However, in the third year of study, the trend is reversed; the clinical and immunological characteristics of MTC students exceed the corresponding criteria for students who do not have an additional educational load. Obviously, during this period, the process of psychological adaptation of military medical students is completed.

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ОЦЕНКА ГУМОРАЛЬНОГО ИММУННОГО ОТВЕТА У ДЕТЕЙ ПРИ ИММУНИЗАЦИИ РАЗНЫМИ ТИПАМИ ИНАКТИВИРОВАННЫХ ГРИППОЗНЫХ ВАКЦИН В СЕЗОН 2019-2020 ГОДА

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Резюме. Грипп является одной из наиболее распространенных респираторных инфекций, вызывающей миллионы случаев заболевания во всем мире. Эффективность вакцинации против гриппа и характер иммунного ответа на препарат могут варьировать в разных возрастных группах и в зависимости от состава вакцины. Поскольку дети подвержены наиболее высокому риску заболевания и являются основными распространителями гриппозной инфекции, исследования иммунологической эффективности вакцин у детей имеют большое значение для контроля эпидемического процесса в целом. Целью данного исследования стала оценка особенностей формирования гуморального иммунного ответа у детей после иммунизации различными типами инактивированных гриппозных вакцин.

Наблюдательное исследование было проведено в сезоне 2019-2020 г. и включало 230 детей в возрасте до 18 лет, а также 87 участников в возрасте от 18 до 60 лет в качестве группы сравнения. Добровольцы, давшие информированное согласие на участие, были привиты одним из трех препаратов: «Гриппол Плюс», «Совигрипп» или «Ультрикс», в открытом режиме. Оценку гуморального иммунного ответа проводили по титру антигемагглютинирующих антител в парных сыворотках добровольцев, взятых до и через три недели после вакцинации.

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Иммуногенность вакцин, проанализированная суммарно по всем препаратам в возрастной группе до 18 лет, удовлетворяла критериям CPMP для оценки инактивированных гриппозных вакцин по показателям кратности прироста антител и доле лиц с сероконверсией в отношении всех трех компонентов (A/H1N1pdm09, A/H3N2 и B/Victoria). У детей в возрасте от 6 до 18 лет наблюдали более активный ответ к компоненту B/Victoria по сравнению со взрослыми участниками (от 18 до 60 лет), которого тем не менее было недостаточно для обеспечения 70%-ной иммунной прослойки лиц с условно защитным титром антител.

Сравнительный анализ иммуногенности препаратов, проведенный для подгруппы детей в возрасте от 6 до 18 лет с исходно низким уровнем антител на момент вакцинации, показал, что сплит-вакцина «Ультрикс» имела преимущество по сравнению с адъювантной вакциной «Гриппол плюс» в формировании антительного ответа в отношении компонента B/Victoria и не отличалась в отношении компонентов A/H1N1pdm09 и A/H3N2. У детей младше 6 лет наблюдалась тенденция к менее выраженному гуморальному иммунному ответу на вакцинацию по сравнению со старшей возрастной группой, что может быть связано с возрастными особенностями иммунной системы у детей младшего дошкольного возраста.

Ключевые слова: инактивированная гриппозная вакцина, дети, подростки, антитела, реакция торможения гемагглютинации, критерии иммуногенности

ASSESSMENT OF THE HUMORAL IMMUNE RESPONSE IN CHILDREN AFTER IMMUNIZATION WITH DIFFERENT TYPES OF INACTIVATED INFLUENZA VACCINES IN THE 2019-2020 SEASON

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Abstract. Causing millions of cases worldwide every year, influenza is one of the most common respiratory infections. The effectiveness of influenza vaccination and the nature of the resulting immune response may vary depending on the vaccine composition and age group. Since children are at the highest risk of disease and act as the main carriers of influenza, the assessment of the immunological efficacy of vaccines in this group is crucial for controlling the epidemic. Therefore, this study aimed to evaluate the characteristics of the humoral immune response in children after immunization with various types of inactivated influenza vaccines.

An observational study was conducted in the 2019-2020 season and involved 230 children (< 18 years old) and a comparison group of 87 adults aged 18 to 60 years. The subjects, who provided informed consent to participate, were vaccinated with one of three vaccines (Grippol Plus, Sovigripp, or Ultrix) in an open-label fashion. The humoral immune response was assessed by measuring the hemagglutination inhibition (HI) titer in the paired sera taken before and three weeks after vaccination.

The immunogenicity of the vaccines in the age group under 18, met the CPMP criteria for the assessment of inactivated influenza vaccines in terms of the fold increase in antibody titers and the proportion of individuals with seroconversion to all three components (A/H1N1pdm09, A/H3N2, and B/Victoria). Although 6 to 18-year-old participants showed a more robust immune response to the B/Victoria component compared to the adult participants (aged 18 to 60), it was insufficient to ensure that 70% of the participants have a protective antibody titer.

A comparative analysis of the vaccines' immunogenicity was carried out for a subgroup of children aged 6-18 who had initially low antibody levels at the time of vaccination. The analysis showed that the split vaccine Ultrix outperformed the adjuvanted vaccine Grippol Plus in generating an antibody response to the component

B/Victoria; however, the antibody responses to the A/H1N1pdm09 and A/H3N2 components did not differ between the two vaccines. The children under 6 years of age demonstrated a less pronounced humoral immune response to vaccination compared with the other age groups, which may be due to the age-related characteristics of the immune system in children of preschool age.

Keywords: inactivated influenza vaccine, children, adolescents, antibodies, hemagglutination inhibition reaction, immunogenicity criteria

The study was carried out within the government contract of the Ministry of Health of the Russian Federation "Assessment of the Intensity of Collective Immunity and Epidemiological Effectiveness of Influenza Vaccines in the Russian Federation (2019-2021)".

List of abbreviations: AB, antibodies; 95% CI, 95% confidence interval; IIV, inactivated influenza vaccine; IQR, interquartile range; HI test, hemagglutination inhibition test; GMT, geometric mean titer.

Introduction

Influenza is one of the most common respiratory infections, causing millions of cases worldwide. Vaccination remains to be the most effective way to prevent influenza-related morbidity and mortality. Children are at higher risk of influenza infection than other age groups [14]. Being the main carriers, children of preschool age are a 'reservoir' for viruses that cause flu and other seasonal acute respiratory viral infections. Influenza causes children to seek outpatient and inpatient treatment and their parents to take sick leave more frequently. The clinical course of influenza infection may vary depending on the age of the child, comorbidities, and the type of virus [for review see 7, 10, 11].

The effectiveness of influenza vaccination and the nature of the resulting immune response may vary depending on the age group and the vaccine composition [5, 6, 9]. In adults, the response to vaccination is often modulated by pre-existing immunity that is formed as a result of multiple vaccinations and past infections [9]. Studying the antibody response in children with a well-documented prior exposure helps identify the factors that influence vaccine efficacy [3]. Although many countries now recommend seasonal vaccination of children, the data on the effectiveness of inactivated influenza vaccines (IIV) in this age group are limited. Therefore, the studies of the immunological efficacy of vaccines in this group are of great importance to control the epidemic.

Antibodies (AB) targeting the hemagglutinin protein of the influenza virus are widely recognized as a crucial component of protection against influenza infection. The antibody titers that inhibit hemagglutination are considered as correlates of protection following the administration of IIV in adults and children [1]. Classic studies conducted by Hobson et al. in 1972, as well as more recent research, established that a titer of 1:40 correlates with 50% protection against influenza infection [4, 12]. This protective titer is a key element in the CPMP criteria for IIV evaluation in adults, proposed by the European Medicines Agency (EMA) (CPMP/BWP/214/96) [2].

The same criteria are typically used for children and adolescents but the question concerning the level of the protective antibody titer in this age group remains unresolved. Children have a reduced ability to develop a cellular immune response and might not have experienced influenza infection or vaccination, so protective titers may differ in children compared to adults. Ng et al. demonstrated that the titers of 1:40 and above corresponded to approximately 50% protection against infection with the A(H1N1)pdm09 and B/Victoria strains in children and adolescents aged 6 to 17 years [8]. However, for children under 6 years of age, a greater titer threshold value of 1:110 may be required to predict 50% protection against clinically confirmed infection [1].

This study, therefore, **aimed** to evaluate the characteristics of the humoral immune response in children from the two age groups after immunization with various types of inactivated influenza vaccines.

Materials and methods

The observational study was conducted during the 2019-2020 epidemic season. The study included 230 participants in total from two age groups: children under 6 years old and those aged 6 to 18. The children were vaccinated with one of the three influenza vaccines (Grippol Plus, Sovigripp, or Ultrix) at two outpatient clinics in St. Petersburg (St. Petersburg State Budgetary Healthcare Institution Municipal Polyclinics N3 and N4). The participants were allowed to choose the preferable vaccine. The comparison group included 87 adults aged 18 to 60 who were vaccinated with the vaccines at the same clinics or at the Smorodintsev Research Institute of Influenza of the Ministry of Health of the Russian Federation. All adult participants signed the informed consent form. For underaged participants, informed consent was obtained from their parents/guardians. The study protocol was approved by the Local Ethics Committee of the Smorodintsev Research Institute of Influenza (protocol No. 145 of 10/4/2019).

There were two types of trivalent inactivated vaccines used for immunization: split or adjuvanted subunit vaccines. Grippol Plus, manufactured by NPO Petrovax, is an inactivated subunit vaccine that contains 5 μg of hemagglutinin of each of the epidemic virus strain subtypes A/H1N1pdm09, A/H3N2, and B and 500 μg of the Polyoxidonium® adjuvant in a 0.5 mL dose. Sovigripp, manufactured by NPO Microgen, is an inactivated subunit vaccine that contains 5 μg of hemagglutinin of each of the epidemic virus strain subtypes A/H1N1pdm09 and A/H3N2, 11 μg of the influenza virus type B, and 500 μg of Sovidon adjuvant in a 0.5 mL dose. Ultrix, manufactured by FORT LLC, is an inactivated split vaccine containing 15 μg of hemagglutinin of each of the virus strain subtypes A/H1N1pdm09, A/H3N2, and B in a 0.5 mL dose. The vaccines' strain compositions were in accordance with the WHO guidelines for the 2019-2020 northern hemisphere influenza season.

The blood sera from children and adults were obtained twice during the study: before vaccination (D0) and on the 21st day after vaccination (D21). The hemagglutination inhibition (HI) test was used to examine the sera as described in the guidelines MU 3.1.3490-17 [13]. To remove nonspecific inhibitors, the serum was treated with a receptor-destroying enzyme (RDE; Denka Seiken, Japan) following the manufacturer's protocol. The antigens used were Dry Influenza Diagnostic Agents for HI (LLC "PPDP", St. Petersburg, Russia; TU 938824-004-4429427-2008) of the three strains corresponding to the vaccine strains. The sera were titrated starting at a dilution of 1:10. A titer < 1:10 was considered equal to 1:5, and a titer > 1:1280 – to 1:1280.

The immunological efficacy of the vaccines in children and adults was assessed following the EMA Note for Guidance on Harmonisation for Requirements for Influenza Vaccines (CPMP/BWP/214/96) for individuals aged 18 to 60 [2]. Seroprotection was defined as the antibody titer of 1:40 or more. Seroconversion was defined as at least a 4-fold increase in titer from pre-vaccination (D0) to post-vaccination (D21). The statistical analysis of the results was carried out using MS Excel 2016, GraphPad Prizm 6.07, and RStudio 2022.12.0. The 95% confidence intervals (CI) for the geometric means were determined using the logarithmic transformation of the data, followed by the calculation of CI for normally distributed data and inverse logarithmic transformation of the values. CI for seroconversion and seroprotection rates were calculated according to the Wald method. For multiple pairwise comparisons between independent samples, the Mann–Whitney test was used without adjustment for multiple comparisons. To compare the sample rates, Fisher's exact test was used without adjustment for multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

Results and discussion

The study of the immune response included 230 children under the age of 18 who were vaccinated with inactivated influenza vaccines during the 2019-2020 epidemic season. The comparison group was randomly selected from 18-60-year-old adults who

TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION

	Age (years)			
	Under 6	**6-18**	**Under 18 (in total)**	**18-60 (comparison group)**
Total number of participants, n	21	209	230	87
Vaccine, n				
– Ultrix	2	109	111	42
– Grippol Plus	19	92	111	42
– Sovigripp	0	8	8	3
Mean age (IQR*)	4 (3.6-4.7)	15 (13.0-17.3)	14 (10.9-17.3)	28 (18.3-36.8)
Sex, n				
– Female	9 (43%)	85 (41%)	94 (41%)	21 (24%)
– Male	12 (57%)	124 (59%)	136 (59%)	66 (76%)
Seropositive, n**				
– A/H1N1pdm09	16 (76%)	125 (60%)	141 (61%)	76 (87%)
– A/H3N2	11 (52%)	92 (44%)	103 (45%)	65 (75%)
– B/Vic	10 (48%)	58 (28%)	68 (30%)	38 (44%)

Note. *, IQR, interquartile range; **, the antibody titer ≥ 1:10 at the time of vaccination.

were vaccinated against influenza in the same season. When selecting the comparison group, the balance between the proportions of those vaccinated with each vaccine type and the corresponding indicators of the combined group of participants under 18 was taken into account (Table 1). This study adopted the CPMP criteria (CPMP/BWP/214/96) used for the assessment of inactivated influenza vaccines in adults since there are currently no standardized criteria for the assessment of influenza vaccine immunogenicity in children [2].

First, the total immunogenicity of the trivalent IIVs was assessed in the combined group of children under 18 years of age. The immunogenicity parameters in children were contrasted with those in the comparison group – adults aged 18 to 60 (Table 2, the first and second rows from the top). The humoral response in children met the CPMP criteria for the antibody titer fold increase and seroconversion rates. The fold increase in antibody titers and the number of seroconversions to the B/Victoria component were statistically significantly higher in children compared to the adult group, even though the groups had been initially comparable in terms of the proportion of the participants with low and high levels of antibodies at the time of vaccination. As for the other two vaccine components, A/H1N1pdm09 and A/H3N2, seroprotection rates at the time of vaccination were statistically significantly lower in the children group, which could contribute to the observed difference in the immunogenicity of these components.

One of the criteria for IIV immunogenicity is that at least 70% of the vaccinated develop a protective antibody titer (seroprotection) after vaccination. Seroprotection rates to the A/H1N1pdm09 and A/H3N2 components were lower in children compared to the adult group both before and after vaccination, although it reached the CPMP threshold value at the latter time point. As for the B/Victoria component, the seroprotection rates after vaccination did not reach the threshold value of 70% in both groups. The obtained data aligns with previous studies on the immunogenicity of trivalent IIV during the 2019-2020 season which demonstrated that children, adolescents, and adults exhibited a less significant response to the influenza B component compared to both influenza A components. Particularly low rates were seen in the adult participants and 14-17-year-old adolescents [5].

TABLE 2. IMMUNOGENICITY OF THE TRIVALENT INACTIVATED INFLUENZA VACCINES IN CHILDREN IN DIFFERENT AGE GROUPS AND ADULTS IN THE 2019-2020 SEASON, BASED ON HEMAGGLUTINATION INHIBITION (HI) TEST

Age (years)	Vaccine	Seroprotection rate, % before vaccination (D0)			Seroprotection rate, % after vaccination (D21)			Antibody fold increase			Seroconversion rate, %		
		A/H1N1 pdm	A/H3N2	B/Vic	A/H1N1 pdm	A/H3N2	B/Vic	A/H1N1 pdm	A/H3N2	B/Vic	A/H1N1 pdm	A/H3N2	B/Vic
under 18 in total[&]	all vaccines[‡]	39°°°°	20°°	7	76°°°	73°°	43	4.2	5.5	3.1[‡‡]	58°	66°	53°°°°
18-60[&]	all vaccines [‡]	76°°°°	37°°	13	93°°°	88°°	34	3.6	7	2.1[‡‡]	42°	79°	26°°°°
under 6	Grippol Plus	32	42	16	63	47	42	2.4	2.1	1.7	47	42	42
6-18	Grippol Plus	5***	24*	10	75	70	30***	2.6***	4.2*	2.7*	50**	58**	49
	Ultrix	28***	11*	5	76	79	55***	7.1***	7.9*	4.1*	69**	77**	61
CPMP threshold value					> 70%			> 2.5			> 40%		

Note: [‡], in total for all three vaccines Grippol Plus, Sovigripp, and Ultrix; [‡‡] $p < 0.01$, the statistically significant difference between the underage group (under 18) and the adult group (aged 18-60), the Mann–Whitney test without adjustment for multiple comparisons; [&], the groups are balanced for the proportion of the participants vaccinated with each type of vaccine; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, the statistically significant difference between the Ultrix and Grippol Plus vaccines in the 6 to 18-year-old age group, Fisher's exact test without adjustment for multiple comparisons; ° $p < 0.05$, °° $p < 0.01$, °°° $p < 0.001$, °°°° $p < 0.0001$, the statistically significant difference between the underage group (under 18) and the adult group (aged 18-60), Fisher's exact test without adjustment for multiple comparisons.

Next, we assessed the immunogenicity of the Grippol Plus and Ultrix vaccines in the participants under 6 years of age and those aged 6 to 18 (Table 2, rows 3, 4, and 5 from above). Young children are known to be more susceptible to influenza [14]. According to the published research, the threshold value for the protective antibody titer in this group is higher than the standard, measuring at 1:110 [1]. In our study, the vast majority of children under the age of 6 (19 out of 21) were vaccinated with the Grippol Plus vaccine, which motivated further analysis of the results obtained for this vaccine. The geometric mean titers (GMT) in this group after vaccination were as follows: to A/H1N1pdm09 – 50 (95% CI: 25-99); to A/H3N2 – 33 (95% CI: 16-68); to B/Victoria – 17 (95% CI: 9-29). Before vaccination, the antibody titers above the threshold value of 1:110 were only identified in isolated cases: in one child (5%) – to the A/H1N1pdm09 component and in another child (5%) – to the A/H3N2 component. After vaccination, the antibody titers above 1:110 were observed in 42% of the children for the A/H1N1pdm09 component, in 26% of the children for the A/H3N2 component, and only in one child (5%) for the B/Victoria component.

At the time of vaccination, the age subgroups (under 6 and 6 to 18 years of age) did not differ in the seroprotection rates. Nonetheless, following vaccination with Grippol Plus, all parameters studied suggested a less pronounced immune response in the younger children compared to the group aged between 6 to 18 years. The differences did not achieve statistical significance, which, however, can be attributed to the insufficient sample size of the younger age group.

The fold increase in antibody titer and seroconversion rates for the A/H1N1pdm09 and A/H3N2 components were higher in the participants aged 6 to 18 who were vaccinated with Ultrix, than in children of the same age who received Grippol Plus. Notably, these subgroups were not comparable in terms of the proportion of the seropositive participants at the time of vaccination, as suggested by the corresponding seroprotection rates. The humoral immune response to vaccination is known to be less pronounced in individuals with high pre-existing antibody titers [3, 9]. Nevertheless, vaccination with Ultrix and Grippol Plus led to similar seroprotection rates for the A/H1N1pdm09 and A/H3N2 components, which met the required seroprotection criterion (more than 70%). The seroprotection rate for B/Victoria after vaccination was statistically significantly higher in the subgroup vaccinated with Ultrix compared to Grippol Plus, despite the absence of differences in the number of seropositive individuals on day 0 between the two groups. This difference may indicate better immunogenicity of the Ultrix vaccine to the influenza B component in comparison with the Grippol Plus vaccine.

A key objective of the study was a comparative assessment of the immunogenicity of the Ultrix and Grippol Plus vaccines in children aged 6 to 18 years who had initially low antibody titers (Table 3). No differences in the immunogenicity of these two vaccines were found regarding the A/H1N1pdm09 and A/H3N2 components. However, the data analysis confirmed that the split vaccine Ultrix exhibited a higher immunogenicity to the B/Victoria component

TABLE 3. CHARACTERISTICS OF THE HUMORAL IMMUNE RESPONSE TO VACCINATION IN CHILDREN WITH INITIALLY LOW TITERS DEPENDING OF THE VACCINE TYPE IN THE 2019-2020 SEASON (HI TEST DATA)

Age (years)	Vaccine	Seroprotection rate, % (95%CI)			Antibody fold increase (95%CI)			Seroconversion rate, % (95%CI)		
		H1N1	H3N2	B/Vic	H1N1	H3N2	B/Vic	H1N1	H3N2	B/Vic
6-18	Grippol Plus	62 (47-78)	66 (54-78)	30** (19-40)	8.3 (5.2-13.0)	8.7 (6.0-12.4)	3.8‡ (2.8-5.2)	76 (62-90)	76 (65-86)	55* (44-67)
	Ultrix	70 (59-80)	81 (73-90)	54** (44-65)	13.6 (9.5-20.0)	11 (8.3-15.0)	5.4‡ (4.3-6.8)	84 (75-93)	88 (80-95)	71* (62-80)
6-21[&]	Flucelvax[&]	70.6	73.3	64.0	11.1 (4.7-26.0)	7.3 (3.5-15.2)	6.6 (4.0-10.9)	N/D	N/D	N/D
CPMP threshold value		> 70%			> 2.5			> 40%		

Note. * $p < 0.05$, ** $p < 0.01$, the statistically significant difference between Ultrix and Grippol Plus in the 6 to 18-year-old age group, Fisher's exact test without adjustment for multiple comparisons; ‡ $p < 0.05$, the statistically significant difference between Ultrix and Grippol Plus in the 6 to 18-year-old age group, the Mann–Whitney test without adjustment for multiple comparisons; [&], comparative data from a similar study of a subunit inactivated vaccine [15]; N/D, no data.

compared to the adjuvanted vaccine Grippol Plus. Importantly, the influenza B virus infection poses the greatest threat to children and is often accompanied by serious complications. Children with influenza B infection require hospitalization in the intensive care unit more often and have a higher mortality rate compared to those with influenza A infection [10, 11].

Furthermore, we compared our data with the results of a similar study that was conducted in the United States on subjects aged 6 to 21 years who had initially low antibody titers in the 2019-2020 season [15]. The analysis showed that Ultrix and Grippol Plus are comparable to the Flucelvax vaccine (Seqirus, USA) in terms of the fold increase of antibody titers against A/H1N1pdm09 and A/H3N2. Moreover, the split vaccine Ultrix showed higher immunogenicity to the influenza A viruses compared to Flucelvax. Both vaccines, however, exhibited a lower fold increase in titer against the B/Victoria component compared to the vaccine produced in the USA. Therefore, it can be advisable to use a vaccine from a foreign manufacturer as a comparator when conducting similar studies. Our data indicate that the vaccine type may impact the antibody response to specific components of the vaccine.

Conclusion

The immunogenicity rates of the studied trivalent inactivated influenza vaccines in children under 18 years of age in the 2019-2020 season met two out of the three CPMP criteria for influenza vaccines: the number of seroconversions > 40% and a significant (at least fourfold) increase in antibody titer. Children (< 18 years old) showed a better antibody response to the B/Victoria component than the adult group (18 to 60 years old) according to the three parameters studied. Yet, this response was not sufficient to ensure 70% persons with HI titer ≥ 40 after vaccination. In the group of participants aged 6 to 18 years, the Ultrix and Grippol Plus vaccines demonstrated similar immunogenicity levels to A/H1N1pdm09 and A/H3N2. The split vaccine Ultrix elicited a better antibody response to the B/Victoria component. The children under 6 years of age tended to have a less pronounced humoral immune response to vaccination, which might be associated with the specifics of the immune system in children of preschool age.

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ЗАКОНОМЕРНОСТИ В РАЗВИТИИ КОЛЛЕКТИВНОГО ИММУНИТЕТА К SARS-CoV-2 В ХОДЕ ПАНДЕМИИ COVID-19

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Резюме. Продолжающаяся на протяжении последних 3 лет пандемия коронавирусной болезни (COVID-19) вызвала пристальное внимание к проблеме популяционного иммунитета, под которым понимается «устойчивость к распространению инфекционного заболевания в популяции». Коллективный иммунитет формируется как в результате заболевания (при естественном распространении возбудителя в популяции восприимчивых индивидуумов), так и в результате применения специфических вакцин. В ходе пандемии COVID-19 реализовались оба варианта формирования популяционного иммунитета. В первую волну происходило естественное формирование невосприимчивости населения к вирусу после перенесенного заболевания COVID-19, обусловленного пандемическим распространением SARS-CoV-2. Начиная с декабря 2020 года в США, Великобритании, Китае, России и ряде других стран началось широкое применение специфических вакцин против SARS-CoV-2. Это запустило процесс формирования поствакцинального популяционного иммунитета, специфичность которого зависит от вида используемой вакцины. В настоящее время в тех странах, где широко проводится вакцинация и ревакцинации переболевших популяционный иммунитет является гибридным.

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Принимая во внимание относительно сходный волнообразный характер эпидемического процесса COVID-19, обусловленный сменой геновариантов возбудителя во всех странах, а также развернутой программой массовой вакцинации населения, можно сделать некоторые выводы об общей для всех стран тенденции формирования популяционного иммунитета в ходе пандемии.

В начале пандемии, в 2020 году, серопревалентность населения в целом не превышала 20%, при этом наибольшие показатели серопревалентности отмечали в детской возрастной группе; выявлялись выраженные территориальные различия, наибольшие показатели отмечали в группе медицинских работников. Популяционный иммунитет развивался вследствие перенесенного заболевания и у большинства серопозитивных волонтеров был представлен антителами к обоим антигенам.

В разгар пандемии, летом 2021 года, серопревалентность населения достигла 50%. Это было обусловлено как значительным числом переболевших лиц, так и началом компании массовой вакцинации населения. Во всех странах практически нивелировались специфические различия в серопревалентности населения (территориальные, возрастные и профессиональные). В этот период наиболее явно можно отметить формирование гибридного иммунитета – увеличилась доля лиц, имеющих антитела только к RBD (вследствие вакцинации векторными вакцинами).

Позднее массовая вакцинация, а также вовлечение большей части населения в эпидемический процесс из-за появления высоко контагиозного штамма «Омикрон», подняли уровень популяционного иммунитета до 80-90%. Это привело к резкому снижению заболеваемости COVID-19 во второй половине 2022 года во всех странах, участвующих в исследовании. На поздних сроках пандемии (2022-2023 годы) практически у 90% серопозитивных волонтеров гуморальный иммунитет являлся гибридным и был представлен антителами к обоим антигенам (Nc+RBD).

Ключевые слова: коллективный иммунитет, популяционный иммунитет, COVID-19, пандемия, вакцинальный иммунитет, эпидемиология

PATTERNS IN THE DEVELOPMENT OF COLLECTIVE IMMUNITY TO SARS-CoV-2 DURING THE COVID-19 PANDEMIC

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Abstract. The ongoing coronavirus disease (COVID-19) pandemic over the past three years has caused close attention to the problem of herd immunity, which is understood as: "resistance to the spread of a contagious disease within a population or herd". Collective immunity is formed both as a result of infection (natural spread of the pathogen in a population of susceptible individuals) and as a result of the use of specific vaccines. During the COVID-19 pandemic, both mechanisms for the formation of collective immunity were realized. In the first wave, there was a natural formation of collective immunity to the virus following recoveries from COVID-19 caused by pandemic spread of SARS-CoV-2. Starting from December 2020, the widespread use of specific vaccines against SARS-CoV-2 began in the USA, Great Britain, China, Russia, and a number of other countries. This launched the process of post-vaccination collective immunity formation; its features have depended on the vaccine types implemented. Currently, in those countries where vaccination and re-vaccination of recovered patients is widely carried out, immunity is "hybrid" in nature.

Several commonalities should be noted in the pandemic experience: a somewhat regular, periodic (wave-like) nature of the COVID-19 epidemic process; changes in pathogen genetics in variants in all countries; and expansive mass vaccination programs in many populations. From these, we can draw some conclusions about the general trend for all countries in the formation of collective immunity during the pandemic:

At the beginning of the pandemic in 2020, overall population seroprevalence did not exceed 20%. Other findings were: the highest seroprevalence rates were noted in the children's age group; pronounced regional

differences were revealed; and the highest indicators were noted among medical workers. Collective immunity developed as a result of infection or illness, and in the majority of seropositive volunteers, it was represented by antibodies to both antigens.

At the height of the pandemic in the summer of 2021, population seroprevalence reached 50%. This was due to both a significant number of convalescents and the start of mass vaccination campaigns. In all countries, specific differences in seroprevalence (by age, region, profession) leveled out, leading to more uniformity. During this period, the formation of "hybrid" immunity is clearly prominent, and the proportion of individuals with antibodies to RBD alone increased (due to vaccination with vector vaccines).

Later, mass vaccination, as well as involvement of most of the population in the epidemic process due to the emergence of the highly contagious Omicron strain, raised the level of collective immunity to 80-90%. This led to a sharp decrease in COVID-19 incidence in the second half of 2022 in all countries participating in the study. In the later stages of the pandemic (2022-2023), almost 90% of seropositive volunteers had hybrid immunity, reflected as antibodies to both antigens (Nc, RBD).

Keywords: collective immunity, herd immunity, COVID-19, pandemic,vaccine-induced immunity, public health

Introduction

The ongoing coronavirus disease (COVID-19) pandemic over the past three years has caused close attention to the problem of herd immunity, which is understood as: "resistance to the spread of a contagious disease within a population or herd" [16]. Collective immunity is formed both as a result of infection (natural spread of the pathogen in a population of susceptible individuals) and as a result of the use of specific vaccines. During the COVID-19 pandemic, both mechanisms for the formation of collective immunity were realized. In the first wave, there was a natural formation of collective immunity to the virus following recoveries from COVID-19 caused by pandemic spread of SARS-CoV-2. Starting from December 2020, the widespread use of specific vaccines against SARS-CoV-2 began in the USA, Great Britain, China, Russia, and a number of other countries. This launched the process of post-vaccination collective immunity formation; its features have depended on the vaccine types implemented. Currently, in those countries where vaccination and re-vaccination of recovered patients is widely carried out, immunity is "hybrid" in nature [2]. In the recent time period with epidemic increases in case numbers alongside a lack of effective specific COVID-19 treatments, collective immunity has served as the only tool for controlling and managing the epidemic.

Materials and methods

At the beginning of the local epidemic in May 2020, Rospotrebnadzor developed a multi-stage program for seromonitoring of the population's immunity to SARS-CoV-2. It was implemented in 2020-2021 in Russia [5, 7, 14]. Since 2021-2023, neighboring countries have participated in a partnership program (Armenia, Belarus, Kyrgyzstan, Tajikistan) [6, 10, 11, 12]. The program has included the formation of volunteer cohorts, the volume and structure of which has made it possible to obtain representative data for the populations in the study region (countries) by age and professional group. Volunteers included in the cohort were divided into seven age groups (years old): 1-17, 18-29, 30-39, 40-49, 50-59, 60-69, and ≥ 70.

Due to their high representation during infectious and post-vaccination processes, two SARS-CoV-2 antigens are especially relevant to analytical methods: nucleocapsid (Nc) and S protein receptor-binding domain (RBD). Depending on vaccine design, antibodies to one, or both, antigens are formed. The presence of serum antibodies as a result of a previous infection makes it possible to use seromonitoring to detect not only clinical, but also subclinical cases of infection that would otherwise go unnoticed.

Levels of IgG antibodies (anti-Nc, anti-RBD) in volunteers were quantified by enzyme immunoassay using Russian assay systems: "Reagent set for enzyme immunoassay quantitative determination of human IgG Abs to SARS-CoV-2 N protein (N-Cov-2-IgG PS)" (Saint Petersburg Pasteur Institute) and "SARS-CoV-2-ELISA-IgG-screen" (LabPack). All studies were carried out according to a single algorithm, which included the use of cloud service (internet) technology to form a cohort of volunteers, collect test results, and assist analysis [8].

In Russia, the study by Rospotrebnadzor involved eight research institutes and twenty regional departments (centers of hygiene and epidemiology, etc.). It involved volunteers from 26 regions (located in all of the country's federal districts), whose population accounted for 54.7% of the total national population. Along with megacities featuring high populations and density (such as Moscow and St. Petersburg), regions with populations below 800,000 (Murmansk and Amur regions, the Republic of Crimea) and low population density (Krasnoyarsk region 1.21 km^{-2}) also took part. The total number of volunteers was 74,158 people and was representative of the total Russian population. The study was conducted in 2020-2021 in 5 stages, with an interval of 3-9 months (Figure 1) [5, 7, 14]. In Armenia, Belarus, Kyrgyzstan and Tajikistan, 32,128 volunteers were examined for population dynamics in 2021-2023; from 2 to 4 stages were carried out in each country (Figure 2).

The multi-stage studies, wherein the levels of Abs to various SARS-CoV-2 antigens were assessed at

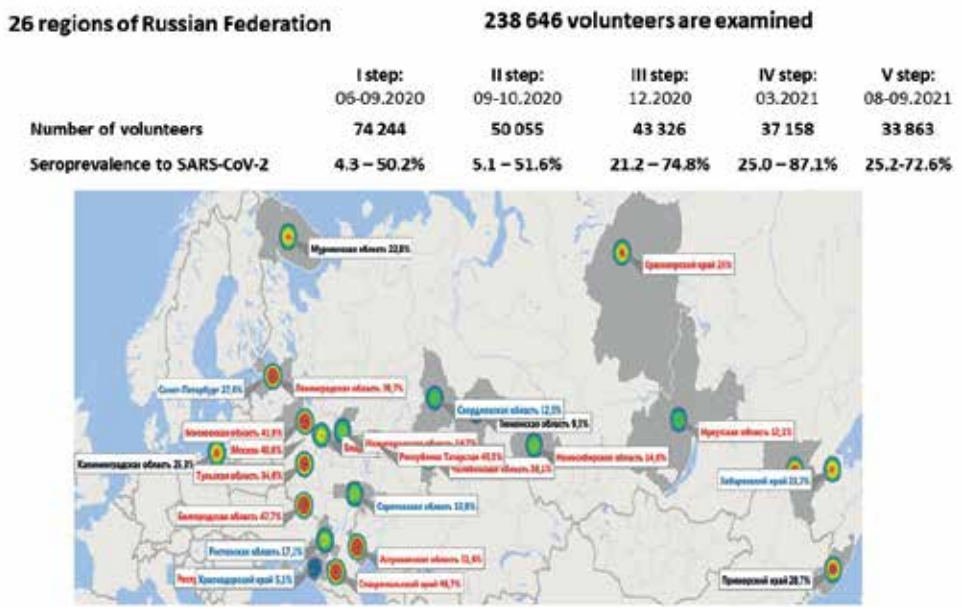

Figure 1. Russian regions included in the study of SARS-CoV-2 collective immunity, 2020-2021. The seroprevalence values presented on the map correspond to the first stage of the study

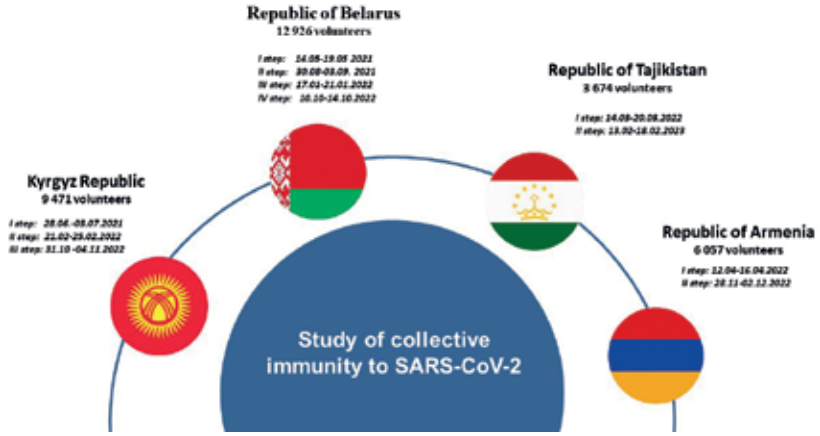

Figure 2. Neighboring countries included in the study of SARS-CoV-2 collective immunity, 2021-2023

different periods of the pandemic, made it possible to identify some patterns in the formation of collective immunity to coronavirus infection, common to populations of all countries included in the study.

Results and discussion

At the beginning of the pandemic in 2020 (stages 1-2 of the study in Russia), seroprevalence in the population varied significantly by region of the country. Average SARS-CoV-2 seroprevalence in the summer of 2020 was 18.0% (95% CI: 10.0-23.9), while the range of variation across regions exceeded 10-fold. The highest seropositivity levels were found in the Kaliningrad (50.2%; 95% CI: 48.4-52.0%) and Amur regions (45.4%; 95% CI: 43.6-47.2). The lowest were seen in the Republic of Crimea (4.3%; 95% CI: 3.6-5.1) and the Krasnodar region (8.0%; 95% CI: 7.1-9.0). No correlations were found between seroprevalence and local morbidity; nor between seroprevalence and local population density.

For the first half of 2020, overall seroprevalence in Russia could be considered low, which posed a certain threat of a further increase in incidence. This is exactly what has been happening everywhere since mid-October 2020 [https://coronavirus-monitor.ru]. Subsequently (2020-2021), following increased COVID-19 incidence, seroprevalence in the Russian population steadily increased, exceeding 70% in December 2021. At the same time, fluctuations in indicators across Russian regions gradually leveled off [5, 7, 14].

At the beginning of the pandemic (summer-autumn 2020), there were age differences in seroprevalence almost throughout Russia. Despite the fact that the number of children with COVID-19 was small, the seroprevalence of SARS-CoV-2 Abs in children was higher than the average value for the entire cohort or the value in adults [9]. One of the most likely reasons for this phenomenon could be a high frequency of asymptomatic or mildly-symptomatic infections in the children's age group. Such infections may have

proceeded under the guise of a common cold without serious clinical manifestations, yet accompanied by serological changes.

Thus, in the process of seromonitoring conducted in 2020-2021, among seropositive children, the proportion of individuals with asymptomatic infection exceeded 90% [9]. In addition, age-related reduced ACE-2 receptor density, and cross-immunity to closely related seasonal coronaviruses, may have markedly reduced susceptibility to SARS-CoV-2 [1, 3]. It has also been hypothesized that in children, mesenchymal stem cells may be a factor in limiting the virus in the body [13]. Such cells can suppress the pathological activation of immune responses (manifested in severe cases as a "cytokine storm") and also promote the regeneration of affected tissues. Mild and asymptomatic forms of infection led to the fact that children were less likely to come to the attention of a doctor, yet remaining a source of pathogen transmission. By March 2021, age-specific seroprevalence differences in the Russian population had completely evened out.

In a later period of pandemic development (spring 2021 – spring 2023), countries adjacent to Russia joined the study: the Republic of Belarus, the Kyrgyz Republic, the Republic of Tajikistan and the Republic of Armenia. By the time the studies began in these countries, significant portions of the population had already experienced infection in a manifest or asymptomatic form. Therefore, like Russia during this period, some statistically significant differences (by age, profession, region) in population seroprevalence were no longer identified in these countries. This is likely the result of the preceding epidemic phases (rising, intense) as well as the vaccination campaign initiated mid-2021 [6, 10, 11, 12].

In general, in those countries where the study began in 2020-2021 (Russian Federation, Republic of Belarus, Kyrgyz Republic), there was a steady upward trend in the level of collective immunity (percentage seropositive individuals) during the pandemic. In countries that joined the study at the height of the pandemic in 2022 (Republic of Tajikistan, Republic of Armenia), already in the first stage of the study, population seroprevalence was almost absolute (approaching 100%), both due to high incidence and high vaccination coverage (more than 70% of the population). This situation continued in 2023 (Figure 3, see 2nd page of cover).

Vaccines proposed for vaccination against SARS-CoV-2 can be divided into two main groups according to their antigenic composition and, accordingly, antibody types elicited. The first group induce the production of antibodies to RBD alone: vector vaccines (AstraZeneca, Sputnik V, Sputnik Light); and mRNA vaccines (Moderna, Pfizer). The second group includes vaccines that generate a response to both Nc and RBD antigens (whole-virion preparations Sinopharm/BIBP, CoronaVac, CoviVac). In Russia and Belarus, the Sputnik vector vaccines (Sputnik V, Sputnik Light) were most widely used. Until recently, they have accounted for up to 80% of vaccinations. To a much lesser extent, the EpiVacCorona peptide vaccine and the CoviVac whole-virion vaccine have been used [15]. In Russia, total vaccination coverage of the population by April 19, 2023 reached 61.4%. In Belarus, coverage had reached almost 70% by May 2022. In Kyrgyzstan, more than 30% of the population had been vaccinated by the end of 2022, with more than 70% of volunteers receiving the Sinopharm/BIBP whole-virion vaccine. In Armenia and Tajikistan, all volunteers who participated in the study were vaccinated by the beginning of 2023. In Armenia, vector and whole-virion vaccines were used equally. In Tajikistan, up to 70% were vaccinated with vector vaccines.

By design, specific vaccines induce the production of antibodies to various SARS-CoV-2 antigens in various ways. With this in mind, it is possible to draw certain conclusions about the nature and structure of collective immunity. At the beginning of the pandemic (summer/autumn of 2020 in the absence of specific prophylaxis), the structure of collective immunity was represented approximately evenly by individuals with: antibodies to Nc alone; or antibodies to both antigens (Nc, RBD) as a result of a previous SARS-CoV-2 infection (Figure 4).

With the start of vaccination of the population in 2021, and in the context of a slight decrease in COVID-19 incidence, there was a shift in the structure of seropositivity towards an increase in the proportion of people who had anti-RBD antibodies only. Given the specificity of vector vaccines (mainly used in Russia and Belarus), the presence of antibodies to RBD antigen alone, to a large extent although not exclusively, indicated post-vaccination immunity. It is obvious that, at that time, it was possible to say with a high degree of certainty that: collective immunity was hybrid in nature; and in half of the seropositive volunteers, it was due to vaccination (Figure 4).

Vaccination against coronavirus (both primary and booster) launched in the first half of 2021 and actively carried out in all countries, as well as genetic changes in the pathogen (such as the highly contagious Omicron variant), undoubtedly became the main reasons for rising collective immunity, reaching 80-90%. In 2022 and 2023, more than 80% of seropositive volunteers had humoral immunity with antibodies to both antigens (Nc, RBD). Obviously, in the late stages of a pandemic, under conditions of high incidence of a highly transmissible strain and high vaccination coverage, collective immunity is going to be "hybrid". Considering the variety of vaccines obtained from various platforms (vector, mRNA, whole-virion), as well as vaccination schedules including booster re-vaccination, it is currently difficult to quantify the exact contribution of vaccination to 'hybrid' immunity, although its valuable contribution is undisputed [6, 10, 11, 12].

The current pandemic has shown that a characteristic feature of COVID-19 is a large number of asymptomatic forms. The manifestations of this

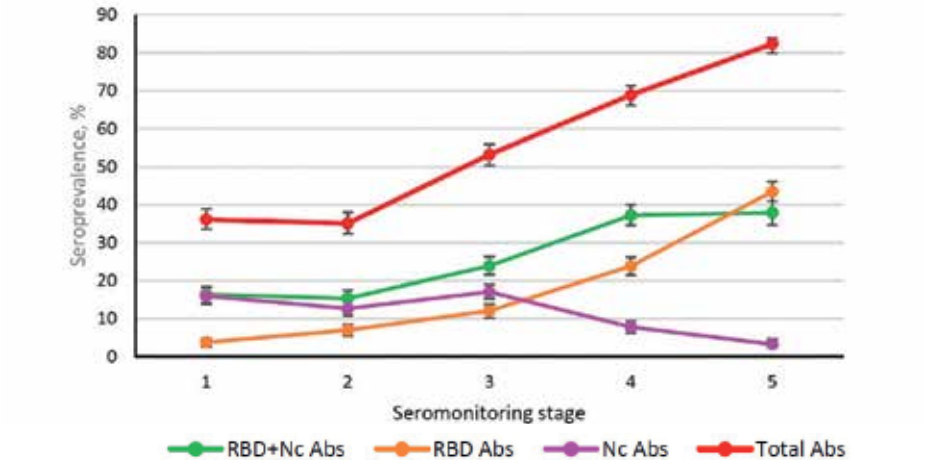

Figure 4. Seroprevalence dynamics from August 2020 (point 1) to September 2021 (point 5) [14]

Note. The status of antibodies to various SARS-CoV-2 antigens and their combinations are shown: "RBD+Nc Abs", double-positive volunteers with both Abs; "RBD Abs", those with only RBD Abs; "Nc Abs", those with only Nc Abs; "Total Abs", total number of seropositive volunteers (RBD+Nc, RBD, Nc) relative to all examined; vertical black lines, 95% CI.

phenomenon can be various, and the very definition of "asymptomatic" needs to be clarified. One possible form may be termed "carrier", in which only viral RNA is detected in blood by PCR in the absence of any other symptoms. In this regard, it can be expected that the host organism will respond to the circulation of viral RNA with an immune response in one form or another. The results obtained fully confirmed this assumption. The study included asymptomatic volunteers who did not have any experienced symptoms of overt COVID-19 in their anamnesis. According to available data, the share of asymptomatic forms of infection, both in the early period and during development of the pandemic, reached very high levels: in Russia (2020) 93.5%; in Belarus (2021) up to 50%; in Kyrgyzstan (2021) up to 70%; in Armenia (2022) 75.0%; and in Tajikistan (2022) above 90%. Moreover, the highest rates of asymptomatic cases in all countries were noted among children [5, 6, 7, 10, 11, 12, 14].

Conclusion

Several commonalities should be noted in the pandemic experience: a somewhat regular, periodic (wave-like) nature of the COVID-19 epidemic process; changes in pathogen genetics in variants in all countries; and expansive mass vaccination programs in many populations. From these, we can draw some conclusions about the general trend for all countries in the formation of collective immunity during the pandemic:

1. At the beginning of the pandemic in 2020, overall population seroprevalence did not exceed 20%. Other findings were: the highest seroprevalence rates were noted in the children's age group; pronounced regional differences were revealed; and the highest indicators were noted among medical workers. Collective immunity developed as a result of infection or illness, and in the majority of seropositive volunteers, it was represented by antibodies to both antigens.

2. At the height of the pandemic in the summer of 2021, population seroprevalence reached 50%. This was due to both a significant number of convalescents and the start of mass vaccination campaigns. In all countries, specific differences in seroprevalence (by age, region, profession) leveled out, leading to more uniformity. During this period, the formation of 'hybrid' immunity is clearly prominent, and the proportion of individuals with antibodies to RBD alone increased (due to vaccination with vector vaccines).

3. Later, mass vaccination, as well as involvement of most of the population in the epidemic process due to the emergence of the highly contagious Omicron strain, raised the level of collective immunity to 80-90%. This led to a sharp decrease in COVID-19 incidence in the second half of 2022 in all countries participating in the study. In the later stages of the pandemic (2022-2023), almost 90% of seropositive volunteers had hybrid immunity, reflected as antibodies to both antigens (Nc, RBD).

In summarizing the presented data, we can agree with existing opinions about the leading role of collective immunity in the course and outcome of the coronavirus epidemic. The combination of "post-infectious immunity", the natural immune response to pathogen contact, and 'artificial immunity', formed as a result of vaccine usage, together form a hybrid immunity that can serve as a major factor in ending the global COVID-19 pandemic.

The data obtained in our study are consistent with other authors [4] and indicate that achieving a 70% level of collective immunity may be sufficient to prevent severe COVID-19 and stop the transmission of SARS-CoV-2 strains with low or moderate transmissibility. However, in order to stop the spread of Omicron strains, or other new highly transmissible SARS-CoV-2 variants, the level of collective immunity must reach 90%. In addition, any vaccines used must be as effective as possible in preventing infection with highly transmissible strains.

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ЦИТОКИНОВАЯ ДИАГНОСТИКА В ПРОГНОЗЕ КРИТИЧЕСКИХ СОСТОЯНИЙ У НОВОРОЖДЕННЫХ, РОДИВШИХСЯ ОТ МАТЕРЕЙ, ИНФИЦИРОВАННЫХ COVID-19

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Резюме. Статья посвящена разработке способа цитокиновой диагностики для прогнозирования развития критических состояний у новорожденных, родившихся от матерей с COVID-19, что имеет большое значение для органов здравоохранения при организации специализированной неонатологической и педиатрической службы.

Цель – разработать метод неинвазивной цитокинодиагностики для прогнозирования развития критических состояний у новорожденных, рожденных от матери с COVID-19. Предложенный способ позволяет ранней диагностики и профилактики развития критических состояний у новорожденных, что имеет важное практическое значение. Контроль цитокинов мочи в динамике определяет прогноз развития критических состояний, как в ранний, так и в поздний период адаптации новорожденных. Являясь маркером воспалительного процесса и ключевым цитокином костной резорбции, IL-17A играет сложную роль в процессе адаптации новорожденных, родившихся от матери с COVID-19. Новорожденные, рожденные от матери с COVID-19, имеют повышение IFNγ и IFNα в крови в первый день жизни на фоне повышения IL-17A в 1,48 раза, что свидетельствует о риске развития как инфекции, так и нарушений остеогенеза. Установлена активация интерферонового статуса к 7-му дню жизни у новорожденных на фоне повышения ключевого цитокина костной резорбции (IL-17A). У новорожденных детей, рожденных от матери, инфицированной COVID-19, обнаружено снижение в моче молекулярных маркеров повреждения эндотелия сосудов – MSR-1 в 4,25 раза. Было обнаружено снижение VEGF в моче новорожденных как при заражении COVID-19 у матери, так и при его отсутствии. Неинвазивная уроцитокинодиагностика позволяет достичь экономической эффективности за счет сокращения больничных коек, а также эффективности лечения за счет минимальной травматизации новорожденных.

Ключевые слова: новорожденные, цитокины, COVID-19, критические состояния, прогноз, дети

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CYTOKINE DIAGNOSTICS IN THE PROGNOSIS OF CRITICAL CONDITIONS IN NEWBORNS BORN TO MOTHERS INFECTED WITH COVID-19

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Abstract. The article is devoted to the development of a cytokine diagnostic method for predicting the development of critical conditions in newborns born to mothers with COVID-19, which is of great importance for health authorities when organizing specialized neonatology and pediatric services.

Objective: to develop a method of noninvasive cytokine diagnostics for predicting the development of critical conditions in newborns born from a mother with COVID-19.

The proposed method allows early diagnosis and prevention of the development of critical conditions in newborns, which is of great practical importance. The control of urine cytokines in dynamics determines the prognosis of the development of critical conditions, both in the early and late period of adaptation of newborns. As a marker of the inflammatory process and a key cytokine of bone resorption, IL-17A plays a complex role in the adaptation process of newborns born from a mother with COVID-19. Newborns born from a mother with COVID-19 have an increase in IFNγ and IFNα in the blood on the first day of life against the background of an increase in IL-17A by 1.48 times, which shows the risk of developing both infection and osteogenesis disorders. Activation of interferon status by the 7th day of life in newborns was established against the background of an increase in the key cytokine of bone resorption (IL-17A). A decrease in the urine of molecular markers of vascular endothelial damage – MSR-1 by 4.25 times was found in newborn children born from a mother infected with COVID-19. A decrease in VEGF in the urine of newborns was found, both with COVID-19 infection in the mother and in its absence. Non-invasive urine cytokine diagnostics allows achieving economic efficiency by reducing hospital beds, as well as medical efficiency due to minimal traumatization of newborns.

Keywords: newborns, cytokines, COVID-19, critical conditions, prognosis, children

Introduction

In modern perinatology, extensive data have been accumulated explaining the processes occurring in the mother – placenta – intrauterine child system both during the physiological course of pregnancy and childbirth, and in the case of pathological changes in the gestational period [1]. Rendering assistance to newborn children in critical condition at the stage of inter-hospital transportation is one of the most acute problems of modern neonatology and neonatal resuscitation.

Newborns in critical condition, delivered to the ICU of a level III hospital three days after birth, had a more severe course of the pathological process with a high probability of developing multiple organ failure syndrome [1]. Proinflammatory cytokines (TNFα, IFNγ and IL-6) differentially increase in newborns with sepsis depending on gestational age. As expected, these cytokine values were high for newborns ≥ 32 weeks during sepsis; however, they were insignificant for newborns born < 32 weeks [2].

Limited data are available on COVID-19 during pregnancy, but studies published to date do not show an increased risk of developing severe diseases in late pregnancy or a significant risk for the newborn. Neither a congenital infection nor a virus was detected in the materials of the afterbirth, which confirms the relevance of this area of scientific research [4].

Objective: to develop a method of noninvasive cytokine diagnostics for predicting the development of critical conditions in newborns born from a mother with COVID-19.

Materials and methods

The medical histories of 37 full-term and 22 premature newborns born to a mother with COVID-19 and hospitalized in inpatient treatment at the Department of Neonatology of the Bukhara Children's Multidisciplinary Medical Center in the periods from 2020 to May 2022 were retrospectively studied. During their stay in the hospital, all patients were subjected to general clinical, laboratory, functional, biochemical, radiographic studies.

Among all (59) newborns born to a mother with COVID-19 and who died in the first months of life, there were 22 premature newborns (37.3%), 37 full-term (62.7%). For the convenience of comparing the main indicators of the severity of newborns, they were divided into 2 groups depending on the gestation period:

Group 1: premature newborns – 22;

Group 2: full-term newborns – 37.

The minimum gestation period of group 1 newborns was 26 weeks; the maximum period was 37 weeks, which averaged 33.55±0.66 weeks. At the same time, the minimum weight of newborns in this group was 1090 g, and the maximum weight was 2880 g, which averaged 2023.23±114.64 g.

The analysis of the days of life lived by newborns showed that the minimum day of life was 1.0 days, the maximum lived up to 93 days, that on average the days of life of premature newborns born from a mother with COVID-19 is 23.09±5.37 days.

The maximum gestation period of full-term infants was 42 weeks. The average gestation period was 38.61±0.57 weeks. At the same time, the weight of full-term children was a maximum of 4,400 grams. On average, newborns were born weighing 2819.12±151.55 grams.

Results and discussion

The structure of the morbidity of premature newborns showed a predominance of congenital malformations-13 (59.1%), in particular, patients with congenital heart defects-5 (22.7%), with anomalies of the kidneys and urinary tract-3 (13.6%), with malformations of the gastrointestinal tract-5 (22.7%). The second place in the structure of morbidity is occupied by intrauterine infections (TORCH infection) with the development of neonatal sepsis-8 (36.4%). The third place is occupied by perinatal lesions of the central nervous system of hypoxic genesis-1 (4.5%) (Figure 1).

To determine the indications for antibacterial therapy and evaluate its effectiveness in systemic inflammatory reaction syndrome in newborns, a study was conducted to determine the level of PCTs in the blood. A fluctuation in the concentration of MPC was detected in the range from 0.1 to 11.8 pg/mL, which on average is 2.49 = 0.5 pg/mL. Consequently, the results of general laboratory and biochemical blood parameters in newborns with critical conditions indicate the beginning of the development of a systemic inflammatory reaction syndrome.

For a comparative assessment of the significance of cytokine status indicators in the prognosis of the development of critical conditions of newborns, a clinical and laboratory examination of 94 newborns was carried out: 33 newborns born from a mother with COVID-19 (group 1), 30 newborns with perinatal central nervous system lesion (PPCNS), born from a mother with somatic diseases (group 2) and 31 healthy newborns born from a healthy mother.

As a result of the analysis of the cytokine content in the blood of newborns on the 2nd day of life, it was found that the concentrations of IL-17A in group 1 exceed the upper limit of the concentration range of these indicators in the group of healthy newborns (Table 1).

The interferon status of newborns of Group 1 and 2 is characterized by a significant increase in IFNγ to 23.64±0.81 and 29.20±1.28 pg/mL, respectively, against the indicators of the control group-20.96±0.66 pg/mL ($p < 0.05$).

At the same time, IFNα has a statistically significant tendency to increase in newborns of the 1st group – 33.71±1.22 pg/mL ($p < 0.05$), in relation to the indicators of the control group – 26.49±1.20 pg/mL.

And in newborns of the 2nd group, its value was at the level of the control indicators.

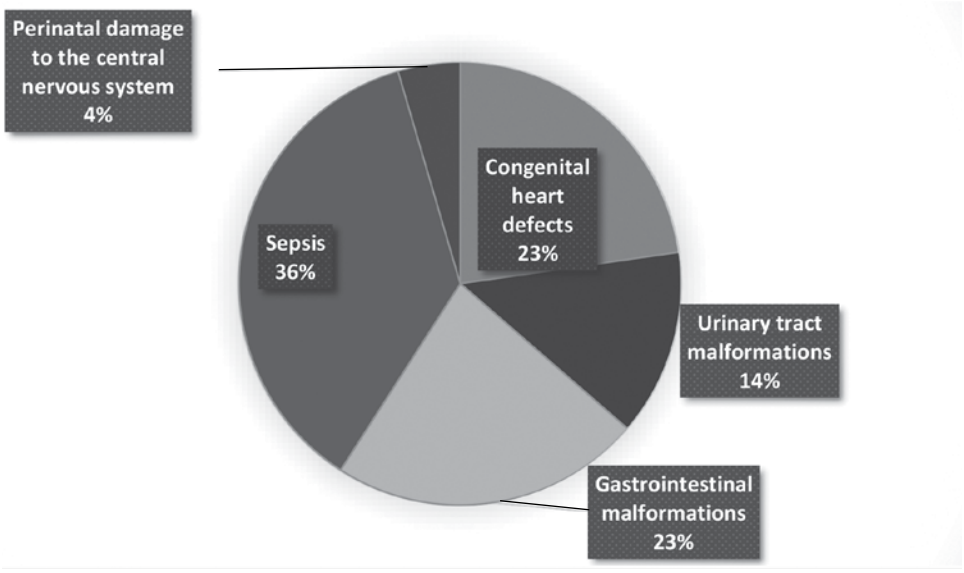

Figure 1. Nosological structure of newborns with critical conditions born from a mother with COVID-19

TABLE 1. CONTENT OF CYTOKINES IN THE BLOOD OF NEWBORNS

Cytokines in pg/mL	Healthy newborns		1st group		2nd group	
	min-max	average	min-max	average	min-max	average
IFNγ	14.48-27.35	20.96±0.66	15.27-32.25	23.64±0.81*	17.05-39.63	29.20±1.28*
IFNα	15.83-38.21	26.49±1.20	21.79-47.37	33.71±1.22*	15.83-38.21	24.43±1.36
IL-17A	29.93-64.97	46.99±1.70	55.34-92.06	69.68±1.70*	24.22-56.17	38.74±2.07*
MCP-1	98.29-305.71	196.69±9.92	422.15-1058.15	765.66±33.07**	98.29-305.71	116.47±7.86*
VEGF	19.21-59.93	38.47±2.23	25.17-54.67	40.05±1.49	19.21-59.93	42.15±1.82

Note.*, significantly relative to the healthy group (*, $p < 0.05$; * $p < 0.01$).

TABLE 2. CONTENT OF CYTOKINES IN THE URINE OF NEWBORNS

Cytokines in pg/mL	Healthy newborns		1st group		2nd group	
	min-max	average	min-max	average	min-max	average
IFNγ	4.25-8.75	5.94±0.23	10.08-24.11	16.84±0.66***	6.94-15.42	10.21±0.41*
IFNα	3.68-8.06	5.78±0.23	4.33-11.87	7.60±0.39*	5.48-14.33	9.49±0.43*
IL-17A	20.05-38.48	30.75±0.93	41.15-92.50	65.48±2.30**	23.55-54.67	37.07±1.43*
MCP-1	74.51-130.20	99.25±2.63	16.72-33.10	23.36±0.75***	39.45-71.27	54.75±1.80**
VEGF	18.36-34.97	26.99±0.87	10.26-30.15	19.79±1.02*	14.48-33.05	22.72±0.96*

Note.*, significantly relative to the healthy group (*, $p < 0.05$; * $p < 0.01$).

In our studies, IL-17A in group 1 newborns was increased to 69.68±1.70 pg/mL, compared to the control group – 46.99±1.70 pg/mL ($p < 0.05$), and in group 2 it has a significant tendency to decrease to 38.74±2.07 pg/mL ($p < 0.05$), against the control values of -46.99±1.70 pg/mL.

MCP-1 is produced by many types of cells, including mononuclear cells, mast cells, T cells, osteoblasts, fibroblasts, endothelial cells, bone marrow cells, epithelial cells, astrocytes. The synthesis of MSR-1 is induced by IL-1β, TNFα, IFNγ, IL-6, IL-4. Under the influence of MCP-1, proliferation of vascular smooth muscle cells also occurs with their secretion of proinflammatory cytokines that contribute to the progression of the disease due to vascular damage [1].

As a result, a 3.89-fold increase in MSR-1 was found in group 1 newborns – 765.66±33.07 pg/mL, against the control – 196.69±9.92 pg/mL. In group 2 newborns, a statistically significant decrease in MSR-1 was found to 116.47±7.86 pg/mL, against the control – 196.69±9.92 pg/mL.

The study of another vascular endothelial growth factor, VEGF, showed that there was no connection between its synthesis and the development of critical conditions in newborns on the first day of life.

The results of the studies showed a significant increase in the synthesis of IFNγ in the first day of life in newborns, regardless of the presence of somatic and infectious diseases of the mother. At the same time, IL-17A protects the mother's body from extracellular bacterial and fungal infections, thereby resorption of bone. On the other hand, osteoporosis is promoted by the use and use of anticoagulant drugs in the treatment of COVID-19.

Consequently, an increase in the concentration of IL-17A by 1.48 times in the blood of newborns born from a mother with COVID-19 shows the risk of developing both infection and osteogenesis disorders.

In order to assess the dynamics of cytokine synthesis, the above cytokines were studied in the urine of newborns of the examined groups on the 7th day of life.

As a result, an increase in IFNγ was found to be 2.84 times in group 1 newborns, 1.72 times in

group 2 newborns ($p < 0.001$), against the control – 5.94±0.23 pg/mL ($p < 0.05$). With respect to IFNα, an increase to 7.60±0.39 pg/mL was also detected in urine. in newborns of the 1st group, up to 9.49±0.43 pg/mL in newborns of the 2nd group, the indicators of the control group were 5.78±0.23 pg/mL, the results obtained were reliable in the range of $p < 0.05$.

At the same time, there is an increase in the level of IL-17A in the urine of group 1 newborns by 2.2 times (65.48±2.3 pg/mL), up to 37.07±1.43 pg/mL in group 2 newborns against the control values of -30.75±0.93 pg/mL ($p < 0.05$).

In contrast to the indicators VEGF in blood, in urine studies VEGF compared to control -26.99±0.87 pg/mL, was reduced to 19.79±1.02 pg/mL and 22.72±0.96 pg/mL in newborns of the 1st and 2nd groups, respectively ($p < 0.05$). All the obtained results of the study of cytokines in the urine of newborns had statistical significance in the ranges from $p < 0.05$ to $p < 0.0001$.

Thus, the obtained results of the study of cytokines in urine show activation of interferon status by the 7th day of life against the background of an increase in the key cytokine of bone resorption (IL-17A).

At the same time, there is a decrease in urine of the leading molecular markers of vascular endothelial damage – MSR-1 by 4.25 times and by 1.82 times at the birth of children from mothers infected with COVID-19 (group 1) and with other somatic diseases (group 2), respectively. And VEGF also decreases significantly in newborns, both with COVID-19 infection in the mother and in her absence.

When comparing the results of the cytokine status with clinical and biochemical data, symptoms of systemic inflammation are noted in parallel on day 7 in patients with group 1 newborns: an increase in body temperature, leukocytosis, tachycardia, an increase in reactive protein and a change in the prothrombin index.

Some differences in the values of this indicator in the blood and urine of group 2 newborns have been established. Thus, with an increase in IL-17A and MCP-1 in the blood on the first day of life, VEGF tends to increase to 42.15±1.82 pg/mL, which shows the risk of developing a systemic inflammatory response syndrome at the level of blood vessels with endothelial damage. On the 7th day of life, there was a tendency to increase IL-17A in urine to 37.07±1.43 pg/mL, than in the healthy group – 30.75±0.93 pg/mL $p < 0.05$.

The obtained results of the study show the accumulation of cytokines in the focus of inflammation and indicate the activity of the inflammatory process, requiring correct anti-inflammatory therapy. Thus, the advantages of noninvasive immunodiagnostics in neonatology have been established by the determination of cytokines in the urine of newborns. The control of urine cytokines in dynamics determines the prognosis of the development of critical conditions in newborns both early and in the late period of adaptation.

Consequently, IFNγ in urine acts as an indicator of the severity of the condition and the development of critical conditions in premature newborns at birth prematurely from a mother with COVID-19.

Thus, the clinical and laboratory assessment of the condition of newborns shows the importance of taking into account the state of cytokine synthesis. To reduce invasive procedures and preserve the blood volume of premature newborns, it is recommended to study cytokines, in particular IFNγ in urine, which allows early prediction of the development of critical conditions in newborns.

Based on the conducted scientific studies, it was found that newborns born from a mother with COVID-19 in the first day of life have an increase in IFNγ and IFNα in the blood against the background of an increase in IL-17A by 1.48 times, which shows the risk of developing both infection and osteogenesis disorders. At the same time, in response to the synthesis of IL-17A, activation of interferon status is noted by the 7th day of life in premature newborns.

Consequently, the study of interferons in urine reduces the risk of sepsis from invasive procedures and predicts the development of critical conditions. In this case, the threshold concentration is IFNγ in urine < 10.1 pg/mL and IFNγ in blood < 15.3 pg/mL. A decrease in IFNγ below the indicated concentrations in blood and urine shows the risk of developing severe critical conditions with the development of coagulopathy, hemorrhagic syndrome.

Conclusions

1. Newborns born from a mother with COVID-19 have an increase in IFNγ and IFNα in the blood on the first day of life against the background of an increase in IL-17A by 1.48 times, which shows the risk of developing both infection and osteogenesis disorders.
2. Activation of interferon status by the 7th day of life in newborns was established against the background of an increase in the key cytokine of bone resorption (IL-17A).
3. A decrease in the urine of molecular markers of vascular endothelial damage – MSR-1 by 4.25 times was found in newborn children born from a mother infected with COVID-19.
4. A decrease in VEGF in the urine of newborns was found, both with COVID-19 infection in the mother and in its absence.
5. Non-invasive urine cytokine diagnostics allows achieving economic efficiency by reducing hospital beds, as well as medical efficiency due to minimal traumatization of newborns.

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СНИЖЕНИЕ MDC/CCL22 ПРИ COVID-19 И В ПОСТКОВИДНОМ СИНДРОМЕ

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Резюме. В этой статье мы исследуем роль макрофагального хемокина MDC/CCL22 в иммунитете против COVID-19.

Материалом для исследования послужили образцы плазмы от 289 пациентов с подтвержденным COVID-19, получавших лечение в специализированных инфекционных стационарах, развернутых во время пандемии. Образцы крови отбирались при поступлении, на 7-10-е сутки от начала инфекции. Для этих же пациентов проводилось генотипирование варианта вируса в носоглоточных мазках. Также в исследование вошли образцы крови 69 реконвалесцентов пациентов, перенесших COVID-19 более чем за месяц до начала исследования. Кроме того, в качестве контроля в исследование вошел 51 здоровый донор. Концентрацию MDC/CCL22 и других цитокинов и хемокинов измеряли с помощью мультиплексного анализа с использованием технологии Luminex MagPix.

Результаты показали, что у пациентов с COVID-19 уровень макрофагального хемокина MDC/CCL22 в плазме был значительно ниже, независимо от штамма SARS-CoV-2, по сравнению со здоровыми донорами. Кроме того, у реконвалесцентов так же до сих пор отмечались сниженные уровни MDC/CCL22, что указывает на то, что истощение этого хемокина может сохраняться даже после выздоровления.

В рамках нашей работы мы предлагаем два механизма, которые могут объяснить причины, приводящие к снижению MDC/CCL22. Во-первых, связывание и инактивация этого хемокина пептидами SARS-CoV-2 может снижать его фунциональну активность. Другим предполагаемым механизмом снижения этого хемокина является «выключение» его эффекторных клеток (например, дендритных клеток и макрофагов) из иммунного процесса.

Лимфопению после COVID-19 потенциально можно объяснить отсутствием MDC/CCL22. Это может привести к сдвигу воспалительной реакции в сторону гиперактивации, что потенциально может объяснить тяжесть течения COVID-19 относительно других респираторных инфекций, особенно на начальных этапах пандемии.

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Наше исследование подчеркивает важность макрофагального хемокина MDC/CCL22 в иммунитете к COVID-19. Понимание механизмов концентраций этого хемокина может дать новое представление о патогенезе COVID-19.

Ключевые слова: макрофагальный хемокин, COVID-19, хемокины, мультиплексный анализ, постковидный синдром, дендритные клетки

MDC/CCL22 DEPLETION IN COVID-19 AND POST-COVID

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Abstract. In this article, we explore the role of macrophage-derived chemokine (MDC/CCL22) in COVID-19 immunity. The study included plasma samples of 289 patients with PCR-verified COVID-19 from specialized hospitals. The blood samples were collected at admission, approximately 7 days after the start of infection. Genetic testing of the virus was performed in nasopharyngeal swabs to determine the viral strain for each patient. We also included blood plasma of 69 convalescent patients who had recovered from COVID-19 more than a month prior to the study. Additionally, 51 healthy donors were included in the study as controls.

The concentrations of MDC/CCL22 and other cytokines and chemokines were measured with multiplex analysis using Luminex MagPix Technology. The results showed that COVID-19 patients had significantly lower MDC levels in their plasma, regardless of the SARS-CoV-2 strain, compared to healthy donors. This finding suggests that MDC/CCL22 depletion may play a role in COVID-19 immunity. Furthermore, convalescent patients still showed decreased concentrations of MDC/CCL22 more than a month after infection, indicating that this depletion may persist even after recovery.

We propose two mechanisms that can explain the reasons leading to MDC/CCL22 depletion. The first is binding and inactivation of this chemokine with SARS-CoV-2 peptides, making it not only undetectable for commercial kits, but also less functionally active. Another mechanism is the dysfunction of its effector cells (e.g., DCs and macrophages). Lymphopenia following COVID-19 can potentially be explained by the absence of MDC/CCL22. This may lead to a shift towards hyperactivation in the inflammatory response, potentially explaining the severity of COVID-19.

This research sheds light on the importance of MDC/CCL22 in COVID-19 immunity and highlights the need for further investigation into its role in the disease. Understanding the mechanisms behind MDC/CCL22 depletion could provide new insights into the pathogenesis of COVID-19 and inform the development of potential treatments.

Keywords: macrophage derived chemokine, COVID-19, chemokines, multiplex analysis, post-COVID, dendritic cells

Introduction

COVID-19 is an acute infectious disease caused by the RNA-based SARS-CoV-2 virion of the genus Betacoronavirus. The COVID-19 pandemic has affected millions of people worldwide, causing significant morbidity and mortality [8]. COVID-19 enters cells through the ACE 2 receptor, which is found in various human cells [10]. The virus primarily targets cells in the respiratory system, causing inflammation and inhibiting the ACE 2 receptor, leading to increased angiotensin II secretion and subsequent activation of inflammatory transcription factors [4]. These conditions can potentially trigger a cytokine storm, resulting in enhanced inflammation [3].

Understanding the immune response to SARS-CoV-2, the virus that causes COVID-19, is crucial in developing effective vaccines and therapeutics. In this article, we review the current knowledge

on COVID-19 immunity from the chemokine standpoint. Specifically, we address our findings in terms of macrophage-derived chemokine in blood plasma and its changes in acute COVID-19 and convalescent patients.

Materials and methods

Study population

The study included 289 patients from 2 hospitals in Saint Petersburg with PCR-verified COVID-19. Blood samples were collected at admission and approximately 7 days after the start of infection. Patients with comorbidities or previous infections were excluded from the study. We also included 69 convalescent patients (n = 69) who had donated their blood plasma in earlier stages of the pandemic. Additionally, 51 blood samples were collected from healthy donors.

Ethics approval

The study was approved by the Local Ethics Committee of Pasteur Institute, Saint Petersburg. All patients were consenting adults and gave their permission for participation in the study.

Blood sample collection

Blood samples were collected from patients and healthy donors using standard venipuncture techniques. Samples were collected in EDTA tubes and immediately transported to the laboratory for processing, where, after centrifugation, the blood plasma was stored at -70 .

Cytokine and chemokine measurements

Concentrations of MDC/CCL22, among other cytokines and chemokines, were measured using Luminex MagPix Technology with the Millipore kit.

Genetic testing

Genotyping of SARS-CoV-2 isolates collected from patients was performed using near-complete genome sequences on the Illumina MiSeq automatic platform. Nasopharyngeal swabs were collected from COVID-19 patients and stored at -20 °C until analysis. Total nucleic acid samples were obtained by extraction and purification using the RIBO-prep DNA/RNA Extraction Kit. Reverse transcription was performed using random hexanucleotide primers and the Reverta-L Kit. Libraries were prepared using the TruSeq Nano DNA Kit and the TruSeq DNA CD Indexes Kit, and sequencing was performed using the Illumina MiSeq System. The quality of Illumina reads was assessed using the FastQC program, and genome assembly was carried out by mapping to the SARS-

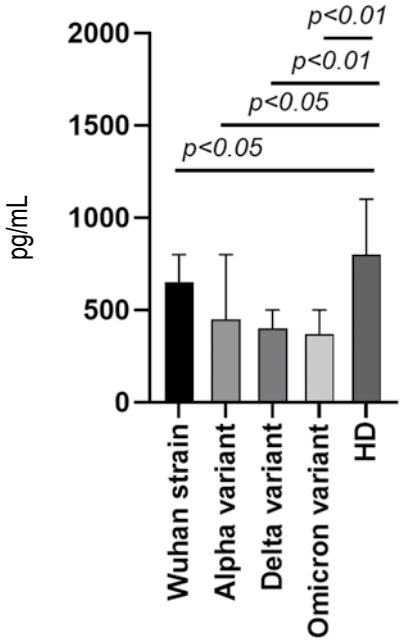

Figure 1. Levels of MDC/CCL22 in the blood plasma of COVID-19 patients infected with different viral strains, including the Wuhan strain (n = 51), Alpha (n = 95), Delta (n = 98), and Omicron variants (n = 57)

Note. The results for healthy donors (HD) are presented as well. The bars in the graph represent the median concentrations of MDC/CCL22 in each group, while the whiskers represent the 75th quartile.

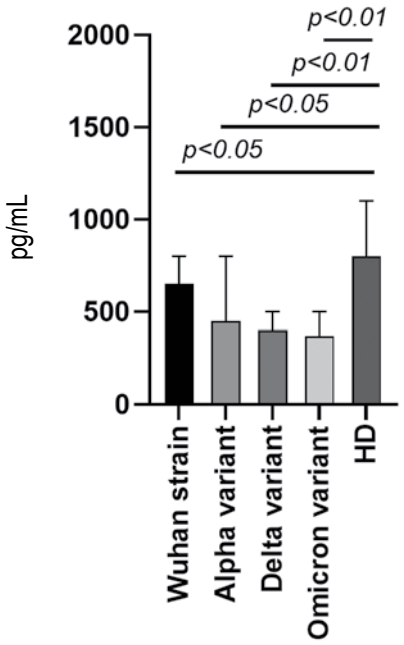

Figure 2. MDC/CCL22 concentrations in the blood plasma of COVID-19 convalescents (n = 69) in comparison to infected patients in the acute phase (n = 51) and healthy donors (n = 56)

Note. Bars represent median concentrations (pg/mL). Whiskers represent the 75th quartile.

CoV-2 reference genome using Bowtie 2. Variant calling and consensus generation were performed using samtools and bcftools software, and the Nextclade tool was used to assess the quality of assembled sequences and to assign genomes to lineages. All sequencing was performed retrospectively.

Data analysis

Data was analyzed using GraphPad Prism version 8.0.2 software. Descriptive statistics were used to summarize the data, and comparisons between groups were made using t-tests or ANOVA as appropriate. A p-value < 0.01 was considered statistically significant.

Results and discussion

The cytokine detection kit identified various biological substances, with the macrophage-derived chemokine (MDC/CCL22), a CC chemokine, having a particularly significant and surprising role. Notably, COVID-19 patients had notably lower MDC levels in their plasma, regardless of the SARS-CoV-2 strain. This was noteworthy since other chemokines tended to increase in COVID-19 patients' blood plasma compared to healthy donors (HD) [7].

The results of the study can be found in Figure 1.

Interestingly, convalescents of the original Wuhan viral strain had significantly lower MDC/CCL22 concentrations, not only compared to healthy donors, but also in comparison with those in phase of infection [1]. The results of previous studies on the matter are presented in Figure 2.

The levels of MDC/CCL22 were found to be lower in COVID-19 patients infected with different strains compared to healthy donors, as shown in the first figure. The decrease in MDC/CCL22 levels in COVID-19 patients may suggest a more profound impact of the virus on the immune system than previously thought, and may contribute to immune dysregulation and severe pulmonary pathology. Further research is needed to fully understand the complex interactions between MDC/CCL22, DCs, platelets, and immune regulation in COVID-19. The observed decrease in MDC/CCL22 levels may be specific to COVID-19, as it is rarely seen in other inflammation-prone illnesses, even those affecting the respiratory tract.

Macrophage derived chemokine belongs to the CC family. Via this classification, it holds the double name MDC/CCL22. MDC/CCL22 is produced by macrophages and dendritic cells, with or without external stimuli like bacterial lipopolysaccharide [12]. Its function is directly linked to the CCR4 molecule, a receptor widely present on Th2 cells. CCR4 receptors on $CD4^+$ lymphocytes in the bone marrow, like MDC/CCL22, mediate cellular growth and maturation. Activation of cellular migration and Th1/Th2 polarization are coordinated with the help of MDC/CCL22.

It is possible that MDC/CCL22 deficiency can partially explain persistent lymphopenia associated with COVID-19 [6], noticeable even after recovery. Several *in vitro* studies have shown the importance of MDC/CCL22 in regulation of inflammation. Its presence complemented regulatory T cell activation and restricted enhanced inflammation with type I helper T cells [9].

A drop in MDC/CCL22 concentration in COVID-19 patients is worthy of attention, and it has been previously noted by other researchers [11]. We present hypothetical explanations for the phenomenon in question below.The first concept implies possible binding of SARS-CoV-2 viral proteins with MDC/CCL22 due to potential affinity with, or mimicry of, MDC/CCL22's main ligands. In such cases, MDC/CCL22 production by producer cells (i.e., DCs and macrophages) is unperturbed. Yet, the selective binding of this chemokine makes it undetectable for commercial kits as it changes its antigenic structure. Moreover, it is possible that its functional activity reduces due to this process. This hypothesis is supported by the fact that other cytokines and chemokines, produced by DCs and macrophages, show enhanced expression when compared to healthy donors.

There is, however, an opposite hypothesis, implying that COVID-19 can actually affect the functional activity of producer cells. Specifically, researchers highlighted a significant shortage of DCs in COVID-19 patients, both in acute and post-recovery periods. Moreover, other studies have highlighted the relationship between disease severity [2] and dendritic cell properties [5], both quantitative and qualitative. This, however, for some reason does not affect other cytokines and chemokines, produced by DCs (IL-1α, IL-1β, IL-6, IL-7, IL-12 (p35 and p40), IL-15, IL-18, TNFα, TGF-β, macrophage CSF, and granulocyte-macrophage CSF, but not IL-2, IL-3, IL-4, IL-5, IL-9, and IFNγ transcripts).

In any case, both hypotheses prove the role of the SARS-CoV-2 infectious process in the suppression of DCs. Theoretically, this may explain a defect in MDC/CCL22 production and its deficiency in the blood plasma of COVID-19 patients in comparison

with healthy donors. While the precise mechanisms by which SARS-CoV-2 suppresses DC function and MDC/CCL22 production are not yet fully understood, the deficiency of MDC/CCL22 in the blood plasma of COVID-19 patients compared to healthy donors suggests that this chemokine may play a critical role in the pathogenesis of the disease. Further research is needed to fully elucidate the complex interactions between SARS-CoV-2, DCs, and MDC/CCL22, with the ultimate goal of developing new therapeutic strategies to combat COVID-19 and other infectious diseases.

Conclusion

In summary, the absence of MDC/CCL22 may lead to a shift towards hyperactivation in the inflammatory response, potentially explaining the severity of COVID-19. Figure 3 (see 2nd page of cover) presents potential mechanisms for this dysfunction. MDC/CCL22 may be a missing link in understanding COVID-19 processes, particularly its role in vaccine-associated immunity and in individuals who have survived severe cases. Further research is needed to fully understand its role in coronavirus infection.

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ОСОБЕННОСТИ ЧАСТОТЫ ВСТРЕЧАЕМОСТИ ПОЛИМОРФИЗМА ГЕНА Т-330G *IL2* У ПАЦИЕНТОВ С COVID-19

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Резюме. Инфекция SARS-CoV-2 является этиопатогенетическим фактором новой коронавирусной инфекции. Восприимчивость к вирусу и, соответственно, заболеваемость отличается у детей и взрослых. С одной стороны, это отражает возрастные особенности иммунного ответа. С другой стороны, реализуется через выработку ряда цитокинов, в том числе IL-2, и отражает генетически-детерминированные особенности продукции цитокинов. Целью исследования был анализ частоты встречаемости полиморфных вариантов Т-330G гена *IL2* у пациентов с новой коронавирусной инфекцией. Всего было обследовано 145 пациентов, из них 31,0% детей (n = 45) и 69,0% взрослых (n = 100). Диагноз «новая коронавирусная инфекция» верифицирован методом ОТ-ПЦР подтверждающего наличие вируса SARS-CoV-2 и выявление клинических симптомов инфекции верхних дыхательных путей. Группу контроля составили 50 здоровых доноров-добровольцев. Для анализа полиморфизма Т-330G гена *IL2* использовали аллель-специфическую ПЦР с электрофоретической детекцией в 3% агарозном геле («Литех», Россия). Для сравнения частот комбинаций аллелей использовали критерий χ^2 и отношение шансов OR и (95% CI).

Доминирующим генотипом у пациентов с COVID-19 был гетерозиготный генотип GT полиморфизма Т-330G гена *IL2*. В группе детей с риском развития новой коронавирусной инфекции был ассоциирован генотип GG полиморфизма Т-330G гена *IL2* (31,1% у детей и 18,0% в группе контроля, $p < 0,05$, OR = 2,047). В то время как гомозиготный генотип TT полиморфизма Т-330G гена *IL2* являлся протективным генотипом (его частота встречаемости составила у пациентов – 26,7%, в группе контроля – 54,0%, $p < 0,05$, OR = 0,315). У взрослых с риском развития новой коронавирусной инфекции был ассоциирован гетерозиготный генотип GT полиморфизма Т-330G гена *IL2* (в группе пациентов – 44,0% против контроля – 28,0%, $p = 0,028$, OR = 2,020). Низкий риск развития заболевания был ассоциирован с гомозиготного вариантом TT полиморфизма Т-330G гена *IL2* (в группе пациентов 37,0% против контроля – 54,0%, $p = 0,024$, OR = 0,500).

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Полиморфизм Т-330G промотроной зоны гена *IL2* по-разному влияет на его продукцию. От уровня IL-2 зависит направление иммунного ответа и его эффективность. Понимание индивидуальных факторов, определяющих особенности иммунного ответа может помочь в понимании механизмов развития COVID-19-ассоциированных заболеваний и подборе подходов к персонализированным методам их лечения.

Ключевые слова: полиморфизм генов, SNP, T-330G IL2, COVID-19, дети, SARS-CoV-2

FEATURES OF THE FREQUENCY OF OCCURRENCE OF T-330G *IL2* GENE POLYMORPHISM IN PATIENTS WITH COVID-19

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Abstract. SARS-CoV-2 infection is the etiopathogenetic factor of the new coronavirus infection. Susceptibility to the virus and, accordingly, the incidence differs in children and adults. On the one hand, this reflects the age-related features of the immune response. On the other hand, it is realized through the production of a number of cytokines, including IL-2, and reflects the genetically determined features of cytokine production. The aim of the study was to analyze the frequency of occurrence of T-330G polymorphic variants of the *IL2* gene in patients with a new coronavirus infection. A total of 145 patients were examined, including 31.0% of children (n = 45) and 69.0% of adults (n = 100). The diagnosis of a new coronavirus infection was verified by RT-PCR confirming the presence of the SARS-CoV-2 virus and identifying clinical symptoms of an upper respiratory tract infection. The control group consisted of 50 healthy volunteer donors. Allele-specific PCR with electrophoretic detection in 3% agarose gel (Litech, Russia) was used to analyze the T-330G polymorphism of the *IL2* gene. To compare the frequencies of allele combinations, the χ^2 test and the odds ratio OR and (95% CI) were used.

The dominant genotype in patients with COVID-19 was the heterozygous GT genotype of the T-330G polymorphism of the *IL2* gene. In the group of children at risk of developing a new coronavirus infection, the GG genotype of the T-330G polymorphism of the *IL2* gene was associated (31.1% in children and 18.0% in the control group, $p < 0.05$, OR = 2.047). While the homozygous TT genotype of the T-330G polymorphism of the *IL2* gene was a protective genotype (its occurrence rate was 26.7% in patients, 54.0% in the control group, $p < 0.05$, OR = 0.315). In adults, the heterozygous GT genotype of the T-330G polymorphism of the *IL2* gene was associated with the risk of developing a new coronavirus infection (in the group of patients – 44.0% *versus* control – 28.0%, $p = 0.028$, OR = 2.020). A low risk of developing the disease was associated with the homozygous TT variant of the T-330G polymorphism of the *IL2* gene (in the group of patients 37.0% *versus* control – 54.0%, $p = 0.024$, OR = 0.500).

The T-330G polymorphism of the promoter zone of the *IL2* gene differently affects its production. The direction of the immune response and its effectiveness depend on the level of IL-2. Understanding the individual factors that determine the features of the immune response can help in understanding the mechanisms of development of COVID-19-associated diseases and the selection of approaches to personalized methods of their treatment.

Keywords: polymorphism of gene, SNP, T-330G IL2, COVID-19, children, SARS-CoV-2

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Introduction

Infants and children under 5 years of age have a unique immunophenotype and respond differently to SARS-CoV-2 infection compared to adults who are more often to develop a cytokine storm [4, 9]. For children SARS-CoV-2 infection develops mild (27%) or asymptomatic disease (66%) [2, 5, 7, 8]. Moderate and severe severity is extremely rare in children (5 and 2%, respectively) [6].

Comparison of adult patients and children also demonstrates differences in both the level of

production and the functional characteristics of the immune response. Increasing evidence has demonstrated that interleukins played an important role in the progression of COVID-19. Compared to mild COVID19 cases, serum interleukins levels increased greatly in severe and critical patients [3]. Pro-inflammatory cytokines induce active production of cytokines and the immune response. Interleukin 2 (IL-2) is a monomeric glycoprotein with a molecular weight of approximately 15 kDa, produced by Th-1 cells. IL-2 plays a critical role in the differentiation and survival of regulatory T cells, thus ensuring their importance in the control of the immune response. One of the best achievements in this direction was to highlight the important role of single nucleotide polymorphisms (SNPs) of those genes involved in the immune regulatory mechanism. **The aim of the study** was to analyze the frequency of occurrence of T-330G *IL2* polymorphic genes in patients with COVID-19.

Materials and methods

In total, 145 patients with a new coronavirus infection were examined, represented by a group of "children" and a group of "adults". The group of "children" (n = 45) – patients admitted to the department of the Republican Children's Infectious Diseases Clinical Hospital with a new coronavirus infection of moderate severity at the age of 0 to 14 years (n = 45). Distribution of children depending on the age of the group 0-4 years old – 40.0% (n = 18), 5-9 years old – 22.2% (n = 10) and 10-14 years old – 37.8% (n = 17).

The group of "adults" (n = 100) – patients admitted to the pulmonology department of the Institution of Healthcare of the Republic of Crimea "Academic Research Institute of Physical Methods of Treatment, Medical Climatology and Rehabilitation named after I.M. Sechenov" for the purpose of sanatorium treatment after a new coronavirus infection. Women made up 74.07% (n = 80) of the study population, 25.93% (n = 28) were men.

Inclusion criteria for the study were: previous novel coronavirus infection. Signed informed consent for inclusion in the study. The exclusion criteria were: the presence of complicated forms of viral pneumonia in the presence of severe functional pulmonary and extrapulmonary disorders, age over 75 years.

Verification of the diagnosis of novel coronavirus infection was based on confirmation of the SARS-CoV-2 virus by RT-PCR and the presence of clinically identifiable symptoms or signs of an upper respiratory tract infection, namely throat congestion, sore throat and fever, and on x-ray. All patients signed an informed consent for the study.

The control group consisted of 50 relatively healthy respondents, 32 women (64.0%) and 18 men (36.0%), whose average age was 44.3±5.23 years.

The study was conducted in accordance with the rules of the Helsinki Declaration of 1975, revised in 2013 and approved by the Ethics Committee of V.I. Vernadsky.

Methods

To analyze the T-330G polymorphism of the *IL2* gene, an allele-specific polymerase chain reaction with electrophoretic detection was used. DNA was isolated from the whole blood of patients with a new coronavirus infection and healthy volunteers using the DNA-express blood kit according to the manufacturer's instructions. Allele-specific PCR was performed using "T-330G *IL2*" kits (Liteh, Russia) according to the manufacturer's instructions. Detection of amplification products was carried out by horizontal electrophoresis in 3% agarose gel.

The study was conducted at the Collective Center for the use of scientific equipment "Molecular Biology" CFU named after V.I. Vernadsky.

The data obtained were analyzed using the Statistica 8.0 software package. The expected allele frequency was calculated based on the Hardy–Weinberg law. To compare the frequencies of allele combinations, the χ^2 test was used with the Yates correction for continuity. The association of polymorphisms with novel coronavirus infection was analyzed by determining the odds ratio (OR) test and 95% confidence interval (95% CI), $p < 0.05$.

Results and discussion

We found all the studied IL-2 mutations in accordance with the Hardy–Weinberg law ($p > 0.05$). As a result of the study, it was shown that the distribution of polymorphic variants of IL-2 genotypes differed in all the research groups. The dominant genotype in patients with COVID-19 was the heterozygous GT genotype of the T-330G *IL2* gene polymorphism (Table 1). Its frequency was statistically significantly higher in patients than in the control group ($p < 0.05$). In the group of healthy donors, the most common genotype was homozygous TT of the T-330G *IL2* gene polymorphism. Its frequency of occurrence was statistically significantly lower in children and adults (Figure 1, Table 1, $p < 0.05$). The frequency of occurrence of GG homozygous of the T-330G *IL2* gene polymorphism in the adult group was comparable to that in the control group, while in children it was statistically significantly lower than in adults ($p < 0.05$).

The analysis showed that in children with the risk of a new development of coronavirus infection, the GT genotype of the T-330G *IL2* gene polymorphism is associated. While the TT polymorphism of the *IL2* T-330G gene is a protective genotype (Table 1). In adults, the GT genotype of the polymorphism of the T-330G *IL2* gene is associated with the risk of developing a new coronavirus infection. While the

TABLE 1. MULTIPLICATIVE INHERITANCE MODEL (χ^2 TEST, df = 1)

Genotypes	Case n (%)	Control n (%)	χ^2	p	OR	
					values	95% CI
Children						
TT	12 (26.7)	27 (54.0)	14.0	0.001	0.315	0.1740-0.5690
GT	19 (42.2)	14 (28.0)	3.714	0.054	–	–
GG	14 (31.1)	9 (18.0)	3.892	0.049	2.047	1.054-3.973
Adult						
TT	37 (37.0)	27 (54.0)	5.162	0.024	0.500	0.284-0.880
GT	44 (44.0)	14 (28.0)	4.883	0.028	2.020	1.122-3.640
GG	19 (19.0)	9 (18.0)	0	1.0	–	–

TABLE 2. COMPARISON OF THE FREQUENCY OF ALLELES AND GENOTYPES OF T-330G *IL2* POLYMORPHIC VARIANTS (rs2069762) COMPARED WITH THE FREQUENCY OF OCCURRENCE IN POPULATIONS

Population	Sample size	Reference allele	Alternative allele
Study groups			
Total	145	0.55	44.4
Children	45	0.478	0.522
Adult	100	0.556	0.444
Control	50	0.680	0.320
According to the website rs2069762 RefSNP Report – dbSNP – NCBI (nih.gov)			
African	4876	0.9219	0.0781
African American	4730	0.9207	0.0793
Asian	216	0.671	0.329
European	53642	0.69843	0.30157
Latin American 2	4672	0.6803	0.3197

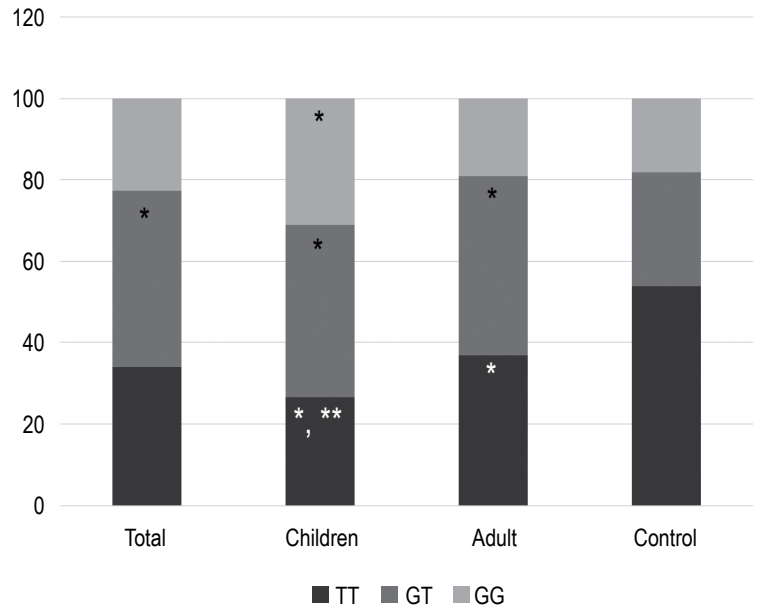

Figure 1. Genotype frequency of T-330G polymorphism of the *IL2* gene in the studied group

Note. *, significant differences from the groups of patients with control; **, significant differences from the group of children with adult.

TT polymorphism of the *IL2* T-330G gene is also protective.

When detecting the frequency of occurrence of alleles with a number of populations, a definite was revealed. The frequency of T alleles of the T-330G *IL2* polymorphism both in the control group and in patients with a new coronavirus infection was considered similar to that in Asians, Caucasians and Latin American, but was lower than in Africans and African Americans (Table 2). Allele C of the T-330G *IL2* polymorphism was marked by an increased frequency of occurrence in Asians, Caucasians and Latin American, and was higher than in Africans and African Americans.

Since the beginning of the COVID-19 outbreak, an increasing number of cases of COVID-19 have been confirmed. Various aspects of COVID-19 disease are described, taking into account risk factors such as age, gender, and pre-existing metabolic conditions such as diabetes, obesity, and hypertension. However, the genetic background has often been overlooked to end up being watched as a major player by COVID-19.

The cytokine storm arose with COVID-19, and interleukins and IFNγ were involved in the process of hyperinflammation. Immune mediators, including interleukins, have been shown to play an important role in the development of COVID-19 [3]. It has been noted that genotypes correlate with susceptibility to COVID-19, mortality, and immune response activity. When comparing our data on the frequency of distribution of genotypes, that in adult patients of the Caucasian race and lived in the Trans-Baikal Territory the chance of developing SARS-COV-2 increased in carriers of the allele T and the TT genotype of *IL2* gene [1].

Conclusion

The dominant genotype in children and adults with COVID-19 was the TT polymorphism T-330G *IL2* gene. The GG genotype of the T-330G *IL2* gene polymorphism in children is statistically significantly lower than in adults, whose level was comparable to the control group. In children, GC genotype of the T-330G *IL2* gene polymorphism is associated with the risk of developing a SARS-COV-2 infection. In adults at risk SARS-COV-2, GT genotype of the T-330G *IL2* gene polymorphism is associated. While TT of the T-330G *IL2* gene polymorphism in both children and adults is protective. T-330G *IL2* SNPs may be associated with a higher risk of COVID-19 infection. Understanding individual-specific polymorphisms may help better explain COVID-19 outcomes in genetic profiling to create personalized COVID-19 therapies.

Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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ОСОБЕННОСТИ ИММУННОГО СТАТУСА БОЛЬНЫХ С ОСТРЫМ КОРОНАРНЫМ СИНДРОМОМ, ПЕРЕНЕСШИХ COVID-19, В ЗАВИСИМОСТИ ОТ ЧИСЛА ЦИТОТОКСИЧЕСКИХ Т-ЛИМФОЦИТОВ ($CD8^+$)

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Резюме. Пандемия коронавирусной болезни 2019 года (COVID-19) оказала беспрецедентное воздействие на здоровье и экономику во всем мире. Прямое повреждение миокарда и цитокиновый шторм, приводящий к дестабилизации ранее существовавших бляшек и ускоренному образованию новых бляшек, являются двумя механизмами, провоцирующими острый коронарный синдром при COVID-19. Недостаточно данных об иммунном статусе пациентов с острым коронарным синдромом, перенесшим COVID-19. Целью работы явилось исследование Т- и В-клеточного, гуморального звеньев иммунитета в зависимости от числа цитотоксических Т-лимфоцитов ($CD8^+$) у больных с острым коронарным синдромом (ОКС), перенесших COVID-19.

Обследовано 65 мужчин с нестабильной стенокардией и острым инфарктом миокарда (острым коронарным синдромом) от 40 до 65 лет, которые ранее болели COVID-19. Проведено исследование периферической крови: общий анализ крови (прибор Medonic – Швеция), общие и специфические IgM, IgG, IgA, фрагменты комплимента («Вектор Бест», Россия). Субпопуляции Т- и В-лимфоцитов определены методом проточной цитометрии. У лиц с острым коронарным синдромом, перенесших COVID-19 преимущественно с нормальным и повышенным уровнями цитотоксических Т-клеток наблюдалось более тяжелое течение заболевания – превалировали больные с острым инфарктом миокарда, у них была больше смертность, продолжительность лечения, отмечались чаще тромбозы стентов. У больных с повышенными цитотоксическими Т-клетками наблюдалось максимальное увеличение эритроцитов, гемоглобина, гематокрита, лимфоцитов как общего числа, так и субпопуляций – Т-хелперов, Т-NK-лифоцитов, NK-лимфоцитов, Т-лимфоцитов ранней и поздней активации,

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В1- и В2-лимфоцитов, индекса НСТ-индуцированного теста. У пациентов с нормальным уровнем NK-клеток в сравнении с другими группами наблюдалось повышение НСТ спонтанной активности и индекса, значимое снижение С3а и С5а фрагментов комплемента. Превалирование тромбоза стентов и смертности в группе больных с нормальным уровнем цитотоксических Т-клеток может свидетельствовать о торпидности иммунной системы у этих пациентов с неблагоприятными исходами. Полученные данные свидетельствуют о значительной вариабельности ответов клеток иммунной системы у постковидных пациентов ОКС с различным уровнем цитотоксических клеток. Все это следует в дальнейшем рассмотреть подходы и к иммунокоррекции выявленных нарушений.

Ключевые слова: цитотоксические Т-лимфоциты, COVID-19, острый коронарный синдром, лимфоциты, иммунная система, комплемент

FEATURES OF THE IMMUNE STATUS OF PATIENTS WITH ACUTE CORONARY SYNDROME WHO UNDERWENT COVID-19, DEPENDING ON THE NUMBER OF CYTOTOXIC T LYMPHOCYTES (CD8$^+$)

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Abstract. The 2019 coronavirus disease (COVID-19) pandemic has had an unprecedented impact on health and economies around the world. Direct myocardial injury and cytokine storm, leading to destabilization of pre-existing plaques and accelerated formation of new plaques, are two mechanisms that trigger the acute coronary syndrome in COVID-19. There is insufficient data on the immune status of patients with acute coronary syndrome who have undergone COVID-19. The aim of the study was to study T and B cell, humoral immunity depending on the number of cytotoxic T lymphocytes (CD8$^+$) in patients with acute coronary syndrome who underwent COVID-19. Materials and methods of research: 65 men with unstable angina pectoris and acute myocardial infarction (acute coronary syndrome) from 40 to 65 years old, who had previously had COVID-19, were examined. A study of peripheral blood was carried out: complete blood count (Medonic device, Sweden), general and specific IgM, IgG, IgA, compliment fragments (Vector Best, Russia). Subpopulations of T and B lymphocytes were determined by flow cytometry. In persons with acute coronary syndrome who underwent COVID-19 with predominantly normal and elevated levels of cytotoxic T cells, a more severe course of the disease was observed: patients with acute myocardial infarction prevailed, they had longer mortality, longer treatment duration, and stent thrombosis was more common. In patients with elevated cytotoxic T cells, there was a maximum increase in erythrocytes, hemoglobin, hematocrit, lymphocytes of both the total number and subpopulations – T helpers, T-NK lymphocytes, NK lymphocytes, T lymphocytes of early and late activation, B1 and B2 lymphocytes, index of NBT-induced test. In patients with normal levels of NK cells, compared with other groups, there was an increase in spontaneous NBT activity and index, a significant decrease in C3a and C5a complement fragments. Prevalence of stent thrombosis and mortality in the group of patients with normal levels of cytotoxic T cells may indicate torpidity of the immune system in these patients with poor outcomes.

Keywords: cytotoxic T lymphocytes, COVID-19, acute coronary syndrome, lymphocytes, immune system, complement

Introduction

The 2019 coronavirus disease pandemic (COVID-19) has had an unprecedented impact on health and the economy around the world. In patients with a new coronavirus infection (COVID-19), manifestations of lymphopenia occur early and are prognostic, most often potentially associated with a decrease in the number of T helpers and some cytotoxic T lymphocytes. This leads to an imbalance of the innate and acquired immune response, delayed

viral clearance and hyperstimulation of macrophages and neutrophils [1]. Inflammation in the vascular system can lead to diffuse microangiopathy with thrombosis. Inflammation in the myocardium can cause myocarditis, acute coronary syndrome, as well as rapid deterioration and sudden death [6]. The influx of T helpers into the cardiac vascular system leads to an increase in the production of cytokines, which stimulate the migration of smooth muscle cells into intima and the production of collagen and other fibrous products, which causes pronounced atherosclerotic damage. Direct myocardial injury and cytokine storm leading to destabilization of pre-existing plaques and accelerated formation of new plaques are two mechanisms provoking acute coronary syndrome in COVID-19 [10].

In the work of Khusainova L.N. et al. [4] it was shown that in patients with acute coronary syndrome (ACS), the number of T lymphocytes decreased, the number of lymphocytes affecting apoptosis increased ($CD25^+$, $CD95^+$).

According to Lebedeva O.K. et al. [5] the development of acute heart failure in patients with acute myocardial infarction (AMI) is associated with an increase in CD16(-) monocytes and a decrease in the number of CD16(+) T-NK cells (natural killers).

According to other data [8], in patients with ACS, in comparison with the control, the relative indices of subpopulations of T helper cells, T lymphocytes of early and late activation, as well as B lymphocytes are statistically higher. At the same time, there is a tendency to increase the absolute values of these parameters. There was a statistically significant increase in the T-NK lymphocyte subpopulation in relative and absolute terms, and total T lymphocytes ($p < 0.01$), as well as T cytotoxic lymphocytes ($p < 0.001$) decreased. As a result of an increase in T helper cells and a decrease in cytotoxic T lymphocytes, the CD4/CD8 lymphocyte index increased by more than 2 times ($p < 0.001$).

There is insufficient data in the available literature on the immune status of patients with ACS who have undergone COVID-19, which determines the relevance of the study.

The aim of the study was to study the T and B cell, humoral links of immunity depending on the number of cytotoxic T lymphocytes ($CD8^+$) in patients with acute coronary syndrome who underwent COVID-19.

Research objectives:

1. To identify clinical differences in the course of the disease in patients with ACS who underwent COVID-19, depending on the number of T cytotoxic lymphocytes.

2. To identify differences in the content of the main populations of T and B lymphocytes, humoral immunity, blood cell composition in patients with ACS who underwent COVID-19, depending on the number of T cytotoxic lymphocytes.

Materials and methods

Sixty-five men with unstable angina pectoris and acute myocardial infarction (acute coronary syndrome) aged 40 to 65 years, who had previously had COVID-19, were examined. All persons also have hypertension. All patients required stenting of the coronary arteries in the next 3 days after admission to the hospital, since according to coronary angiography (CAG), they had coronary artery stenosis of 70% or more. CAG was performed on an Innova JE device, stents with a drug coating from the company Xience Alpine were implanted. Before this procedure, all patients signed an informed consent (protocol of the Ethical Committee of the Southern State Medical University of the Ministry of Health of the Russian Federation No. 9 dated 11.09.2006 and protocol of the Ethical Committee of the GAU OTKZ GKB No. 1 of Chelyabinsk No. 12 dated 10.10.2022).

The following examinations were performed: a general blood test (25 parameters were examined): leukocyte, erythrocyte and platelet sprouts of hematopoiesis, quantitative and qualitative composition of hematopoietic sprouts was carried out by a standardized method on a Medonic M20 hematological analyzer (Sweden).

From immunological indicators, the phagocytic activity of latex particles with a diameter of 1.7 microns was evaluated by neutrophils (phagocytosis activity, phagocytosis intensity, phagocytic number); spontaneous and induced NBT activity of neutrophils was determined by morphological method (light microscopy using Olympus microscopes (Japan). The phagocytic activity of neutrophils was determined by their ability to absorb latex particles [2].

Specific immunoglobulins M and G to COVID-19 and general immunoglobulins A, M, G were determined by VectorBest kits using standard enzyme immunoassay (ELISA) methods.

From immunological parameters, the following were determined by flow cytometry on the Navios cytofluorimeter (Beckman Coulter, USA) using a standardized technology for assessing the lymphocytic link of immunity [14]: $CD45^+$, $CD3^+$ (T lymphocytes), $CD45^+$, $CD3^+$, $CD4^+$ (helper inducers), $CD45^+$, $CD3^+$, $CD8^+$ (cytotoxic T lymphocytes,), $CD45^+$, $CD3^+CD16^+$, $CD56^+$ (T-NK cells) $CD45^+$, $CD3^-$, $CD16^+$, $CD56^+$ (natural killers), $CD45^+$, $CD3^+$, $CD4^+$, $CD25^+$, $CD127^-$ (T regulatory cells/suppressors), $CD45^+$, $CD3^+$, $CD4^+$, $CD25^+$ (activated helpers, early activation of lymphocytes), $CD45^+$, $CD3^+$, HLA-DR (activated T lymphocytes – late activation of lymphocytes. Phagocytosis and NBT activity of neutrophils were evaluated by standard

methods. C1q, C3a, and C5a complement fragments were also determined by the standard ELISA method.

The patients also underwent a study of the lipid profile and troponin level using a standard technique.

The clinical condition of patients was assessed at the time of examination and during the entire postoperative period in the hospital, and some indicators (such as mortality and thrombosis) in the next 6 months after surgery.

With the help of IBM SPSS Statistics 19, StatPlus 2009 Professional programs, statistical processing of the material was carried out. Calculated: arithmetic mean (M), arithmetic mean error (m), determined the Student's criterion for independent samples.

Results and discussion

All ACS patients who underwent COVID-19 were divided into 3 groups depending on the number of T cytotoxic lymphocytes [14]: with reduced indicators of T cytotoxic lymphocytes (23 people), normal (36 patients) and elevated – 6 patients. In the group with elevated T cytotoxic lymphocytes (group 1), all patients had AMI.

AMI was diagnosed among patients with normal T cytotoxic lymphocytes (group 2) in 58%, and with reduced T cytotoxic lymphocytes (group 3) – in 47%. The rest of the individuals had unstable angina. The maximum number of stents was implanted in patients of group 1; they also had the highest risk of Grace and the longest duration of hospitalization. At the same time, stent thrombosis and mortality were maximal in patients of group 2 – with normal T cytotoxic lymphocytes.

As for the general clinical blood test, it should be noted that there were no significant differences in the number of leukocytes, but there was a tendency to increase them in group 2. Red blood cells were significantly higher in group 1 compared to group 2 ($p < 0.05$) and group 3 ($p < 0.001$). Erythrocytes were also quantitatively larger in group 2 individuals compared to group 3. The concentration of hemoglobin is maximal in individuals of group 1 in comparison with group 2 ($p < 0.05$) and group 3 ($p < 0.01$). Statistically significant differences in hematocrit were observed in groups 1 and 3 ($p < 0.01$): higher in group 1. The average concentration of corpuscular hemoglobin was higher in patients with reduced T cytotoxic lymphocytes compared to their normal level ($p < 0.05$). In patients with normal T cytotoxic lymphocytes, the percentage of segmented neutrophils was higher than in group 1 ($p < 0.01$). The absolute number of lymphocytes was observed more in group 1 compared with groups 2 and 3 ($p < 0.001$), and in patients with normal T cytotoxic lymphocytes compared with their reduced level ($p < 0.001$). The relative number of lymphocytes was higher in patients with elevated T cytotoxic lymphocytes compared with normal and reduced T cytotoxic lymphocytes ($p < 0.001$). The relative number of segmented neutrophils was recorded higher in group 3 compared to group 2 ($p < 0.05$) and group 1 ($p < 0.001$).

The width of the distribution of red blood cells was higher in group 3 compared to group 2.

NBT spontaneous activity and index were significantly higher ($p < 0.05$) in patients with normal T cytotoxic cell content compared with reduced. The NBT-induced index was maximal in group 1 and significantly differed ($p < 0.05$) from that in patients of groups 2 and 3. The relative number (%) of T lymphocytes ($CD45^+CD3^+CD19^-$) was maximal in group 2 and significantly ($p < 0.05$) differed from the same indicator in groups 1 and 3.

The absolute number of T lymphocytes ($CD45^+CD3^+CD19^-$) was significantly higher in group 1 compared to group 2 ($p < 0.01$) and group 3 ($p < 0.001$). Also, this parameter was higher in individuals with normal T cytotoxic lymphocytes in comparison with the group with a reduced level of T cytotoxic lymphocytes ($p < 0.001$).

As for the absolute values of T helpers, it should be noted that they were minimal in group 3 ($p < 0.001$) in comparison with 2 and 3 groups. The immunoregulatory index (CD4/CD8) was maximal in group 3 and significantly differed from that of group 1 ($p < 0.05$) and group 2 ($p < 0.01$).

T-NK lymphocytes relative values were significantly higher ($p < 0.05$) in individuals with elevated T cytotoxic lymphocytes in comparison with this parameter in patients of groups 2 and 3. The absolute number of T-NK lymphocytes was higher in group 1 compared to groups 2 and 3 ($p < 0.001$). In patients of group 2, this indicator exceeded that of group 3 ($p < 0.01$).

The absolute number of B lymphocytes ($CD45^+CD3^+CD19^+$) was minimal in group 3 and significantly differed from this parameter in group 1 ($p < 0.001$) and group 2 ($p < 0.01$).

T lymphocytes of early activation (absolute number) were maximal in group 1 and had a statistical difference from 3 groups ($p < 0.001$). At the same time, this indicator was higher in group 2 than in group 3 ($p < 0.01$).

T lymphocytes of late activation were higher in individuals with elevated T cytotoxic lymphocytes compared to group 2 ($p < 0.001$) and group 3 ($p < 0.0001$). In the group with normal T cytotoxic lymphocytes, this parameter was recorded higher than in group 3 ($p < 0.01$).

The content of C5a and C3a complement fragments was higher in group 3 patients compared with that of group 2 individuals ($p < 0.05$).

Total B1 lymphocytes (absolute number) prevailed in group 1 patients and significantly ($p < 0.001$) differed from group 3 patients. In individuals with

normal cytotoxic T cells, this indicator was higher ($p < 0.01$) than in patients with reduced. B2 lymphocytes (absolute values) were significantly increased ($p < 0.01$) in patients of group 1 relative to group 3. B2 lymphocytes were higher in group 2 individuals compared to group 3 ($p < 0.05$).

T regulatory cells (absolute values) were the largest in group 2 patients and significantly larger ($p < 0.05$) than in group 3 individuals (minimum values in this group). T regulatory cells of late activation (absolute values) were minimal in the group with reduced T cytotoxic lymphocytes and significantly differed ($p < 0.05$) from those in the group with normal T cytotoxic lymphocytes.

According to a study by Lebedeva O.K. et al. [5], lower T and NK cells were recorded in patients with acute myocardial infarction.

Other authors [11] note that 51.5% of ACS patients with implanted stents had higher helper T lymphocytes with $CD3^+CD4^+$ phenotype and 45.4% had T cytotoxic lymphocytes with $CD3^+CD8^+$ phenotype. This indicates immune activation. 63.6% of patients had an increase in NK lymphocytes, which could be explained by an increase in the activity of antitransplantation immunity. In our patients, high NK cells were observed in the group with an increased content of T cytotoxic lymphocytes.

According to Liu Y. et al. [7], a pathological autoreactive immune response is responsible for plaque rupture and the subsequent onset of acute coronary syndrome (ACS). Naturally occurring regulatory T cells ($CD45^+CD3^+CD4^+CD25^+CD127$-) are necessary to suppress the pathological autoreactive immune response and maintain immune homeostasis. In patients with ACS, a decrease in the number and suppressive function of T regulatory cells was revealed. This is also observed in our patients who underwent COVID-19 and had a reduced level of T cytotoxic cells. According to research, Tian X. et al. [12], in patients with ACS, compared with patients with stable angina pectoris and the control group, a violation of the formation of T regulatory cells is closely associated with hyperreactivity of the sympathetic system.

In Gang H. et al. [3], it was shown that T cytotoxic lymphocytes from patients with AMI showed increased cytotoxicity compared to the control group, which was manifested by increased cytolytic activity against target cells, increased secretion of IFNγ and TNFα. Dysregulation of cytotoxic T lymphocytes in patients with ACS and COVID-19 was noted by Shafeghat M. et al. [9].

In this research analysis, Zidar D.A. et al. [13] it was shown that naive T cytotoxic lymphocytes of patients with ACS demonstrate phenotypic and functional characteristics of immune depletion: impaired IL-2 production and activation of programmed cell death-1. Exposure to oxidized low-density lipoproteins repeats these features *in vitro*. These data suggest that oxidized low-density lipoproteins may play a role in immune depletion, and this immunophenotype may be a biomarker of ACS. It is no coincidence that the most clinically severe patients (all with acute myocardial infarction) were in the group with an increased content of T cytotoxic lymphocytes. In many ways, the studies of these authors confirm the data we have obtained.

Conclusions

1. People with acute coronary syndrome who had COVID-19 mainly with normal and elevated levels of cytotoxic T cells had a more severe course of the disease: patients with acute myocardial infarction prevailed, they had more mortality, duration of treatment, stent thrombosis was more common.
2. In individuals with ACS and COVID-19 with elevated cytotoxic T cells, there was a maximum increase in erythrocytes, hemoglobin, hematocrit, lymphocytes of both the total number and subpopulations – T helper cells, T-NK lymphocytes, NK lymphocytes, T lymphocytes of early and late activation, B1 and B2 lymphocytes, NBT-induced test index.
3. In patients with normal levels of NK cells, compared with other groups, there was an increase in spontaneous activity and index of NBT, a significant decrease in C3a and C5a complement fragments.
4. The prevalence of stent thrombosis and mortality in the group of patients with normal levels of cytotoxic T cells may indicate torpidity of the immune system in these patients with adverse outcomes.

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ПОСТКОВИДНЫЙ СИНДРОМ ИММУНОПАТОЛОГИИ. ХАРАКТЕРИСТИКА ФЕНОТИПИЧЕСКИХ ИЗМЕНЕНИЙ ИММУННОЙ СИСТЕМЫ У ПОСТКОВИДНЫХ ПАЦИЕНТОВ

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Резюме. В данном исследовании рассматриваются долгосрочные последствия инфекции SARS-CoV-2 в отношении иммунного статуса. Учитывая длительную и глубокую иммунную дисрегуляцию, наблюдаемую во время острой инфекции SARS-CoV-2, необходимо определить, переходят ли эти изменения в последующую дисфункцию иммунной системы у выздоравливающих людей. В связи с этим целью исследования явилось изучение параметров иммунной системы у пациентов, перенесших SARS-CoV-2-инфекцию.

Было проведено обследование 150 пациентов, перенесших SARS-CoV-2 инфекцию по 96 параметрам методом проточной цитометрии. Общий анализ крови проводился на приборе Medonic (Швеция); методом иммуноферментного анализа определены уровни общих и специфических IgM, IgG, IgA, фрагменты комплимента (АО «Вектор-Бест», Россия); согласно общепринятой методике исследована активность фагоцитов.

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В ходе исследования установлено, что у пациентов выявляется минимум 4 фенотипа нарушений иммунной системы. Первые два фенотипа относятся к нарушению врожденных факторов иммунной системы и связаны со снижением количества $CD46^+$ и NK-клеток. Отмечено, что снижение $CD46^+$ сохраняется у значительного числа переболевших пациентов на протяжении длительного времени, что подчеркивается нарушениями экспрессии этого маркера на различных субпопуляциях лимфоцитов. Снижение уровня натуральных киллеров сопровождалось компенсаторным повышением количества Т-лимфоцитов, преимущественно за счет Т-хелперов и TNK-лимфоцитов и роста общих В-клеток памяти. Два других выявленных фенотипа характеризуются повреждением факторов приобретенного иммунного ответа и связаны с повреждением В-клеток и Т-цитотоксических клеток. Показана связь таких нарушений с повреждением эритроцитарного и тромбоцитарного ростков кроветворения, способствующих появлению гипоксии и возможному нарушению системы свертывания крови.

Таким образом, полученные результаты свидетельствуют о длительном выраженном повреждении иммунной системы постковидных пациентов, требующих иммунокоррекции данных нарушений. При этом нарушения врожденных и приобретенных параметров иммунной системы (4 новых фенотипа постковидной иммунопатологии) сопровождаются повреждениями эритроидного и тромбоцитарного ростков кроветворения. Данные изменения могут способствовать формированию как нарушений, связанных с гипоксэмическими процессами, так и способствовать тромбообразованию у постковидных пациентов. Длительное сохранение таких повреждений всех ростков кроветворения может приводить к нарушениям и других систем организма (сердечно-сосудистой, нервной и эндокринной), что требует дополнительных исследований в этих направлениях.

Ключевые слова: SARS-CoV-2, постковидные пациенты, CD-типирование, иммунная система, иммуноглобулины, комплемент, фагоциты

POST-COVID IMMUNOPATOLOGY SYNDROME: CHARACTERISTICS OF PHENOTYPICAL CHANGES IN THE IMMUNE SYSTEM IN POST-COVID PATIENTS

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Abstract. This study examines the long-term effects of SARS-CoV-2 infection on immune status. Given the prolonged and profound immune dysregulation observed during acute SARS-CoV-2 infection, it remains to be determined whether these changes translate into subsequent immune system dysfunction in recovering individuals. In this sense, the aim of the study was to study the parameters of the immune system in patients who had undergone SARS-CoV-2 infection.

150 patients who underwent SARS-CoV-2 infection were examined according to 96 parameters using flow cytometry. A complete blood count was performed using a Medonic device (Sweden); ELISA method determined the levels of general and specific IgM, IgG, IgA, compliment fragments (JSC Vector-Best, Russia). The activity of the phagocytes was studied according to the generally accepted method.

The study found that at least four phenotypes of immune system disorders are detected in patients. The first two phenotypes are related to the impairment of innate immune system factors and are associated with a

decrease in the number of $CD46^+$ and NK cells. It has been observed that a decrease in $CD46^+$ persists for a long time in a significant number of recovered patients, highlighted by the impaired expression of this marker in various subpopulations of lymphocytes. The decrease in the level of natural killers was accompanied by a compensatory increase in the number of T lymphocytes, mainly due to T helpers and TNK lymphocytes, and the growth of total memory B cells. Two other identified phenotypes are characterized by damage to acquired immune response factors and are associated with damage to B cells and T cytotoxic cells. The relationship of such disorders with damage to hematopoiesis erythrocyte and platelet sprouts, which contribute to the appearance of hypoxia and possible violation of the blood coagulation system, has been shown.

Therefore, the results obtained indicate a long-term pronounced damage to the immune system in post-COVID patients that requires immunocorrection of these disorders.

Keywords: SARS-CoV-2, post-COVID patients, CD typing, immune system, immunoglobulins, complement, phagocytes

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Introduction

Recent studies have significantly improved our understanding of SARS-CoV-2 infection by highlighting profound and disease-specific changes in the innate and adaptive immune compartments [2, 3, 4]. Lymphopenia and altered lymphocyte function in COVID-19 patients are correlated with disease severity, indicating the key role of T and B cells in pathology [2, 3, 4].

Given the profound and long-term immune dysregulation observed during acute SARS-CoV-2 infection, it is necessary to determine whether these changes translate into long-term immune changes and subsequent dysfunction in recovering individuals. However, the long-term consequences of SARS-CoV-2 infection are still poorly understood. The literature suggests significant and long-term changes in T cell populations and key events associated with the pathogenesis of COVID-19 in affected patients [6]. Furthermore, specific neutralizing antibodies against SARS-CoV-2 and T cell responses have been found to persist even 12 months after initial infection, according to the literature [7]. Some patients may also experience a variety of mental and somatic symptoms, including chronic fatigue, myalgia, altered memory, and emotional state, as well as signs of fibrotic lesions of the lungs and diseases of the pulmonary vessels [8].

Therefore, this study aims to investigate the parameters of the immune system in patients who have undergone SARS-CoV-2 infection.

Materials and methods

A survey was carried out in 150 patients 6-12 months after being diagnosed with COVID-19 in Chelyabinsk, Russian Federation, on 1 May 2021 to 30 December 2022. The sample size was determined based on previous work [1, 5, 12]. The inclusion criteria for the study groups were a confirmed diagnosis of SARS-CoV-2 infection by polymerase chain reaction (PCR), the presence of IgM, IgG, and IgA in the SARS-CoV-2 virus, and data from the computed tomography from previous pneumonia. The study was carried out no earlier than 6 months after pneumonia caused by SARS-CoV-2 infection.

All patients were previously examined by a general practitioner and an immunologist-allergist to identify concomitant diseases. The groups were randomly assigned by sex, age, and comorbidities according to the χ^2 test. Depending on the examination, the patients were divided into groups with altered immune system parameters and normal immune parameters. The data presented in the monograph by Zurochka et al. (2018) [9] were used. All studies were approved by the Independent Local Ethics Committee at the City Clinical Hospital No. 1, Chelyabinsk, Protocol No. 8 dated April 11, 2022.

Flow cytometry was used to determine $CD45^+$ and $CD46^+$ (panleukocyte markers for gating lymphocytes), $CD45^+$ and $CD46^+$, $CD3^+$ (T lymphocytes), $CD45^+$ and $CD46^+$, $CD3^+$, $CD4^+$ (helper inducers), $CD45^+$ and $CD46^+$, $CD3^+$, $CD8^+$ (cytotoxic T lymphocytes), $CD45^+$ and $CD46^+$, $CD3^+$, $CD56^+$ (TNK cells), $CD45^+$ and $CD46^+$, $CD3^-$, $CD56^+$ (natural killers), $CD45^+$ and $CD46^+$, $CD3^-$, $CD19^+$ (B lymphocytes), $CD45^+$ and $CD46^+$, $CD3^+$, $CD4^+$, $CD25^+$ (activated helpers, early activation of lymphocytes), $CD45^+$ and $CD46^+$, $CD3^+$, HLA-DR (activated T lymphocytes, late activation of lymphocytes). A general blood test was also carried out on Medonic Device (Sweden), and the levels of general and specific IgM, IgG, IgA, complement fragments ("Vector-Best", Russia) were determined by the enzyme immunoassay method. The activity of phagocytes was studied according to the generally accepted method.

Results and discussion

The present study selected patients with a violation of natural killers among those who underwent COVID-19, as described in published works [9, 10]. Among the 150 patients examined, 50.9% had a

markedly reduced level of natural killers when gated with the CD45 panleukocyte marker, and an even lower level of natural killers when gated with the CD46 panleukocyte marker. Additionally, an increase in T lymphocytes, T helper cells and TNK lymphocytes (likely compensatory), as well as memory B cells, was observed against the background of disturbances in the natural level of killers, accompanied by a decrease in the level of total IgM. Platelet levels, thrombocrit, and erythroidhemotopoietic germs also showed decreased levels of blood cortisol. These data indicate that patients with post-COVID syndrome exhibit a phenotype associated with impaired innate immunity systems.

Furthermore, the study found a pronounced decrease in $CD46^+$ expression (the receptor for the complement fragment) in T lymphocytes in 57.9% of patients with post-COVID syndrome. This indicates the involvement of CD46 in the immunopathogenesis of the disease. According to the literature, CD46 controls at least three key metabolic events, including the translocation of the γ-secretase-treated intracellular domain of CYT-1 CD46 to the nucleus, where it induces the expression of carrier proteins (GLUT1, LAT1 and CAT1) and the assembly of mTORC1. Activation of CD46 also induces increased expression of metabolic enzymes, such as fatty acid synthases and GAPD, as well as activation of intracellular C5 pools with intracellularly generated C5a, which stimulates mitochondrial C5aR1 and drives ROS production and activation of the NLRP3 inflammasome in $CD4^+$T cells.

These changes contribute to increased glycolysis and OXPHOS and ROS production, which are necessary for induction of IFNγ production and granzyme B expression, and ultimately lead to the implementation of protective effector responses of Th1 and T killers [11]. However, more research is needed to determine whether the observed changes are caused by the direct interaction of the virus and CD46. In particular, the identified complex of changes persists for a long time in a significant number of recovered patients, as highlighted by the impaired expression of this marker in various subpopulations of lymphocytes.

Additionally, the study detected two types of phenotypic changes in the immune status of patients with post-COVID syndrome that are related to acquired immune defense mechanisms. One of the most common disorders in patients with acute COVID-19 is the violation of the formation of cytotoxic T cells. This disorder was detected in 29.9% of patients with post-COVID syndrome, consistent with data from the literature [13]. In addition to a decrease in cytotoxic lymphocyte levels, these patients also showed lower levels of total T lymphocytes, TNK lymphocytes, and late activated T lymphocytes bearing HLA-DR.

The study found that in patients with COVID-19 with impaired natural killer cell function, the majority of patients had reduced levels of natural killer cells. This was accompanied by an increase in T lymphocytes, particularly T helpers and TNK lymphocytes, as well as an increase in memory B cells, but a decrease in total IgM. Platelet and erythroidhemotopoietic germ levels were also reduced, and blood cortisol levels were lower. These changes suggest that patients with post-COVID syndrome have an immune phenotype associated with impaired innate immune systems.

Furthermore, the study found that almost all changes in immune system parameters were found in the acquired immune system and did not depend on the CD45 or CD46 gated immune system. However, a subset of patients (17%) had a special type of immune system disorder characterized by a disorganized switch of B lymphocytes from IgM to IgG and IgA synthesis, leading to a marked decrease in the subpopulations of B2 lymphocytes. These patients also had a decrease in hemoglobin and platelet parameters, which may contribute to hypoxia and blood coagulation problems. This phenotype of immune system disorders is difficult to determine and requires non-standard approaches to assess immune status.

Conclusion

In general, the study identified four phenotypic immune system disorders associated with damage to $CD46^+$, NK cells as innate immunity factors, and B lymphocytes and cytotoxic cells as acquired defense factors in COVID-19 survivors. These findings suggest pronounced long-term immune system damage and the need for immunocorrection in patients with post-COVID syndrome.

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ОСОБЕННОСТИ Т-КЛЕТОЧНОГО ЗВЕНА ИММУНИТЕТА И УРОВЕНЬ НАТУРАЛЬНЫХ КИЛЛЕРОВ У БОЛЬНЫХ, ПЕРЕНЕСШИХ COVID-19 С НАРУШЕНИЯМИ УГЛЕВОДНОГО ОБМЕНА

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Резюме. Пандемия новой коронавирусной инфекции COVID-19 создала чрезвычайную ситуацию в области общественного здравоохранения в РФ в 2020-2022 годах. Последствия COVID-19 многообразны и часто проявляются дисфункцией органов эндокринной системы. SARS-CoV-2 оказывает прямое цитотоксическое и опосредованное повреждающее действие на островки поджелудочной железы, что приводит к развитию гипергликемии. Установлено, что гипергликемия ассоциируется с провоспалительным уровнем иммунного статуса, увеличением количества циркулирующих маркеров воспаления, что приводит к изменениям врожденного и адаптивного иммунитета. Среди первых, кто реагирует на вирусные инфекции, клетки-натуральные киллеры (NK) обладают огромным терапевтическим потенциалом, образуя мост между врожденными и адаптивными реакциями. В целом Т-клеточный ответ в когорте long-COVID претерпевает как фенотипические, так и функциональные изменения. Актуальностью исследования являются недостаточные данные о Т- и NK-клеточном иммунитете у больных с гипергликемией после перенесенного COVID-19. Целью данного исследования явилось выявление особенностей Т-клеточного иммунитета у лиц с постковидным синдромом и нарушениями углеводного обмена, в зависимости от числа NK-клеток.

Пациенты с нарушениями углеводного обмена (НУО) в постковидном периоде (всего 64 человека) в зависимости от числа NK-клеток разделены на три группы: со сниженными показателями NK-клеток, нормальными и повышенными. НУО включали нарушенную толерантность к глюкозе (n = 36) и сахарный диабет 2 типа (n = 28). Группу сравнения составили лица в постковидном периоде без НУО в анамнезе (всего 60 человек). Оценка лимфоцитарного звена иммунитета включала определение: $CD45^+CD3^+$ (Т-лимфоциты), $CD45^+CD3^+CD4^+$ (хелперы индукторы), $CD45^+CD3^+CD8^+$ (цитотоксические Т-лимфоциты), $CD45^+CD3^+CD16^+CD56^+$ (Т-NK-клетки), $CD45^+CD3^-CD16^+CD56^+$ (натуральные киллеры), $CD45^+CD3^+CD4^+CD25^+$ (Т-лимфоциты – ранняя активация), $CD45^+CD3^+HLA\text{-}DR^+$ (Т-лимфоциты – поздняя активация).

Снижение количества натуральных киллеров сопровождалось более высоким уровнем Т-хелперов в группе с нарушениями углеводного обмена, последнее, вероятно, связано с компен-

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саторным повышением Т-лимфоцитов и нарушением регуляции Т-клеточного звена иммунной системы. Также можно сделать вывод, что у пациентов с long-COVID и НУО при нормальных показателях NK-клеток сохраняется измененный субпопуляционный состав, а именно, значимое повышение общих Т-лимфоцитов. Полученные данные о значимом снижении Т-NK-лимфоцитов многие авторы связывают со снижением противовирусной активности иммунной системы, что может приводить к некачественному ответу на новые вирусные агенты или способствовать активации хронических вирусных инфекций. Повышение значений Т-лимфоцитов ранней активации, вероятно, связано с каскадом реакций, направленных на активацию транскрипционных факторов NFAT, NF-κB и AP-1, которые отвечают за регуляцию продукции множества генов (в том числе гена IL-2), контролирующих пролиферацию и дифференцировку активированных лимфоцитов. Нарушения регуляции Т-клеточного звена у лиц с long-COVID и нарушениями углеводного обмена требует более детального изучения, в том числе с оценкой цитокинового профиля у данной категории пациентов.

Ключевые слова: Т-лимфоциты, натуральные киллеры, постковидный синдром, нарушенная толерантность к глюкозе, сахарный диабет 2 типа, иммунная дизрегуляция

CHARACTERISTICS OF T CELL IMMUNITY AND LEVEL OF NATURAL KILLER CELL CONTENT IN COVID-19 CONVALESCENTS WITH CARBOHYDRATE METABOLISM DISORDERS

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Abstract. The pandemic of the new COVID-19 coronavirus infection has created a public health emergency in the Russian Federation in 2020-2022. COVID-19 causes various consequences, often manifested by the endocrine system dysfunction. The rationale for our study is insufficient data on T and NK cell immunity in patients with hyperglycemia after COVID-19. The study was aimed at the features of T cell immunity in individuals with post-COVID syndrome and disorders of carbohydrate metabolism, depending on the NK cells count. Materials and methods: Sixty-four post-COVID patients with carbohydrate metabolism disorders were divided into three groups: with reduced, normal, or elevated NK cell counts. Carbohydrate metabolism disorders included impaired glucose tolerance (n = 36) and type 2 diabetes mellitus (n = 28). The comparison group comprised 60 post-COVID persons with no history of carbohydrate metabolism disorders. The assessment of the lymphocytic link of immunity included the definition of: $CD45^+CD3^+$ (T lymphocytes), $CD45^+CD3^+CD4^+$ (T helpers), $CD45^+CD3^+CD8^+$ (T cytotoxic), $CD45^+CD3^+CD16^+CD56^+$ (T-NK cells), $CD45^+CD3^-CD16^+CD56^+$ (natural killers), $CD45^+CD3^+CD4^+CD25^+$ (T lymphocytes – early activation), $CD45^+CD3^+$HLA-DR^+ (T lymphocytes – late activation). Results and Discussion. As the study showed, a decrease in the number of natural killers was accompanied by a higher level of T helpers in the group with carbohydrate metabolism disorders, the latter is probably associated with a compensatory increase in T lymphocytes and dysregulation of the T cell link of the immune system. It can also be concluded that in patients with long-COVID and CMD, with normal NK cell counts, an altered subpopulation composition remains, namely, a significant increase in total T lymphocytes. Many authors associate the obtained data on a significant decrease in T-NK lymphocytes with a decrease in the antiviral activity of the immune system, which can lead to a poor response to new viral agents or contribute to the activation of chronic viral infections. Dysregulation of the T cell link in individuals with long-COVID and disorders of carbohydrate metabolism requires a more detailed study, including an assessment of the cytokine profile in this category of patients.

Keywords: T lymphocytes, natural killers, long-COVID, impaired glucose tolerance, diabetes mellitus type 2, immune dysregulation

Introduction

The pandemic of the new COVID-19 coronavirus infection has created a public health emergency in the Russian Federation in 2020-2022. The wave-like spread of COVID-19 has led to high morbidity in all regions of the Russian Federation. COVID-19 causes various consequences, often manifested by the endocrine system dysfunction. SARS-CoV-2 has a direct cytotoxic and indirect damaging effect on the pancreatic islets, which leads to hyperglycemia [3]. Prediabetes and diabetes mellitus (DM) are often diagnosed in patients infected with SARS-CoV-2, who had no history of carbohydrate metabolism disorders (CMD) or of glucocorticoid treatment. Hyperglycemia has been found to be associated with an increase in proinflammatory immune status and in the circulating markers of inflammation, leading to changes in innate and adaptive immunity.

Among the first to respond to viral infections, natural killer cells (NK) have enormous therapeutic potential, bridging the innate and adaptive immune responses [5]. M. Galan et al. showed a 1.7-fold ($p = 0.032$) increase in the NK cells population expressing the CD16 marker on the surface ($CD3^-CD56^+CD16^+$) in the long-COVID group [2]. M. Dobrynina et al. showed a sharp three-fold decrease in the NK cells count in over a third of the patients with post-covid syndrome of immunopathology. This decrease was accompanied by a higher relative level of T lymphocytes and T helper cells [1]. The T cell response in the long-COVID cohort undergoes both phenotypic and functional changes. The rationale for our study is insufficient data on T and NK cell immunity in patients with hyperglycemia after COVID-19. The study was **aimed** at the features of T cell immunity in individuals with post-COVID syndrome and disorders of carbohydrate metabolism, depending on the NK cells count.

Materials and methods

Sixty-four post-COVID patients with carbohydrate metabolism disorders were divided into three groups: with reduced, normal, or elevated NK cell counts. Carbohydrate metabolism disorders included impaired glucose tolerance ($n = 36$) and type 2 diabetes mellitus ($n = 28$). The comparison group comprised 60 post-COVID persons with no history of carbohydrate metabolism disorders. Diagnosis of diabetes mellitus met the criteria of Algorithms of specialized medical care for patients with diabetes mellitus (2021).

The post-COVID syndrome was diagnosed based on polymerase chain reaction confirmed SARS-CoV-2 infection, detection of IgA, IgM, or IgG to the SARS-CoV-2 virus, and computed tomography-confirmed pneumonia. The study was conducted at least 6 months after the pneumonia caused by SARS-CoV-2 infection. Before the inclusion, a general practitioner and an endocrinologist examined each patient to identify concomitant diseases. Lymphocyte immunological indices were studied by flow cytometry on the Navios cytofluorimeter (Beckman Coulter, USA) using a standardized technology: $CD45^+CD3^+$ (T lymphocytes), $CD45^+CD3^+CD4^+$ (T helpers), $CD45^+CD3^+CD8^+$ (T cytotoxic), $CD45^+CD3^+CD16^+CD56^+$ (T-NK cells) $CD45^+CD3^-CD16^+CD56^+$ (natural killers), $CD45^+CD3^+CD4^+CD25^+$ (T lymphocytes – early activation), $CD45^+CD3^+$HLA-DR^+ (T lymphocytes – late activation).

The study was approved by the Independent Local Ethics Committee at the Autonomous Healthcare Institution Order of the Red Banner of Labor City Clinical Hospital No. 1, Chelyabinsk, Record No. 8 of April 11, 2022, the base of the studies. IBM SPSS Statistics, Version 19 software was used for statistical data processing. Correlation analysis within the groups was performed by calculating Spearman's rank correlation. The differences in the distribution of values between the groups were calculated using the Mann–Whitney U test. The differences between the groups were considered significant at $p < 0.05$.

Results and discussion

As the study showed, a decrease in the number of natural killer cells was accompanied by a higher level of T helper cells in the group with carbohydrate metabolism disorders; the latter is probably associated with a compensatory increase in T lymphocytes and dysregulation of the T cell link of the immune system (Table 1). It can also be concluded that in patients with long-COVID and CMD, with normal NK cell counts, an altered subpopulation composition remains, namely, a significant increase in total T lymphocytes.

Many authors associate the obtained data on a significant decrease in T-NK lymphocytes with a decrease in the antiviral activity of the immune system, which can lead to a poor response to new viral agents or contribute to the activation of chronic viral infections [6]. An increase in the values of early activation T lymphocytes is probably associated with a cascade of reactions aimed at activating the

TABLE 1. COMPARISON OF T CELL IMMUNITY INDICES IN PATIENTS WITH POST-COVID SYNDROME AND DISORDERS OF CARBOHYDRATE METABOLISM (CMD), DEPENDING ON NK CELL COUNT

Measures	Group of patients with post-COVID syndrome and CMD (n = 64)			p
NK cells	1 reduced (n = 16)	2 normal (n = 30)	3 elevated (n = 18)	
NK cells ($CD45^+CD3^-CD16^+CD56^+$), 10^6 cells/L	86.130±7.586	246.110±14.466	546.440±25.089	
T lymphocytes ($CD45^+CD3^+CD19^-$), %	79.688±2.735	74.716±1.427	62.000±1.909	$p_{2,5} = 0.029$
T lymphocytes ($CD45^+CD3^+CD19^-$), 10^6 cells/L	1668.500±198.340	1781.630±145.475	1625.890±150.595	$p_{2,5} = 0.041$
T helpers ($CD45^+CD3^+CD4^+$), %	53.330±2.751	49.730±2.353	41.590±2.424	$p_{1,4} = 0.019$
T helpers ($CD45^+CD3^+CD4^+$), 10^6 cells/L	1121.380±149.424	1089.680±93.715	1108.110±141.759	$p_{1,4} = 0.006$
T cytotoxic ($CD45^+CD3^+CD8^+$), %	24.263±2.295	25.484±2.658	20.000±1.432	
T cytotoxic ($CD45^+CD3^+CD8^+$), 10^6 cells/L	496.380±52.527	621.160±98.659	522.330±57.936	
Immunoregulatory index (Tx/Tc)	2.400±0.339	2.416±0.336	2.200±0.249	
T-NK lymphocytes ($CD45^+CD3^+CD16^+CD56^+$), %	2.233±0.643	5.626±0.649	5.911±1.281	$p_{1,4} = 0.010$
T-NK lymphocytes ($CD45^+CD3^+CD16^+CD56^+$), 10^6 cells/L	54.140±14.647	126.110±15.616	149.670±30.769	$p_{1,4} = 0.048$
T lymphocytes $CD45^+CD3^+CD4^+CD25^+$ (early activation), %	7.367±0.916	5.642±0.682	7.000±0.991	$p_{1,4} = 0.026$
T lymphocytes $CD45^+CD3^+CD4^+CD25^+$ (early activation), 10^6 cells/L	79.290±14.615	58.210±7.556	79.110±15.478 norm	$p_{1,4} = 0.010$
T lymphocytes $CD45^+CD3^+CD4^+HLA\text{-}DR^+$ (late activation), %	3.350±0.563	4.974±0.839	7.567±2.213	
T lymphocytes $CD45^+CD3^+CD4^+HLA\text{-}DR^+$ (late activation), 10^6 cells/L	67.250±31.099	51.680±9.564	80.560±20.843	

Таблица 1 (окончание)
Table 1 (continued)

Measures	Group of patients with post-COVID syndrome without CMD (n = 60)			p
NK cells	4 reduced (n = 14)	5 normal (n = 38)	6 elevated (n = 8)	
NK cells ($CD45^+CD3^-CD16^+CD56^+$), 10^6 cells/L	62.290±10.167	229.110±14.561	657.75±189.051	
T lymphocytes ($CD45^+CD3^+CD19^-$), %	79.414±2.608	70.961±1.125	57.975±6.143	$p_{2,5} = 0.029$
T lymphocytes ($CD45^+CD3^+CD19^-$), 10^6 cells/L	1427.140±294.744	1342.440±77.867	1636.000±247.748	$p_{2,5} = 0.041$
T helpers ($CD45^+CD3^+CD4^+$), %	40.800±3.327	48.560±1.501	34.050±2.282	$p_{1,4} = 0.019$
T helpers ($CD45^+CD3^+CD4^+$), 10^6 cells/L	548.600±58.391	1018.840±83.168	966.500±146.301	$p_{1,4} = 0.006$
T cytotoxic ($CD45^+CD3^+CD8^+$), %	28.871±5.271	22.826±1.638	22.9500±5.8487	
T cytotoxic ($CD45^+CD3^+CD8^+$), 10^6 cells/L	467.290±72.987	480.740±55.944	634.250±149.956	
Immunoregulatory index (Tx/Tc)	2.300±0.731	2.384±0.229	1.700±0.274	
T-NK lymphocytes ($CD45^+CD3^+CD16^+CD56^+$), %	8.350±1.601	6.653±0.942	5.450±1.648	$p_{1,4} = 0.010$
T-NK lymphocytes ($CD45^+CD3^+CD16^+CD56^+$), 10^6 cells/L	116.400±22.997	145.580±35.064	142.000±32.432	$p_{1,4} = 0.048$
T lymphocytes $CD45^+CD3^+CD4^+CD25^+$ (early activation), %	4.033±0.457	6.053±0.571	8.775±2.051	$p_{1,4} = 0.026$
T lymphocytes $CD45^+CD3^+CD4^+CD25^+$ (early activation), 10^6 cells/L	28.200±2.518	61.420±8.103	88.250±32.082	$p_{1,4} = 0.010$
T lymphocytes $CD45^+CD3^+CD4^+HLA\text{-}DR^+$ (late activation), %	4.700±1.549	3.705±0.474	6.850±1.687	
T lymphocytes $CD45^+CD3^+CD4^+HLA\text{-}DR^+$ (late activation), 10^6 cells/L	33.860±8.860	39.370±6.393	68.250±25.477	

transcription factors NFAT, NF-κB and AP-1, which are responsible for regulating the production of many genes (including the IL-2 gene) that control proliferation and differentiation of activated lymphocytes [4]. Dysregulation of the T cell link in individuals with long-COVID and disorders of carbohydrate metabolism requires a more detailed study, including an assessment of the cytokine profile in this category of patients.

Conclusions

1. Decreased and normal values of NK cells are accompanied by a change in the subpopulation composition of T lymphocytes in the group of people with long-COVID and concomitant pathologies, both in impaired glucose tolerance and in type 2 diabetes mellitus.

2. In individuals with impaired carbohydrate metabolism in the post-COVID period, a decrease in the level of NK cells is accompanied by a significantly higher level of T helper cells.

3. With normal NK cells, the level of total T lymphocytes is significantly higher with concomitant disorders of carbohydrate metabolism in the long-COVID group.

4. In the group of people with post-COVID syndrome and disorders of carbohydrate metabolism, a significant increase in T lymphocytes of early activation was revealed.

The obtained data showed that in the post-COVID period, in the group of patients with disorders of carbohydrate metabolism, dysregulation of the immune system is observed, which in the future can lead to an inadequate immune response, which requires a more detailed study, including an assessment of the cytokine profile.

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ПРОВОСПАЛИТЕЛЬНЫЕ ЦИТОКИНЫ VEGFA, IL-6, IL-8 КАК МАРКЕРЫ ГЕПАТОТОКСИЧНОСТИ ПОСЛЕ COVID-19

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Резюме. Механизм гепатоцелюллярного повреждения печени после COVID-19 – многофакторный процесс. В литературе наиболее обсуждаемыми причинами являются прямые цитолитические повреждения печени вследствие воспалительной реакции после COVID-19, лекарственно индуцированной гепатотоксичности и прямого цитотоксического действия вируса. Существуют наблюдения, что инфекция SARS-CoV-2 вызывает реактивацию вируса гепатита В, однако взаимодействие между вирусом гепатита С и SARS-CoV-2 описано мало. Течение коронавирусной инфекции связано с выраженной экспрессией провоспалительных цитокинов – участников мультисистемного воспалительного ответа – IL-1β, IL-6, IL-8, IL-18, MCP-1, TNFα, что вносит существенный вклад в наблюдаемые ранние и поздние нарушения функции печени. Цель исследования – оценить роль провоспалительных цитокинов (VEGFA, IL-8, IL-6, MCP-1, TNFα, IL-18) как дополнительных маркеров гепатотоксичности после COVID-19. Исследование выполнено в период с марта по август 2022 года на базе поликлиики № 2 ЦК МСЧ г. Ульяновска. Пациенты были разделены на 2 группы: 1-я группа – с повышением аминотрансфераз на фоне лечения от COVID-19 и/или в последующие 3-6 месяцев после перенесенного заболевания без вирусного поражения печени (n = 42), 2-я группа – пациенты с ко-инфекцией (хроническим вирусным гепатитом С (HCV) и COVID-19 (n = 26). Методом иммуноферментного анализа оценивались уровни цитокинов – VEGF-A, IL-6, IL-8, MCP-1, IL-18, TNFα в сыворотке крови. Статистический анализ проводился с использованием программы StatTech v.3.1.4. Выявлено сопоставимое повышение уровня трансаминаз и С-реактивного белка в обоих группах, значимо отличающееся от референсных значений. Установлены прямые корреляционные связи умеренной силы (линейная корреляция по Спирмену) между следующими цитокинами: TNFα-MCP-1 (R = 0,559; p = 0,001), TNFα-VEGFA (R = 0,400; p = 0,002), TNFα-IL-6 (R = 0,503; p = 0,001). Нами установлено значимое повышение уровня VEGFA в сыворотке крови пациентов 1-й группы (гепатотоксичность после COVID-19) (Me ($Q_{0,25}$-$Q_{0,75}$): 522 (250-1002), p = 0,001) и у пациентов 2-й группы (HCV + COVID-19) (Me 1196, $Q_{0,25}$-$Q_{0,75}$: (73-432). Аналогичная тенденция наблюдается с сывороточными уровнями IL-6 и IL-8 в 1-й группе пациентов, выражено превышающими значения цитокинов у здоровых доноров и достоверно выше, чем у пациентов 2-й группы. Выявленные корреляционные

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связи между воспалительными цитокинами доказывают однонаправленность изменений в функционирований регуляторной сети, контролирующей иммунные вирус-индуцированные реакции.

Ключевые слова: гепатотоксичность, COVID-19, воспалительные маркеры, васкулоэндотелиальный фактор роста, IL-6, IL-8

PROINFLAMMATORY CYTOKINES VEGFA, IL-6, IL-8 AS MARKERS OF HEPATOTOXICITY AFTER COVID-19

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Abstract. The mechanism of hepatocellular liver damage after COVID-19 is a multifactorial process. The most widely discussed causes are cytolytic liver damage due to the inflammatory response after COVID-19, drug-induced hepatotoxicity and direct cytotoxic effect of the virus. There are observations that SARS-CoV-2 infection causes hepatitis B virus reactivation, but little has been described about the interaction between hepatitis C virus and SARS-CoV-2. The course of coronavirus infection is associated with marked expression of proinflammatory cytokines, participants in the multisystem inflammatory response, IL-1β, IL-6, IL-8, IL-18, MCP-1, TNFα, which contribute significantly to the observed early and late liver function impairment. The aim of the study was to evaluate the role of proinflammatory cytokines (VEGFA, IL-8, IL-6, MCP-1, TNFα, IL-18) as additional markers of hepatotoxicity after COVID-19. The study was performed between March and August 2022. Patients were divided into 2 groups: Group 1 – with increased aminotransferases against the background of treatment from COVID-19 and/or in the following 3-6 months after the disease without viral liver damage (n = 42), Group 2 – patients with co-infection (chronic viral hepatitis C (HCV) and COVID-19 (n = 26). The levels of cytokines – VEGF-A, IL-6, IL-8, MCP-1, IL-18, TNFα in blood serum were estimated by enzyme immunoassay method. Statistical analysis was performed using StatTech v. 3.1.4. The results of the study revealed a comparable increase in the level of transaminases and C-reactive protein in both groups, significantly different from the reference values. Direct correlations of moderate strength (linear Spearman correlation) were found between the following cytokines: TNFα-MCP-1 (R = 0.559; p = 0.001), TNFα-VEGFA (R = 0.400; p = 0.002), TNFα-IL-6 (R = 0.503; p = 0.001). We diagnosed a significant increase in serum VEGFA levels in group 1 patients (hepatotoxicity after COVID-19) (Me ($Q_{0.25}$-$Q_{0.75}$): 522 (250 to 1002), p = 0.001) and in group 2 patients (HCV + COVID-19) (Me 1196, $Q_{0.25}$-$Q_{0.75}$: (73 to 432). Similar trend with the level of IL-6, IL-8, exceeding the values of cytokines in healthy donors and significantly higher than in group 2 patients. Identified correlations between inflammatory cytokines prove unidirectional changes in the functioning of the regulatory network controlling immune virus-induced reactions.

Keywords: hepatotoxicity, COVID-19, inflammatory markers, vascular endothelial growth factor, IL-6, IL-8

Introduction

Post-COVID syndrome – a condition occurring after COVID-19, caused in particular by multisystem inflammatory reaction in the body, having a variety of pathological symptoms, and in particular hepatotoxic manifestations [9]. One of the key reasons for the development of hepatotoxicity after a new coronavirus infection is direct cytolytic liver damage due to a severe inflammatory reaction after COVID-19, drug-induced damage and direct cytotoxic effect of the virus [1, 6].

Patients with SARS-CoV-2 infection and without previous liver disease showed signs of cytolytic liver damage proportional to the severity of COVID-19. Patients with cirrhosis were at higher risk of developing severe COVID-19 and worse liver-related outcomes compared to patients without cirrhosis [3]. A series of meta-analyses on hepatotoxicity after COVID-19 have been published with preliminary conclusions

about the insufficiency and ambiguity of laboratory and clinical data considering transaminases, albumin, bilirubin levels in the evaluation of liver status in post-COVID syndrome [2, 10, 11]. The following questions remain debatable – should all patients after COVID-19 be routinely screened for chronic liver disease and should COVID-19 patients with chronic hepatitis B continue antiviral therapy started before SARS-CoV-2 infection, what are the risks of inter-drug drug interactions between CG and COVID-19.

The course of SARS-CoV-2 infection is associated with marked expression of proinflammatory cytokines – participants in a multisystem inflammatory response such as IL-1β, IL-6, IL-8, IL-18, MCP-1, TNFα, which probably contribute significantly to the early and late liver function impairments observed. In some patients with severe disease, laboratory studies indicate an unregulated inflammatory response similar to cytokine release syndrome, characterized by plasma leakage, increased vascular permeability, diffuse intravascular clotting, and immunodeficiency. High serum levels of proinflammatory cytokines, particularly interleukin-6, have been found in these patients. There may be signs of secondary hematophagocytic syndrome [4].

The question of the associated toxic effect of the SARS-CoV-2 virus on patients with chronic hepatitis remains poorly studied. Therefore, there is a need to search for new promising prognostic markers of hepatotoxicity, taking into account the existing pathogenetic mechanisms of virus-induced systemic inflammation.

The aim of the study was to investigate the role of proinflammatory cytokines (VEGFA, IL-8, IL-6, MCP-1, TNFα, IL-18) as additional markers of hepatotoxicity after COVID-19.

Materials and methods

The study was performed in the period from March to August 2022 at the Polyclinic No. 2 of the Central Clinical Medical and Sanitary Unit, named after Honored Doctor V.A. Egorov and the Research Medical and Biological Center of Ulyanovsk State University. Criteria for inclusion in the study – the age of the patient from 18 to 70 years, a confirmed case of COVID-19 infection from 3 to 12 months ago; increase in aminotransferases (AST, AST) 2-4 times higher than normal during acute and early long-term periods; having informed consent. The patients were divided into 2 groups: Group 1 – with increased aminotransferases against the background of treatment for COVID-19 and/or in the following 3-6 months after the disease without viral liver damage (n = 42, median age – 52) and Group 2 – patients with co-infection (chronic viral hepatitis (HCV) and COVID-19 (n = 26, median age – 46). Exclusion criteria – no elevation of aminotransferases, age less than 18 years. All patients were determined during the period of follow-up after COVID-19 with levels of AST, ALT, C-reactive protein. Determination of cytokines VEGFA, IL-6, IL-8, MCP-1, IL-18, TNFα in blood serum was performed by ELISA (Vector-Best, Novosibirsk, Russia). Statistical analysis was performed using StatTech v. 3.1.4 (Stattech, Russia). Quantitative indices were assessed for their correspondence to normal distribution using the Kolmogorov-Smirnov criterion. If there was no normal distribution, quantitative data were described using median (Me) and lower and upper quartiles ($Q_{0.25}$ to $Q_{0.75}$). Comparison of the two groups for a quantitative indicator whose distribution differed from normal was performed using the Mann–Whitney U criterion.

Results and discussion

When assessing the general condition of patients by questionnaire at follow-up after COVID-19, it was found that more than 74% of patients included in the study complained of shortness and asthenia, 15% had edema, and 23% had muscle and joint pain. Seventy-three percent were vaccinated against COVID-19. 63% of the patients included in the study had a mild form of the disease.We found that

TABLE 1. SERUM LEVELS OF TRANSAMINASE AND C-REACTIVE PROTEIN IN PATIENTS OF THE STUDIED GROUPS

Group	ALT (U/L)	AST(U/L)	C-reactive protein (mg/L)
1st group	64.3 (46.8-78.1)	54.5 (32.1-75.8)	11.5 (7.5-14.8)
2nd group	84.6 (33.5-130.7)	77.3 (35.5-101.6)*	8.3 (3.6-13.5)

Note. *, the differences in the indicators are statistically significantly different between the groups ($p < 0.05$).

TABLE 2. SERUM LEVELS OF PROINFLAMMATORY CYTOKINES IN PATIENTS OF THE STUDY GROUPS

Indicator	1st group (n = 42)	2nd group (n = 26)	Level of difference (Mann–Whitney, U test)
VEGFA, pg/mL	522 (250-1002)	196 (73-432)*	p = 0.001
IL-6, pg/mL	4.8 (2.3-15.7)	2.9 (1.4-7.5)*	p = 0.043
IL-8, pg/mL	106.8 (25.4-250.0)	42.5 (8.8-115.2)*	p = 0.014
MCP-1, pg/mL	190 (124-352)	151 (119-185)	p = 0.093
TNFα, pg/mL	6.7 (2.5-12.3)	4.2 (3.5-5.2)	p = 0.218
IL-18, pg/mL	393 (257-550)	380 (273-510)	p = 0.668

within 2-10 months after COVID-19, both groups of patients had elevated levels of C-reactive protein and transaminases (Table 1). At the same time, it should be noted that C-reactive protein values were higher in group 1 patients.

We revealed a significant difference in VEGFA, IL-8, and IL-6 levels in serum of group 1 patients (post-COVID, with elevated transaminases) in comparison with group 2 HCV+COVID-19 (Table 2). Such an increase in cytokines indicates a manifestation of a systemic inflammatory response caused by viral infection. It should also be taken into account that Group 1 patients had more comorbid conditions (AH, DM, CHC) than patients in the HCV group. Maximal high levels of VEGFA and IL-8 in the serum of patients persisted for 2-6 months after COVID-19.

The dynamics of transaminase levels in both groups were not linear and were characterized by a wave-like pattern, independent of the period of COVID-19 (Figure 1).

Study of levels of CRP, IL-18, MCP-1, TNFα between the groups showed that there were no statistically significant differences between them, but higher levels of the studied parameters were noted in patients with liver damage after COVID-19.

We established direct correlations of moderate strength (linear Spearman correlation) between the following cytokines: TNFα-MCP-1 (R = 0.559; p = 0.001), TNFα-VEGFA (R = 0.400; p = 0.002), TNFα-IL-6 (R = 0.503; p = 0.001), which proves unidirectional changes of inflammatory markers during development of post-COVID syndrome in hepatotoxicity patients.

Lu X.N. et al. (2021) showed that patients with COVID-19 and HBV infection had a lower risk of severe events, including ICU hospitalization or

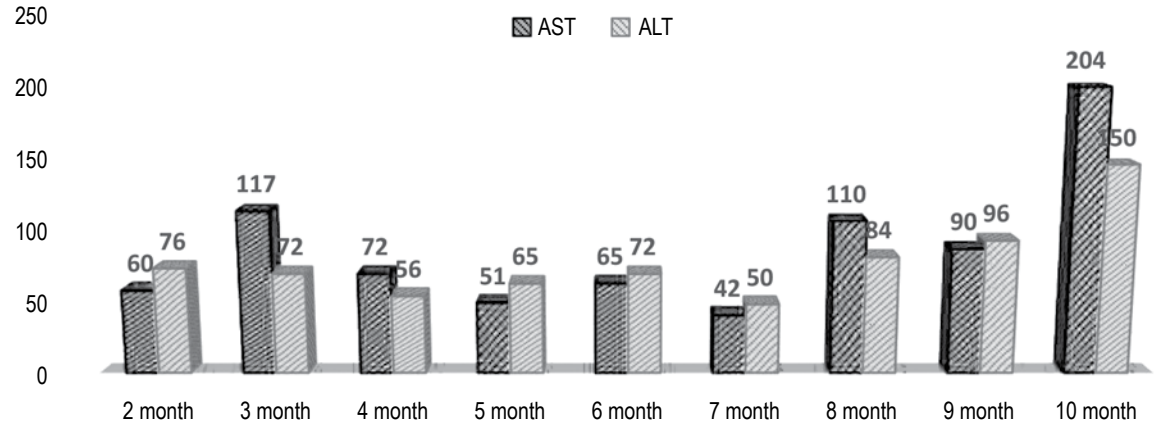

Figure 1. Serum levels of transaminase (U/L) in patients of the studied groups as the time of recovery from COVID-19

death. Factors such as whether patients had other comorbidities, the use of antiviral medications, and the combination of multiple medications may also have influenced patient outcomes [9]. The use of antibodies to block COVID-19-induced cytokine storms that neutralize various cytokines such as IL-1, IFNγ, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) is an undeniable approach to treating severe patients [7]. However, persistent elevated levels of proinflammatory cytokines after the acute period and their modulation of the multisystem inflammatory response appear to be a leading factor in longCOVID.

Activation of VEGF as an angiogenic factor is a consequence of impaired permeability and local hypoxia caused by infection. "Silent hypoxia" in the lungs of patients in the acute period COVID-19 caused by a combination of biological dysfunctions (microvascular thrombosis, redistribution of capillary blood flow, collapse of the air sac (atelectasis), interstitial edema may be an aggravating factor contributing to systemic complications during rehabilitation [5].

Conclusion

Thus, elevated levels of AST and ALT persisted in some patients after coronavirus infection for 6 months. In patients with hepatotoxicity after COVID-19, we found a statistically significant increase in levels of coagulation mediators – VEGFA, IL-6, IL-8 compared to the HCV + COVID-19 group. The identified correlations between the main cytokines implementing the multisystem inflammatory response in postviral syndrome prove unidirectional changes in the functioning of this regulatory network controlling immune virus-induced responses. Postviral liver dysfunction probably has multifactorial causes, but some of the responses are immune-mediated. The use of additional markers to assess and monitor systemic inflammation, such as – VEGFA, IL-6, IL-8, may be recommended for screening patients at risk of developing liver dysfunction, especially those with comorbid conditions, after COVID-19.

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СОХРАНЕНИЕ ИММУНОМОДУЛИРУЮЩЕГО ДЕЙСТВИЯ ИНТЕРВАЛЬНОЙ ГИПОКСИТЕРАПИИ ПОСЛЕ КОРОНАВИРУСНОЙ ИНФЕКЦИИ В ОТДАЛЕННОМ ПЕРИОДЕ

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Резюме. Новая коронавирусная инфекция COVID-19 ввиду сложного патогенеза заболевания, системного воздействия на органы, развития осложнений и стойких нарушений после перенесенной инфекции остается важной проблемой медицины. С каждым годом увеличивается число пациентов с постковидным синдромом, нуждающихся в своевременной и полноценной реабилитации. Недавно стали появляться единичные работы по применению интервальной гипокситерапии для лечения пациентов с коронавирусной инфекцией. Целью нашего исследования было выявление отдаленных результатов влияния интервальной гипокситерапии на иммунологический и коагуляционный статус пациентов после перенесенной коронавирусной инфекции COVID-19. Обследовано 170 пациентов в возрасте от 45 до 59 лет после перенесенной коронавирусной инфекции средней степени тяжести до, после и через три месяца после интервальной гипокситерапии. Определялось количество лимфоцитов, иммуноглобулинов A, M, G, E и цитокинов (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNFα) в крови, D-димера крови, протромбинового времени, фибриногена в крови, антитромбина III, С-реактивного белка и ферритина в крови. Проведенные исследования выявили изменения иммунологической реактивности после перенесенной коронавирусной инфекции COVID-19, требующие коррекции. Интервальная гипокситерапия оказала иммуномодулирующее действие и привела к нормализации основных иммунологических показателей, которые сохранились через три месяца после гипокситерапии: отмечалось достоверное ($p < 0,05$) повышению количества Т-лимфоцитов $CD3^+$, Т-лимфоцитов $CD4^+$, Т-лимфоциты $CD8^+$. Об улучшении иммунного статуса также свидетельствовали нормализация иммунорегуляторного индекса, повышение уровня иммуноглобулинов A и G. Снижение иммуноглобулинов E в крови являлось показателем уменьшения выраженности сенсибилизации организма. Курс гипокситерапии привел к снижению содержания провоспалительных цитокинов: IL-1β, IL-2, IL-6, IL-8, TNFα и повышению противовоспалительных цитокинов: IL-4

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и IL-10 в крови, что свидетельствовало о затухании воспалительного процесса в легочной ткани и в организме в целом. Отмечалось снижение содержания ферритина в крови через 3 месяца после курса гипокситерапии. Как показали проведенные исследования, эффект от гипокситерапии сохраняется в течение трех месяцев после курса лечения. Таким образом, интервальная гипокситерапия может быть рекомендована для реабилитации больных после перенесенной коронавирусной инфекции средней степени тяжести. Повторный курс гипокситерапии может быть проведен через три месяца после первого курса гипокситерапии.

Ключевые слова: коронавирусная инфекция, интервальная гипокситерапия, иммунологический статус, коагуляционный статус, реабилитация, постковидный синдром

PRESERVATION OF THE IMMUNOMODULATORY EFFECT OF INTERVAL HYPOXYTHERAPY AFTER CORONAVIRUS INFECTION IN THE LONG-TERM PERIOD

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Abstract. The new coronavirus infection COVID-19, due to the complex pathogenesis of the disease, systemic impact on organs, the development of complications and persistent disorders after the infection, remains an important medical problem. Every year the number of patients with postcovid syndrome in need of timely and full rehabilitation is increasing. Recently, isolated work began to appear on the use of interval hypoxytherapy for the treatment of patients with coronavirus infection. The purpose of our study was to identify the long-term results of the effect of interval hypoxytherapy on the immunological and coagulation status of patients after suffering coronavirus infection COVID-19. 170 patients aged 45 to 59 years were examined after a moderate coronavirus infection before, after and three months after interval hypoxytherapy. The number of lymphocytes, immunoglobulins A, M, G, E and cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNFα) in blood, blood D-dimer, prothrombin time, fibrinogen in blood, antithrombin III, C-reactive was determined protein and ferritin in the blood. The conducted studies revealed changes in immunological reactivity after the coronavirus infection COVID-19, requiring correction. Interval hypoxytherapy had an immunomodulatory effect and led to the normalization of the main immunological parameters, which remained three months after hypoxytherapy: there was a significant ($p < 0.05$) increase in the number of $CD3^+$T lymphocytes, $CD4^+$T lymphocytes, $CD8^+$T lymphocytes. The improvement in immune status was also evidenced by the normalization of the immunoregulatory index, an increase in the level of immunoglobulins A and G. The decrease in immunoglobulins E in the blood was an indicator of a decrease in the severity of sensitization of the body. The course of hypoxytherapy led to a decrease in the content of pro-inflammatory cytokines: IL-1β, IL-2, IL-6, IL-8, TNFα and an increase in anti-inflammatory cytokines: IL-4 and IL-10 in the blood, which indicated an attenuation of the inflammatory process in the lung tissue and in the body as a whole. Blood ferritin decreased 3 months after hypoxytherapy. As studies have shown, the effect of hypoxytherapy persists for three months after the course of treatment. Thus, interval hypoxytherapy can be recommended for the rehabilitation of patients after a moderate coronavirus infection. A repeated course of hypoxytherapy may be performed three months after the first course of hypoxytherapy.

Keywords: coronavirus infection, interval hypoxic therapy, immunological status, coagulation status, rehabilitation, postcoronavirus syndrome

Introduction

The new coronavirus infection COVID-19, which caused a pandemic in 2020, and currently remains a serious health problem due to the complex pathogenesis of the disease, systemic effects on organs, the development of complications and persistent disorders after infection [1, 14].

Every year, the number of patients with postcovid syndrome after a coronavirus infection who need timely and full-fledged rehabilitation increases [11]. The treatment of such patients is a difficult task, since the therapy of a new coronavirus infection and its complications often causes the development of various side effects that make it difficult to help

patients. The first target of coronavirus infection is the lung tissue and complications primarily affected the bronchopulmonary system with the development of pneumosclerosis and bronchial obstruction. Postcovid complications from the cardiovascular system include specific virus-associated myocardial damage, accompanied by the development of arrhythmias, coronary and heart failure. There are pronounced vascular changes with prolonged thrombotic microangiopathy and persistent hypercoagulation syndrome, as a result, thrombotic complications develop, which increases the risk of pulmonary embolism, stroke, heart attack, deep vein thrombosis. The coronavirus infection caused cognitive and psychological disorders [1, 6]. All this has led to the need to search for new, non-drug methods of treatment and rehabilitation of patients after a coronavirus infection.

Normobaric interval hypoxytherapy has found wide application in the treatment and recovery of many chronic diseases, since properly performed hypoxytherapy does not cause side effects and complications [5]. Recent studies have shown that adaptation to hypoxia during interval hypoxytherapy has a pronounced effect on the cardiovascular system: the efficiency and effectiveness of its work increases due to an increase in the systolic volume of the heart [10]. On the part of the respiratory system, an increase in respiratory and minute breathing volumes provides an improvement in alveolar ventilation and gas exchange processes in the lung tissue. Adaptation to hypoxia has a pronounced stimulating effect on hematopoiesis due to the production of HIF factor (hypoxia–induced factor), which leads to an increase in the content of erythrocytes and hemoglobin in the blood [12]. W.G. Kaelin, G.L. Semenza, P.J. Ratcliffe for the discovery of HIF factor were awarded the Nobel Prize in Physiology and Medicine in 2019. The formation of long-term adaptation to insufficient oxygen supply to cells is genetically determined and associated with the expression of a specific protein factor HIF, which functions as a transcription activator and a key regulator of various cellular and systemic responses to hypoxia [8].

Hypoxytherapy has an effect on morphofunctional changes of the myocardium, manifested by an increase in the capillary reserve of the myocardium, an improvement in blood supply and oxygen supply of the myocardium and an increase in the reserve potential of the heart muscle [3]. In recent years, there has been information about the specific effect of HIF factor on the expression of genes of various types of immune cells and their effector function. HIF-factor increases the life expectancy of neutrophils, enhances the synthesis of various antimicrobial substances and apoptosis [7]. Titova O.N., Kuzubova N.A., Lebedeva E.S. in their works, they proved that hypoxia, due to the action of HIF factor, can actively affect inflammatory processes by regulating oxygen-sensitive signaling pathways in multiple subtypes of immune cells [13]. All of the above adaptive mechanisms caused by hypoxia lead to an improvement in the clinical course of diseases and emergency rehabilitation.

Interval hypoxytherapy cautiously entered the rehabilitation of patients after coronavirus infection, as it caused a lot of controversy about the possibility of using hypoxytherapy after a coronavirus infection. Isolated works on the use of hypoxytherapy for the treatment of patients with coronavirus infection began to appear, in which the effectiveness of the use of normobaric interval hypoxytherapy in the rehabilitation of patients after a coronavirus infection COVID-19 has been proven [15]. Articles on the state of the immune system in patients with a new coronavirus infection began to appear, however, articles on identifying the long-term results of the effect of interval hypoxytherapy on the immunological status of patients after the COVID-19 coronavirus infection was not encountered. There is also no information in the available literature on the persistence of changes in the coagulation status of patients 3 months after interval hypoxytherapy.

The aim of the study was to identify the long-term results (3 months after the course of hypoxytherapy) of the effect of interval hypoxytherapy on the immunological and coagulation status of patients after COVID-19 coronavirus infection.

Materials and methods

The main group consisted of 170 patients aged 45-59 years after a moderate coronavirus infection. The control group was represented by 60 patients after suffering from COVID-19 coronavirus infection, who underwent standard rehabilitation without interval hypoxytherapy. Interval hypoxytherapy and laboratory studies were carried out on the basis of the University Clinic of the Kabardino-Balkarian State University named after H.M. Berbekov. Patients underwent normobaric interval hypoxytherapy in the mode of alternating hypoxic intervals with normoxic ones. The hypoxic effect was carried out using the hypoxytherapy unit "Hypo-Oxy" of the company "Oxyterra" (Russia), which generated a hypoxic mixture with different oxygen content. Hypoxytherapy was performed in the hypoxia-normoxia mode, including alternating 5-minute hypoxic effects with 5-minute hyperoxic (20.9% O_2). To determine individual sensitivity to hypoxia and tolerance of hypoxic mixtures, a hypoxic test was performed for all patients, after which the optimal oxygen content in the hypoxic mixture was selected, the duration of hypoxic effects and the number of procedures were determined. Usually the course of hypoxytherapy was 15 days. As a result, there was a gradual adaptation to hypoxia.

All patients before, after and 3 months after hypoxytherapy underwent a study of D-dimer by immunoturbidimetry (ACL TOP 750, Instrumentation Laboratory, USA), prothrombin (thromboplastin) time – according to the time of plasma recalcification

with the addition of tissue thromboplastin. INR was calculated using the formula: INR (INR) = BY mich(isi), where BY = patient's prothrombin time / normal mean prothrombin time. Determination of activated partial thromboplastin time (APTT) by the clotting time of decalcified plasma after adding kaolin-kefalin-calcium mixture to it. To determine fibrinogen in the blood, the method of detecting lateral light scattering, determining the percentage at the endpoint, was used. Determination of antithrombin III was carried out by colorimetric method (%). All studies were carried out on the device coagulometer analyzer automatic CS-5100 Sysmex (Japan). Determination of C-reactive protein and ferritin in the blood was carried out by immunoturbidimetry on a biochemical analyzer Cobas 6000 (Roche Diagnostics, Switzerland).

The determination of the number of lymphocytes was carried out by flow cytofluorimetry on the XN-9000, Sysmex (Japan), immunoglobulins A, M, G by immunoturbidimetry on the Cobas 6000, Roche Diagnostics (Switzerland); the content of immunoglobulins E and cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNFα) in the blood was determined by electrochemiluminescent immunoassay on the Cobas 6000 apparatus, Roche Diagnostics (Switzerland). Studies were conducted only after obtaining the personal consent of patients in accordance with ethical principles.

Statistical processing of the results was carried out in accordance with the rules of mathematical statistics using the program "Microsoft Excel" and "Statistica 6.0" for "Windows". During the parametric analysis, the paired and unpaired Student's t-test was used. The nonparametric Mann–Whitney criterion was used to assess the reliability of intergroup differences. All numerical data in the form of the arithmetic mean and the standard error of the mean M±m, and with the nonparametric nature of the distribution of quantities – in the form of a median indicating the 25th and 75th quartiles: Me ($Q_{0.25}$-$Q_{0.75}$). The differences were considered statistically significant at $p < 0.05$.

Results and discussion

Coronavirus infection had a significant impact on the coagulation system of patients, which is consistent with the data of many authors [2, 4]. In patients, there was a moderate decrease in the platelet content in the blood (165.41±10.22 × 10^9/L), which, apparently, was associated with increased consumption due to damage to the vascular wall and the action of the virus [9]. On the part of coagulation hemostasis, there was a statistically significant ($p < 0.05$) decrease in APTT, INR, antithrombin III, an increase in the Quick prothrombin index, fibrinogen, D-dimer, which indicated an increased tendency to thrombosis in patients.

After the course of hypoxytherapy, the coagulogram indicators normalized and approached the indicators of the control group. Three months after the course of hypoxytherapy, the preservation of changes in the hemostasis system was noted: the platelet count increased statistically significantly ($p < 0.05$) to 223.63±11.42 × 10^9/L and remained at the level of 237.54±13.36 × 10^9/L three months after hypoxytherapy. Activated partial thromboplastin time significantly ($p < 0.05$) increased to 25.89±1.46 sec after hypoxytherapy and remained at the level of 26.15±1.12 sec 3 months after hypoxytherapy. INR significantly ($p < 0.05$) increased to 0.82±0.04 after hypoxytherapy and remained at the achieved level after 3 months.

There was a statistically significant ($p < 0.05$) increase in the content of antithrombin III in the blood 3 months after hypoxytherapy (87.57±3.15%). The prothrombin index for Quick decreased to 125.61±3.15% after hypoxytherapy and remained at 124.84±3.57% 3 months after hypoxytherapy. The reduced fibrinogen content in the blood was noted 3 months after hypoxytherapy and amounted to 9.52±1.03 g/L. An important result of hypoxytherapy was a decrease in the content of D-dimer in the blood serum after hypoxytherapy and 3 months after its implementation at the level of 1.52±0.03 mg/L. The revealed changes testified to the normalization of coagulation hemostasis after the course of hypoxytherapy and the preservation of these changes 3 months after hypoxytherapy. A statistically significant ($p < 0.05$) persistent decrease in C-reactive protein in the blood indicated the suppression of the inflammatory process and the preservation of the effect of hypoxytherapy in the long term.

The conducted studies of immunological reactivity after coronavirus infection revealed changes in cellular immunity, manifested in a decrease in the number of $CD3^+$T lymphocytes to 48.6 (37.3-57.5) %, the level of $CD4^+$T helper cells to 28.4 (20.8-40.8) %, which led to a violation of humoral and cellular immunity. There was also a significant ($p < 0.05$) decrease in $CD8^+$T lymphocytes to 14.5 (10.3-19.8) %. The number of B-lymphocytes was not significantly changed and was 19.6 (12.7-24.2) %.

There was a significant ($p < 0.05$) decrease in the level of immunoglobulins A and M. Reduction of immunoglobulin A levels to 0.88 (0.67-0.98) g/L resulted in decreased resistance to various infections against the background of developing lymphopenia after a coronavirus infection COVID-19. The results of our studies revealed a significant ($p < 0,01$) increase in the content of immunoglobulins E to 97.43 (78.7-121.5) IU/L, which indicated increased sensitization of patients. There was a statistically significant ($p < 0.05$) increase in the content of circulating immune complexes to 96.57 (86.4-99.5) standard units and the immuno-regulatory index up to 2.08 (2.05-2.20) standard units as a result of increased immunological reactivity. A decrease in the content of $CD3^+$T lymphocytes, $CD4^+$T helper cells, the level of immunoglobulins A and an increase in the content of immunoglobulins E and circulating immune

complexes led to a change in the immunological status of patients after a coronavirus infection and the maintenance of the inflammatory process in the bronchopulmonary system even during recovery.

Interval hypoxytherapy had an immunomodulating effect and led to the normalization of the main immunological parameters. There was a significant ($p < 0.05$) increase in the number of $CD3^+$T lymphocytes to 59.3 (37.5-67.1) % after hypoxytherapy and to 60.1 (45.3-68.4) % 3 months after hypoxytherapy. The increase in $CD4^+$T lymphocytes after hypoxytherapy persisted 3 months after hypoxytherapy at the level of 41.7 (29.6-49.6) %. Normalization of cellular immunity was also observed in the long-term periods after hypoxytherapy; the number of $CD8^+$T lymphocytes was 24.4 (13.7-28.5) % 3 months after hypoxytherapy.

The improvement of the immune status was evidenced by the normalization of the immune regulatory index to 1.77 (1.83-1.91) units, an increase in the level of immunoglobulins A to 1.37 (1.11-1.55) g/L and immunoglobulins G to 9.64 (6.32-12.68) g/L, which increased the body's resistance to re-infection. The retention of a statistically significant ($p < 0.05$) decrease in immunoglobulin E to 289.45 (272.4-374.3) IU/L in the blood indicated a decrease in the sensitization of the body. These changes persisted 3 months after the course of hypoxytherapy.

The study of the cytokine status of the blood revealed changes even during the period of convalescence. The increased content of pro-inflammatory and decreased anti-inflammatory cytokines remained, which indicated the depletion of sanogenetic mechanisms and contributed to the maintenance of the inflammatory process in the bronchopulmonary system and in the body. Hypoxytherapy had a positive effect on the cytokine profile of the blood: in the long-term periods (after 3 months), there was a significant ($p < 0.05$) decrease in the content of proinflammatory cytokines: IL-1β to 3.46±0.84 pg/mL, IL-2 to 3.32±0.04 pg/mL, IL-6 to 3.72±0.11, IL-8 to 4.33±0.13 pg/mL, TNFα to 9.51±0.15 pg/mL and an increase in anti-inflammatory cytokines: IL-4 to 5.83±1.23 pg/mL, IL-10 to 8.63±0.11 pg/mL in the blood, which indicated the activation of sanogenetic mechanisms.

Conclusion

An important result of hypoxytherapy was the preservation of the ferritin content in the blood 3 months after the course of hypoxytherapy at the level of 86.25±5.07 mcg/L, which, along with a decrease in the content of pro-inflammatory interleukins and an increase in anti-inflammatory interleukins, indicated the attenuation of the inflammatory process in the lung tissue and in the body as a whole. Studies have shown that the effect of hypoxytherapy persists for 3 months after the course of hypoxytherapy, which is consistent with the literature data on the effectiveness of hypoxytherapy. Thus, interval hypoxytherapy can be recommended for the rehabilitation of patients after a moderate coronavirus infection. A second course of hypoxytherapy can be carried out 3 months after the first course of hypoxytherapy.

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ПЛЕОТРОПНЫЕ ИММУНОМОДУЛИРУЮЩИЕ ЭФФЕКТЫ ПЕПТИДА ARGINYL-ALPHA-ASPARTYL-LYSYL-VALYL-TYROSYL-ARGININE НА РАЗЛИЧНЫЕ СУБПОПУЛЯЦИИ НЕЙТРОФИЛЬНЫХ ГРАНУЛОЦИТОВ И ИХ ФЕНОТИП У ПАЦИЕНТОВ С COVID-19 В ЭКСПЕРИМЕНТАЛЬНОЙ СИСТЕМЕ *IN VITRO*

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Резюме. Ключевая роль нейтрофильных гранулоцитов (НГ) в патогенезе COVID-19 делает их новыми мишенями для таргетных терапевтических подходов с возможностью влияния на течение и исход болезни, восстановление негативных изменений фенотипа и функций НГ. Наиболее перспективными в лечении COVID-19 являются синтетические пептиды или полипептидные комплексы с физиологическим механизмом действия. Цель – выявить эффекты влияния гексапептида (ГП) – Arginyl-alpha-Aspartyl-Lysyl-Valyl-Tyrosyl-Arginine на фенотип функционально-значимых субпопуляций НГ при среднетяжелой форме COVID-19.

Обследованы пациенты 61 (57-71) года (n = 45) в остром периоде COVID-19 – группа исследования 1 (ГИ1). Кровь пациентов ГИ1 инкубировали *in vitro* с ГП (10^6 г/л, 60 мин, Т 37 °С) – группа исследования 2 (ГИ2). Оценивали количество НГ субпопуляций $CD16^+IFN\alpha/\beta R1^+CD119^+$, $CD16^+IFN\alpha/\beta R1^+CD119^-$, $CD16^+IFN\alpha/\beta R1^+CD119^+$, $CD64^-CD16^+CD32^+CD11b^+$, $CD64^+CD16^+CD32^+CD11b^+$ и фенотип по интенсивности флюоресценции (MFI) (FC 500, Beckman Coulter, США); фагоцитарную активность НГ до и после инкубации с ГП. Группа сравнения (ГС) – 22 добровольца 58 (57-70) лет, обследованных в доковидный период.

Выявлены однонаправленные эффекты ГП *in vitro*, способствующие восстановлению фенотипа субпопуляций $CD16^+IFN\alpha/\beta R1^-CD119^+$НГ и $CD16^+IFN\alpha/\beta R1^+CD119^-$НГ до показателей ГС. Показано снижение MFI CD16 ($p < 0,05$) в обеих субпопуляциях; MFI CD119 ($p < 0,05$) в субпопуляции $CD16^+IFN\alpha/\beta R1^-CD119^+$НГ и MFI IFNα/βR1 рецепторов в субпопуляции $CD16^+IFN\alpha/\beta R1^+CD119^-$НГ. Эффекты влияния ГП на фенотип субпопуляций $CD16^+IFN\alpha/\beta R1^+CD119^+$НГ в 76% случаев прояв-

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лялись снижением MFI CD16 ($p < 0,05$) и повышением MFI IFNα/βR1 и CD119 ($p_{1,2} < 0,05$), а в 24% случаев уменьшением MFI IFNα/βR1 ($p < 0,05$). Под влиянием ГП *in vitro* установлено ремоделирование фенотипов субпопуляций НГ $CD64^-CD16^+CD32^+CD11b^+$ и $CD64^+CD16^+CD32^+CD11b^+$, отвечающих за эффекторные функции, от гиперактивированных до нормальных. Изменялся фенотип НГ в субпопуляции $CD64^-CD16^+CD32^+CD11b^+$ – отмечалось снижение MFI CD16 и CD11b до показателей ГС ($p_{1,2} < 0,05$). Наблюдалась уменьшение количества $CD64^+CD16^+CD32^+CD11b^+$НГ, со сниженным MFI CD16 ($p_{1,2} > 0,05$). Восстановление фенотипа НГ, трансформированного при COVID-19, под влиянием ГП приводило и к нормализации фагоцитарной функции.

Положительные эффекты влияния ГП *in vitro* на фенотипы субпопуляций, участвующих в противовирусной защите, и функции НГ при COVID-19 открывают перспективы для создания новых методов иммунотерапии с включением гексапептида для восстановления дисфункций НГ.

Ключевые слова: COVID-19, нейтрофильные гранулоциты, субпопуляции, фенотип, иммуномодуляция, гексапептид

PLEIOTROPIC IMMUNOMODULATING EFFECTS OF PEPTIDE ARGINYL-ALPHA-ASPARTYL-LYSYL-VALYL-TYROSYL-ARGININE ON VARIOUS SUBSETS OF NEUTROPHILIC GRANULOCYTES AND THEIR PHENOTYPE IN PATIENTS WITH COVID-19 *IN VITRO*

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Abstract. The key role of neutrophilic granulocytes (NG) in the pathogenesis of COVID-19 makes them new targets for therapeutic approaches and of influencing the course and outcome of the disease, restoring changes in the phenotype and functions of NG. Synthetic peptides or polypeptide complexes of action are the most promising in the treatment of COVID-19. Aim: to reveal the effects of the influence of the hexapeptide (HP) – Arginyl-alpha-Aspartyl-Lysyl-Valyl-Tyrosyl-Arginine on the phenotype of functionally significant NG subsets in moderate COVID-19.

The study examined patients 61 (57-71) years old ($n = 45$) in the acute period of COVID-19 – study group1 (SG1). *In vitro*, samples SG1 were incubated with HP (10^6 g/L, 60 min, 37 °C) – study group2 (SG2). The number of NG subsets was evaluated: $CD16^+IFN\alpha/\beta R1^+CD119^+$, $CD16^+IFN\alpha/\beta R1^+CD119^-$, $CD16^+IFN\alpha/\beta R1^+CD119^+$, $CD64^-CD16^+CD32^+CD11b^+$, $CD64^+CD16^+CD32^+CD11b^+$ and phenotype by membrane receptor expression density (MFI) (FC 500, Beckman Coulter, USA); NG phagocytic activity was tested before and after incubation with HP. The comparison group (GS) – of 22 volunteers examined in the pre-COVID period.

It was revealed that unidirectional effects of HP *in vitro* contributing to the restoration of the phenotype of subsets $CD16^+IFN\alpha/\beta R1^-CD119^+$, $CD16^+IFN\alpha/\beta R1^+CD119^-$ to CG indicators. There was a decrease in MFI CD16 ($p < 0.05$) in both subsets; MFI CD119 ($p < 0.05$) in the $CD16^+IFN\alpha/\beta R1^-CD119^+$NG subset, MFI IFNa/βR1 in the $CD16^+IFN\alpha/\beta R1^+CD119^-$NG subset. The effects of HP on the phenotype of $CD16^+IFN\alpha/\beta R1^+CD119^+$NG subsets in 76% of cases were manifested by a decrease in MFI CD16 ($p < 0.05$), an increase in MFI IFNα/βR1 and CD119 ($p_{1,2} < 0.05$), and in 24% of cases a decrease in MFI IFNα/βR1 ($p < 0.05$). HP *in vitro* remodeling of the phenotypes subsets $CD64^-CD16^+CD32^+CD11b^+$ and $CD64^+CD16^+CD32^+CD11b^+$ were established, providing the usefulness of effector functions from hyperactivated to normal. In the $CD64^-CD16^+CD32^+CD11b^+$ subset, there was a decrease in MFI CD16 and CD11b to the indicators CG ($p_{1,2} < 0.05$). Recovery of the NG phenotype under the influence of HP led to the restoration of the phagocytic function of NG.

Positive effects of HP *in vitro* on the phenotypes of subsets actively and NG functions in COVID-19 open up prospects for the creation of new methods of immunotherapy to restore NG dysfunctions.

Keywords: COVID-19, neutrophilic granulocytes, subsets, phenotype, immunomodulation, hexapeptide

Introduction

The ongoing COVID-19 pandemic, the emergence of new strains of SARS-CoV-2 and the lack of a specific treatment for COVID-19 are drawing attention to the search for effective immunotherapy due to the possibility of increasing immunity to the virus and reducing hyperinflammation [4]. It has been shown that the immunological phenotype of COVID-19 is characterized by an increased number of neutrophilic granulocytes (NG) and depletion of lymphocytes, the ratio of NG to lymphocytes correlates with the severity of the disease and respiratory symptoms, being a predictor of an unfavorable outcome [7, 10]. It was found that the pathophysiology of severe COVID-19 is characterized by changes in the number, phenotype and functionality of NG [6]. It is known that NG secrete cytokines and chemokines that contribute to the maintenance of inflammation in COVID-19, while ROS and NETosis are involved in tissue damage [6, 10]. Convincing evidence about the role of NG in the pathogenesis of COVID-19 make them the new targets for targeted therapeutic approaches with the possibility of influencing the development, course and outcome of the disease, restoring negative changes in the NG phenotype.

The most promising for research and use in the treatment of COVID-19 are short synthetic peptides or polypeptide complexes isolated from animal organs and tissues: inhibitors of the SARS-CoV-2 protein, immunomodulators and broncho protectors with a physiological mechanism of action [3]. It has been shown that immunomodulatory peptides contribute to the normalization of innate and adaptive immunity, the hemostasis system and cytokine synthesis and have an anti-inflammatory effect, thereby preventing the development of distress syndrome and multiple organ failure [3].

Thymopentin is an immunomodulatory pentapeptide (Arg-Lys-Asp-Val-Tyr, RKDVY, TP5), which is the active center of the thymus hormone thymopoietin [3], which is previously used to normalize immunological parameters in tumor, immunodeficiency and autoimmune diseases [11], also was used to treat COVID-19 in China. It has been shown that TP5 *in vitro* affects the functions of T cells and monocytes by activating intracellular signaling cascades [1]. The ability of TP5 to normalize the functions of the immune system (IS) in viral diseases also has been established [1]. In the context of SARS-CoV-2, timopentin is considered to be a 3-chymotrypsin-like protease (3CLpro) inhibitor [13].

In Russia, a medicinal product No. P N000106/04 is registered, the main active substance is Hexapeptide (HP) – Arginyl-alpha-Aspartyl-Lysyl-Valyl-Tyrosyl-Arginine, a synthetic analogue of the active center of the thymus hormone – thymopoietin, which has all the biological activities of the native thymus hormone [9, 13].

According to the scientific data, Hexapeptide has an immunoregulatory effect on a defectively functioning immune system, regulating and restoring the T cell link, the number and functional activity of neutrophilic granulocytes, monocytes, normalizing the synthesis of cytokines; also are described effects of hepatoprotective, antioxidant properties, the ability to enhance the effectiveness of antibiotic therapy, inhibit the multidrug resistance of the body [5, 12], while its effect on neutrophilic granulocytes in COVID-19 remains unexplored.

The particular scientific interest, from our point of view, is the simultaneous assessment of NG subsets that are responsible for triggering and regulating antiviral immunity and subsets that provide effector phagocytic and microbicidal properties of NG.

The surface receptors for IFN I and II types: IFNα/β (IFNAR1) and CD119 (IFNγ – IFNGR) were chosen for the study, through which innate antiviral responses are regulated [12]. The receptors which are responsible for the effector functions of NG were also studied: CD64 (FcγRI) is a high-affinity cytoactivating receptor, its cross-linking induces phagocytosis, release of inflammatory mediators, participates in antibody-dependent cell-mediated cytotoxicity and antigen presentation; CD32 (FcγRII) is a low-affinity receptor takes part in activating the processes of phagocytosis and degranulation; CD16 (FcγRIII) is a low-affinity receptor for degranulation, oxidative burst, phagocytosis activation; CD11b (CR3, Mac-1, integrin) regulates adhesion and migration, participates in cell-mediated cytotoxicity, phagocytosis.

Aim: to evaluate the various effects of the influence of the Arginyl-alpha-Aspartyl-Lysyl-Valyl-Tyrosyl-Arginine peptide on the phenotype of 5 functionally significant NG subsets in moderate course of COVID-19 *in vitro*.

Materials and methods

A study was made of peripheral blood samples of patients with COVID-19, in the acute period of the disease (7-9 days of the disease), on the 1st day of hospitalization at the Specialized Clinical Infectious Diseases Hospital of the Ministry of Health of the Krasnodar Territory. Study group 1 (SG1) included 45 patients, aged 61 (57-71) years, 58% men, 42% women with a moderate form of the disease, in accordance with clinical and laboratory severity

criteria (World Health Organization guidelines version 10 (02/08/2021)).

In vitro system, patient samples (SG1) were incubated with Hexapeptide (HP) (10^6g/L), 60 min, at 37 °C – study group 2 (SG2).

The number of NG, % of subsets, was estimated: $CD16^+IFN\alpha/\beta R1^+CD119^+$, $CD16^+IFN\alpha/\beta R1^+CD119^-$, $CD16^+IFN\alpha/\beta R1^+CD119^+$, $CD64^-CD16^+CD32^+CD11b^+$, $CD64^+CD16^+CD32^+CD11b^+$ and phenotype according to the expression density of membrane receptors (MFI) (FC 500, Beckman Coulter, USA); monoclonal antibodies (Mab): IFNαβR1-FITC, CD119-PE, CD16-ECD, CD64-FITC, CD32-PE, CD11b-PC5 (Beckman Coulter International S.A., France); phagocytic activity of NG in relation to S. aureus. The parameters were tested before and after the incubation with HP. The comparison group (CG) was formed from the indicators of 22 volunteers 58 (57-70) years old, examined in the pre-COVID period.

The data were analyzed using the StatSoft Statistica 10.0 program. After checking the normal distribution by the Kolmogorov–Smirnov method, they are presented as a median (Me) and a quartile interval ($Q_{0.25}$-$Q_{0.75}$). To compare groups, nonparametric criteria was used: Mann–Whitney U test. The difference in indicators was considered statistically significant at $p < 0.05$.

Results and discussion

It was established that 3 subsets of NG expressing receptors for IFN I and II types circulate in the peripheral blood of CG volunteers: $CD16^+IFN\alpha/\beta R1^-CD119^+$, $CD16^+IFN\alpha/\beta R1^+CD119^-$, $CD16^+IFN\alpha/\beta R1^+CD119^+$. It is shown that NG subset $CD16^+IFN\alpha/\beta R1^-CD119^+$ make up 93.7 (89.8-96.5) % with MFI expression density of CD16 – 39.8 (20.4-51.3) and $CD119^+$ (IFNγ) – 2.8 (2.5-3.1), which determines the $CD16^{mid}IFN\alpha/\beta R1^-CD119^{dim}$ phenotype. The subset of NG $CD16^+IFN\alpha/\beta R1^+CD119^-$, not expressing the IFNγ receptor, was 1.4 (0.5-2.3) % with an expression density according to MFI IFNα/βR1 of 3.4 (2.6-4.1) and MFI CD16 – 39.9 (22.9-54.5) with $CD16^{mid}IFN\alpha/\beta R1^{dim}CD119^-$ phenotype. The $CD16^+IFN\alpha/\beta R1^+CD119^+$ NG subset simultaneously expressing IFNα/β and IFNγ receptors accounted for only 0.9 (0.4-1.8) % of NG, but had higher MFI values of IFNα/βR1 ($p > 0.05$) and MFI CD119 ($p > 0.05$), which was reflected by the $CD16^{mid}IFN\alpha/\beta R1^{mid}CD119^{mid}$ (Table 1).

In SG1 with a moderate form of COVID-19, in 76% of cases (SG1a, n = 36), the content of NG in the studied subsets did not differ significantly from CG ($p > 0.05$), but a phenotype type changes was observed. There was 2 times increasing in MFI CD16 in all subsets ($p_{1\text{-}3} < 0.05$), MFI CD119 was 1.3 times increasing ($p < 0.05$) in the subset $CD16^+IFN\alpha/\beta R1^-CD119^+NG$, MFI IFNα/βR1 was 1.8 times increasing in the $CD16^+IFN\alpha/\beta R1^+CD119^-NG$ ($p < 0.05$) and 1.6 times increasing in the $CD16^+IFN\alpha/\beta R1^+CD119^+NG$ ($p < 0.05$) (Table 1). The identified changes are characterized by the phenotypes $CD16^{bright}IFN\alpha/\beta R1^-CD119^{mid}NG$, $CD16^{bright}IFN\alpha/\beta R1^{mid}CD119^-NG$, $CD16^{bright}IFN\alpha/\beta R1^{bright}CD119^{mid}NG$. At the same time, in 24% of cases in SG1b (n = 9) there was a 63 times increasing in the content of subset of neutrophilic granulocytes in the $CD16^+IFN\alpha/\beta R1^+CD119^-$ subset up to 88.95 (81.73-92.65) % versus 1.4 (0.53-2.35) % in CG and 1.2 (0.6-1.9) % in SG1a ($p_{1,2} < 0.05$) and 15 times reduction in NG subset in the $CD16^+IFN\alpha/\beta R1^+CD119^-$ with phenotype transformation in the $CD16^{bright}IFN\alpha/\beta R1^-CD119^{mid}NG$ subset, similar to that observed in SG1a, and $CD16^{bright}IFN\alpha/\beta R1^{bright}CD119^-NG$ with a 3 times ($p < 0.05$) increasing in MFI IFNα/βR1 (Table 1).

Innate antiviral responses are largely controlled by type I IFNs signaling through IFNAR. Elevated expression levels of IFN receptors demonstrate the readiness of NG to perceive cytokine signals and respond to them. However, given that type I IFNs enhance NETosis [2], it is possible to suggest that the significant increase in receptor expression noted in SG1b may exacerbate neutrophil infiltration and netosis.

In vitro incubation of NG of study group with COVID-19 with HP revealed unidirectional modulating effects that contributed to the restoration of the phenotype of 2 subsets: $CD16^{mid}IFN\alpha/\beta R1^-CD119^{dim}NG$, $CD16^{mid}IFN\alpha/\beta R1^{dim}CD119^-NG$. A decrease in both SG2a and SG2b expression density by MFI CD16 ($p_{1,2} < 0.05$) was shown in both subsets; MFI CD119 ($p_{1,2} < 0.05$) in subset $CD16^+IFN\alpha/\beta R1^-CD119^+NG$ and MFI IFNα/βR1 receptors in subset $CD16^+IFN\alpha/\beta R1^+CD119^-NG$ to comparison group values (CG) ($p_{1,2} > 0.05$) (Table 1). The effects of HP in SG2a on the phenotype of $CD16^+IFN\alpha/\beta R1^+CD119^+NG$ subsets are consisted in a decrease in MFI CD16 ($p < 0.05$) and an increase in MFI IFNα/βR1 and MFI CD119 ($p_{1,2} < 0.05$) – $CD16^{mid}IFN\alpha/\beta R1^{bright}CD119^{bright}$. At the same time, SG2b is showed a decrease in MFI IFNα/βR1 ($p < 0.05$), an increase in MFI CD119 ($p > 0.05$), and the density of CD16 expression did not differ from the values registered in SG1a in patients with COVID-19 ($p > 0.05$) – $CD16^{bright}IFN\alpha/\beta R1^{dim}CD119^{mid}$ (Table 1).

TABLE 1. EFFECTS OF HEXAPEPTIDE INFLUENCE ON THE CONTENT AND PHENOTYPE OF SUBSETS OF NEUTROPHILIC GRANULOCYTES EXPRESSING RECEPTORS FOR IFN TYPES I AND II IN PATIENTS WITH MODERATE COVID-19, Me ($Q_{0.25}$-$Q_{0.75}$)

Indicators	Comparison group (CG)	Moderate form of COVID-19			
		Study group 1 (SG1) before incubation with HP		Study group 2 (SG2) after incubation with HP	
		SG1a, n = 36	SG1b, n = 9	SG2a, n = 36	SG2b, n = 9
$CD16^+IFN\alpha/\beta R1^-CD119^+NG$					
NG,%	93.7 (89.8-96.5)	95.6 (92.0-98.2)	15.8* ^ (14.8-16.5)	94.9 (93.0-97.0)	16.8* ♦ (16.6-17.0)
MFI CD16	39.8 (20.4-51.3)	79.0* (59.2-91.4)	67.5* (53.2-79.7)	24.8# (20.9-32.1)	13.7♦ (8.3-17.4)
MFI CD119	2.8 (2.5-3.1)	3.6* (3.3-4.3)	3.4* (3.2-3.6)	2.0# (2.3-4.2)	6.6* ♦ (5.9-7.4)
$CD16^+$ $IFN\alpha/\beta R1^+CD119^-NG$					
NG,%	1.4 (0.5-2.4)	1.2 (0.6-1.9)	88.9* ^ (81.7-92.7)	2.2 (1.4-4.0)	82.1* ♦ (32.4-90.1)
MFI CD16	39.9 (22.9-54.5)	74.8* (59.6-109.0)	66.5* (55.9-79.6)	24.8# (20.2-32.1)	68.7* (52.5-73.8)
MFI IFNα/β	3.4 (2.6-4.1)	6.1* (5.0-18.1)	11.22* (7.7-16.1)	3.7^ (2.6-5.0)	3.8 (2.3-5.1)
$CD16^+IFN\alpha/\beta R1^+CD119^+NG$					
NG,%	1.0 (0.4-1.8)	2.8 (1.5-3.0)	0.9 (0.8-2.3)	0.4 (0.2-0.5)	2.6 (1.7-7.6)
MFI CD16	39.1 (26.6-50.3)	77.5* (59.1-109.0)	66.5* (51.9-79.6)	40.3 (35.0-48.0)	80.5 (63.8-107.0)
MFI IFNα/β	5.7 (4.6-6.5)	9.2* (7.8-12.0)	3.4* ^ (3.1-3.6)	13.7* (8.3-17.4)	1.72* ♦ (1.58-1.86)
MFI CD119	3.2 (2.9-5.8)	2.5 (1.7-9.0)	3.7 (2.5-5.0)	6.6* (5.9-7.4)	7.7 (6.1-9.1)

Note. *, significant differences relative to the comparison group, $p < 0.05$; ^, significant differences between SG1a and SG1b, $p < 0.05$; #, significant differences in SG1a before and after incubation with hexapeptide, $p < 0.05$; ♦, significant differences in SG1b values before and after incubation with hexapeptide, $p < 0.05$

Testing in the CG, the number of NG (%) subsets: $CD64^-CD16^+CD32^+CD11b^+$, $CD64^+CD16^+CD32^+CD11b^+$ it was shown that the $CD64^-CD16^+CD32^+CD11b$ NG subset is major and amounts to 94.4 (89.7-95.9) %, has the $CD64^-CD16^{dim}CD32^{dim}CD11b^{mid}$ NG phenotype determined by the density of receptor expression: low MFI CD16 and MFI CD32 and medium MFI CD11b (Table 2). Also, a subset of $CD64^+CD16^+CD32^+CD11b^+NG$ – 1.2 (0.7-2.7) %, additionally expressing the CD64 receptor with an MFI of 6.1 (3.3-9.9) and having equipment of CD11b,CD32,CD16 receptors identical with the major subset – $CD64^{mid}CD16^{dim}CD32^{dim}CD11b^{mid}$ phenotype (Table 2).

There is a 5 times increase ($p < 0.05$) of NG of the $CD64^+CD16^+CD32^+CD11b^+$ subset with a transformed phenotype – $CD64^{mid}CD16^{bright}CD32^{mid}CD11b^{bright}$ versus $CD64^{mid}CD16^{dim}CD32^{mid}CD11b^{mid}$ in CG ($p < 0.05$, Table 2). The altered phenotype had high levels of expression of 2 membrane activation receptors: MFI CD16 -67.2 (44.4-84.4) и CD11b -39.3 (39.1-41.1) ($p_{1,2} < 0.05$), which indicates negative hyperactivation of NG. The number of NG subset $CD64^-CD16^+CD32^+CD11b^+$ did not change ($p > 0.05$), but an altered phenotype was deter-

TABLE 2. EFFECTS OF HEXAPEPTIDE (HP) INFLUENCE ON THE CONTENT AND PHENOTYPE OF $CD64^-CD16^+CD32^+CD11b^+$ AND $CD64^+CD16^+CD32^+CD11b^+$ SUBSETS OF NEUTROPHILIC GRANULOCYTES IN PATIENTS WITH MODERATE COVID-19, Me ($Q_{0.25}$-$Q_{0.75}$)

Indicators	Comparison Group (CG) n = 22	Moderate form of COVID-19 n = 45	
		SG1 before incubation with HP	SG2 after incubation with HP
$CD64^-CD16^+CD32^+CD11b^+$NG			
NG,%	94.4 (89.7-95.9)	96.2 (90.5-97.8)	92.7 (84.5-93.1)
MFI CD16	33.6 (30.0-44.3)	79.4* (64.9-87.6)	57.7* ♦ (54.7-60.0)
MFI CD32	2.7 (2.2-3.1)	2.7 (2.3-3.5)	4.7* ♦ (3.9-5.2)
MFI CD11b	32.3 (24.1-38.4)	19.3* (16.1-26.1)	26.8* (25.7-27.6)
$CD64^+CD16^+CD32^+CD11b^+$NG			
NG,%	1.2 (0.7-2.7)	5.8* (4.2-10.6)	2.7 (1.7-6.5)
MFI CD64	6.1 (3.3-9.9)	3.1 (2.1-6.6)	10.7 (5.7-14.7)
MFI CD16	28.4 (13.8-56.1)	67.2 (44.4-84.4)	15.2 (11.6-26.6)
MFI CD32	3.7 (3.1-4.2)	4.5* (4.4-6.8)	6.2 (4.9-11.1)
MFI CD11b	31.1 (24.2-38.2)	39.3* (39.1-41.1)	29.2 (22.2-32.6)

Note. *, significant differences relative to the comparison group, $p < 0.05$; ♦, significant differences in SG1 values before and after incubation with hexapeptide, $p < 0.05$.

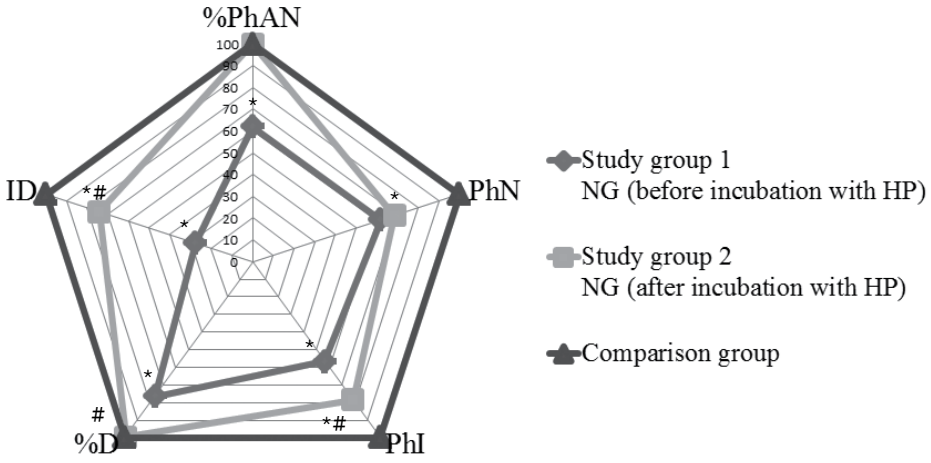

Figure 1. Effect of hexapeptide (HP) on phagocytic activities of NG in COVID-19

Note. *, differences from the CG, $p < 0.05$; #, differences from SG with moderate course COVID-19, $p < 0.05$

mined $CD64^{-}CD16^{bright}CD32^{dim}CD11b^{dim}$ NG versus $CD64^{-}CD16^{dim}CD32^{dim}CD11b^{mid}$ NG in comparison group (CG) reflects the reduced functionality of NG (Table 2). Defects in the phagocytic activity of NG were revealed: a decrease in the percentage of active phagocytic neutrophilic granulocytes (%), absorbing and killing ability (%) ($p_{1-3} < 0.05$) (Figure 1).

The effects of exposure HP *in vitro* are shown in Table 2. There was a decrease in the number of NG in the subset $CD64^{+}CD16^{+}CD32^{+}CD11b^{+}$ to the values of the comparison group ($p > 0.05$). Parallel the phenotype of the subset is changed with a decrease in MFI CD16 to comparison group values ($p > 0.05$) $CD64^{bright}CD16^{dim}CD32^{mid}CD11b^{bright}$. The receptor equipment of NG in the main subset has changed $CD64^{-}CD16^{+}CD32^{+}CD11b^{+}$, there was a decrease in MFI CD16 and an increase in MFI CD32 ($p > 0.05$) and a decrease in MFI CD11b ($p_{1-3} < 0.05$) to the values of CG – $CD64^{-}CD16^{mid}CD32^{mid}CD11b^{mid}$NG. The revealed remodeling of the NG phenotype transformed during COVID-19 under the influence of HP also led to the restoration of the phagocytic function of NG (Figure 1).

Conclusion

Thus, a positive effect of the Arginyl-alpha-Aspartyl-Lysyl-Valyl-Tyrosyl-Arginine peptide on the phenotypes of functionally significant subsets actively involved in antiviral protection and on the function of NG in moderate COVID-19 was shown *in vitro*. Unidirectional modulating effects were revealed that contributed to the restoration of the phenotype of regulatory subsets $CD16^{+}IFN\alpha/\beta R1^{-}CD119^{+}$, $CD16^{+}IFN\alpha/\beta R1^{+}CD119^{-}$, expressing receptors for IFN I II types to the indicators of the comparison group. Subset phenotype remodeling noted $CD64^{-}CD16^{+}CD32^{+}CD11b^{+}$ NG, $CD64^{+}CD16^{+}CD32^{+}CD11b^{+}$ NG ensure the usefulness of effector functions from hyperactivated to normal, which is confirmed by the restoration of the defective phagocytic activity of NG. The data obtained open up prospects for the creation of new methods of immunomodulatory therapy for the restoration of NG dysfunctions in COVID-19 with the inclusion of the hexapeptide Arginyl-alpha-Aspartyl-Lysyl-Valyl-Tyrosyl-Arginine, which is the active ingredient of a Russian registered medicinal product.

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ХОЛЕКАЛЬЦИФЕРОЛ В РОЛИ СРЕДСТВА НЕСПЕЦИФИЧЕСКОЙ ИММУНОПРОФИЛАКТИКИ COVID-19

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Резюме. Актуальным направлением научного поиска последних лет стало исследование иммунобиологических свойств витамина D. Целью данной работы стал анализ результатов перорального применения холекальциферола в целях предупреждения инфицирования вирусом SARS-CoV-2 в первую волну пандемии COVID-19. Исследование выполнено в период с 07 октября по 29 декабря 2020 года, когда отсутствовали иммунобиологические препараты для специфической профилактики COVID-19. Общее количество респондентов составило 73 человека, все однократно перенесли новую коронавирусную инфекцию. Этиологическая диагностика заболевания включала молекулярно-генетическое тестирование полученных общепринятым способом образцов двух локализаций (носоглотка, ротоглотка). Концентрация антител к вирусу определена в среднем через 2 месяца после болезни с использованием набора реагентов SARS-CoV-2-IgG количественный-ИФА-Бест (АО «Вектор-Бест», Россия). Ориентировочную оценку концентрации IgM осуществляли с использованием набора SARS-CoV-2-IgM-ИФА-Бест того же производителя. Среди участников исследования были такие, кто в целях профилактики инфицирования использовал иммунобиологические препараты (риамиловир, умифеновира гидрохлорида моногидрат, интерферон альфа-2b человеческий рекомбинантный, ацетат цинка, витамин С), в частности 28 человек (38,4%) принимали холекальциферол (группа № 1) и 45 человек (61,6%) не использовали его (группа № 2). Статистическая обработка полученных данных произведена с использованием статистического пакета STATISTICA v.12.5.192.5 (StatSoft, Inc., USA). Применен анализ базовых статистик, Linear Discriminant Analysis, Kolmogorov–Smirnov test, Chi-Square test, Wald–Wolfowitz Runs Test, Kruskal–Wallis test.

Выявлены отличия в частоте развития респираторного дистресс-синдрома двух изученных групп: у пациентов, принимавших холекальциферол синдром не развивался совсем, в группе № 2 он регистрировался в 20,0% случаев (Chi-Square = 5,242, p = 0,02). Помимо этого, у пациентов группы № 1 концентрация IgG через 2 месяца после болезни была в 3,8 раз выше значений в группе № 2 (Chi-Square = 9,268, p = 0,003). Сходные отличия выявлены и для уровня IgM (Wilks' Lambda: 0,659 approx. F (7,32) = 2,367 p < 0,045). Было известно, что в обеих группах присутствовали респонденты, применявшие в профилактических целях и другие иммуноактивные вещества. В первой группе таких было 18 человек (24,7% от всех), во второй – 13 человек (17,8% от всех). Установлено, что те, кто использовал другие иммуноактивные вещества и не принимал витамин D перенесли заболевание легче всех остальных. Следующими по степени тяжести перенесенной инфекции были респонденты,

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не использовавшие никаких иммунопрофилактических средств. Респонденты, принимавшие холекальциферол, преимущественно оценили тяжесть инфекции как среднюю. Участники исследования, принимавшие и витамин D и использовавшие другие средства профилактики, наиболее тяжело перенесли COVID-19. Респонденты, принимавшие холекальциферол, чаще других сообщали о длительно сохраняющейся утомляемости, об обострении хронических и появлении новых заболеваний (гипертоническая болезнь, кардиалгия, бронхиальная астма, аллергия, снижение остроты зрения), впервые появившихся мышечных, суставных и позвоночных болях. Феномен артралгий и других поражений крупных суставов при COVID-19 описывался нами ранее. В исследованиях других авторов также сообщается о частых жалобах на повышенную утомляемость и боли в суставах. При этом роль витамина D рассматривается исключительно с позиции его недостаточности при новой коронавирусной инфекции и его потенциальной роли в ингибировании гипервоспалительных реакций, а также ускорении процесса заживления пораженных участков, особенно в легочной ткани.

Установлено, что прием витамина D не влиял на частоту возникновения лихорадочного состояния, частоту развития пневмонии легких, объем поражения тканей легких (на основании данных компьютерной томографии), длительность госпитализации и заболевания в целом, а также не предотвращал развитие аносмии и дисгевзии. Использование витамина D, как протективного средства для предотвращения инфицирования вирусом SARS-CoV-2, оказало влияние на снижение частоты / предотвращение случаев респираторного дистресс-синдрома в процессе заболевания. Также у принимавших витамин D зафиксировано увеличение образования IgG к вирусу SARS-CoV-2 через 2 месяца после инфицирования 3,8 раза выше значений, зарегистрированных у респондентов, не принимавших колекальциферол. Участники, принимавшие холекальциферол, переносили инфекцию тяжелее, особенно, если использовали еще какие-либо протективные вещества. Также при превентивном приеме витамина D после COVID-19 дольше сохранялась повышенная утомляемость, чаще сообщалось о появлении новых и активации хронических заболеваний и впервые появившихся мышечных, суставных и позвоночных болях, что соотносится с полученными нами ранее данными.

Ключевые слова: COVID-19, холекальциферол, риамиловир, умифеновира гидрохлорида моногидрат, аскорбиновая кислота, цинк, IFNα-2b человеческий рекомбинантный, сустав, позвоночник

CHOLECALCIFEROL AS A MEANS OF NONSPECIFIC IMMUNOPROPHYLAXIS AGAINST COVID-19

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Abstract. The current direction of scientific research in recent years has been the study of the immunobiological properties of vitamin D. The purpose of this work was to analyze the results of oral administration of cholecalciferol in order to prevent infection with the SARS-CoV-2 virus in the first wave of the COVID-19 pandemic. The study was performed in the period from October 07 to December 29, 2020, when there were no immunobiological drugs for specific prevention of COVID-19. The total number of respondents was 73 people; all had been ill with coronavirus only once. The etiological diagnosis of the disease included molecular genetic testing of samples of two localizations obtained by the conventional method (nasopharynx, oropharynx). The concentration of antibodies to the virus was determined on average 2 months after the disease using a set of reagents SARS-CoV-2-IgG quantitative-ELISA-Best (JSC Vector-Best, Russia). An approximate assessment of IgM concentration was carried out using a set of SARS-CoV-2-IgM-ELISA-Best from the same manufacturer. Among the study participants were those who used immunobiological drugs for the prevention of infection (riamilovir, umifenovir hydrochloride monohydrate, human recombinant interferon alpha-2b, zinc acetate, vitamin C). In particular, 28 people (38.4%) took cholecalciferol (group No. 1) and 45 people (61.6%) did not use this (group No. 2). Statistical processing of the obtained data was performed using the statistical package STATISTICA v.12.5.192.5 (StatSoft, Inc., USA). We applied the analysis of basic statistics, Linear Discriminant Analysis, Kolmogorov–Smirnov test, Chi-Square test, Wald–Wolfowitz Runs Test, Kruskal–Wallis test.

Differences in the incidence of respiratory distress syndrome of the two studied groups were revealed: in patients taking cholecalciferol, the syndrome did not develop at all; in group No. 2, it was registered in 20.0% of cases (Chi-Square = 5.242, p = 0.02). In addition, in patients of group No. 1, the concentration of IgG 2 months after the disease was 3.8 times higher than the values in group No. 2 (Chi-Square = 9.268, p = 0.003).

Similar differences were found for the IgM level (Wilks' Lambda: 0.659 approx. F (7.32) = 2.367 p < 0.045). It was known that in both groups there were respondents who used other immuno-active substances for preventive purposes. In the first group there were 18 people (24.7% of all); in the second, there were 13 people (17.8% of all). It was found that those who used other immuno-active substances and did not take vitamin D suffered the disease more easily than everyone else. The respondents who did not use any immunoprophylactic agents were the next in terms of the severity of the infection. The respondents who took cholecalciferol mainly assessed the severity of the infection as average. The study participants who took both vitamin D and used other means of prevention suffered the most from COVID-19. Respondents who took cholecalciferol more often than others reported long-term fatigue, exacerbation of chronic and the appearance of new diseases (hypertension, cardialgia, bronchial asthma, allergies, decreased visual acuity), muscle, joint and vertebral pains that appeared for the first time. The phenomenon of arthralgia and other lesions of large joints in COVID-19 was described by us earlier. Studies by other authors also report frequent complaints of increased fatigue and joint pain. At the same time, the role of vitamin D is considered exclusively from the standpoint of vitamin deficiency in a new coronavirus infection and its potential role in inhibiting hyperinflammatory reactions, as well as accelerating the healing process of affected areas, especially in lung tissue.

It was found that vitamin D intake did not affect the incidence of fever, the incidence of pneumonia, the volume of lung tissue damage (based on computed tomography data), the duration of hospitalization and the disease as a whole, and also did not prevent the development of anosmia and dysgeusia. The use of vitamin D as a protective agent to prevent infection with the SARS-CoV-2 virus has had an impact on reducing the frequency/prevention of cases of respiratory distress syndrome during the disease. Also, those who took vitamin D recorded an increase in the formation of IgG to the SARS-CoV-2 virus 2 months after infection 3.8 times higher than the values recorded in respondents who did not take cholecalciferol. The participants who took cholecalciferol suffered the infection more severely, especially if they used any other protective substances. Also, with the preventive intake of vitamin D after COVID-19, increased fatigue persisted longer, the appearance of new and activation of chronic diseases and muscle, joint and vertebral pains that appeared for the first time were reported more often, which correlates with the data we received earlier.

Keywords: COVID-19, cholecalciferol, riamilovir, umifenovir hydrochloride monohydrate, ascorbic acid, zinc, IFNα-2b human recombinant, joint, spine

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Introduction

The current direction of scientific research in recent years has been the study of the immunobiological properties of vitamins, in particular substances belonging to group D [12]. It is no secret that the surge of such interest is associated with the appearance of commercially available test systems on the market. Great hopes were pinned on the use of vitamin D as a means of reducing the spread of the 2019 pandemic. The assumptions were based on the established facts of the involvement of 25(OH)D in the regulatory [7] and protective [1] reactions in a new coronavirus infection. Previously, it was reported that cholecalciferol may have some protective properties in relation to reducing the risk of infection with the SARS-CoV-2 virus, the severity of the infectious process [10], as well as mortality from COVID-19 [5].

Data were presented on the important role of vitamin D in the prevention of the persistence of the pathogen in the human population [13]. A local study aimed at studying the feasibility of using cholecalciferol for the prevention of a new coronavirus infection, regardless of the use of specific immunotropic drugs, in particular vaccines, remains relevant. The purpose of this work was to analyze the results of oral administration of cholecalciferol in order to prevent infection with the SARS-CoV-2 virus in the first wave of the COVID-19 pandemic.

Materials and methods

The study was performed in the period from October 07 to December 29, 2020 (in the first wave of a new coronavirus infection) at the time of the absence of immunobiological drugs developed and approved for clinical use for specific prevention of COVID-19. There were no registered vaccines at the time of the study. This made it possible to evaluate the protective properties of vitamin D in infection caused by the SARS-CoV-2 virus.

The study of the data was based on the fulfillment of two mandatory conditions for respondents. The first condition was: the presence of direct contact with a primarily untreated contingent of patients, among whom there could potentially be and were cases of a new coronavirus infection. The second condition was: compliance with generally accepted preventive measures, primarily barrier measures, to prevent infection with the SARS-CoV-2 virus, namely: the use

of personal protective equipment (disposable medical masks), hand sanitizing liquids / wearing disposable gloves and distancing, such as could be possible in the conditions of performing their work functions.

All study participants personally filled out questionnaires to assess the nature and severity of the course of a new coronavirus infection, premorbid and postmorbid status, and also gave written voluntary informed consent to the use of the information obtained, including medical information. The above gave grounds to assert that the rights of patients specified in the provisions of the Order of the Ministry of Health of the Russian Federation No. 266 of 19.06.2003 "On approval of the Rules of Clinical Practice in the Russian Federation", international documents based on the "Helsinki Declaration of the World Medical Association" and its subsequent editions, documents of the United Nations, were not violated. The life and health of the participants of the clinical and laboratory study were not in danger. Before the analytical work, all open personal data was anonymized. The study and analysis of the collected information was carried out with the approval of the local ethical committee of the medical organization.

All the study participants were employees of a multidisciplinary medical institution that provided round-the-clock emergency care to children in Ekaterinburg. The total number of respondents was 73 people. At the time of receiving the data, all participants had suffered a new coronavirus infection once. This fact was attested in the medical documentation. The causative agent of the disease is the SARS-CoV-2 virus. The diagnosis of a new coronavirus infection has been confirmed by clinical and laboratory studies. The etiological diagnosis of the disease included molecular genetic testing of samples of two localizations obtained by the conventional method (nasopharynx, oropharynx) in accordance with the provisions of the temporary methodological recommendations of the Ministry of Health of the Russian Federation. Ribonucleic acid of the SARS-CoV-2 virus was detected in all cases of infection. The concentration of antibodies to the virus was determined on average 2 months after the disease using a set of reagents SARS-CoV-2-IgG quantitative-ELISA-Best (D-5505, RU No. RZN 2021/14458, JSC Vector-Best, Russia). An approximate assessment of the concentration of IgM was carried out using a set of SARS-CoV-2-IgM-IFA-Best (D-5502, RU No. RZN 2020/10389, JSC Vector-Best, Russia).

The collection of additional information about the study participants included information about the presence of previous diseases (autoimmune, allergic, infectious, cardiovascular, and others) and addictions (tobacco smoking). The changes recorded after the disease were studied: the appearance of new or exacerbation of chronic diseases. The nature of the course of COVID-19 was also investigated: changes in the state of health, syndromes, the use of medicines and others were detected. In total, more than 70 positions were studied.

Among the study participants were those who, on their own initiative (without consulting a doctor), used immunobiological drugs to prevent infection with the SARS-CoV-2 virus. The duration of measures to prevent the disease was at least three weeks. It was found that the following were used orally: riamilovir (250 mg three times a day), umifenovir hydrochloride monohydrate (100 mg twice a week), ascorbic acid (a solution of 250 mg of dry matter in 200 mL of boiled chilled water twice a day), zinc acetate (100 mg once a day [9]), cholecalciferol (625-1250 IU once a day). Human recombinant interferon alpha-2b was also administered intranasally at a dosage of 3000 ME in each nasal passage twice a day. None of the study participants had previously been vaccinated against the SARS-CoV-2 virus. The decision to use non-specific immunoprophylactic agents was made by the participants independently after the WHO announced a pandemic of coronavirus infection. Taking into account the fact that all respondents were employees of a medical institution, including doctors, nursing staff, or had access to consultations on the specifics of taking immunobiological agents, in this study we believed that the implementation of preventive measures for the use of the above substances was carried out exactly in accordance with the described schemes.

Among the total number of participants, 28 people (38.4%) took cholecalciferol in order to prevent infection with the SARS-CoV-2 virus (group No. 1) and 45 people (61.6%) did not use it (group No. 2). In group No. 1 there were 8 (28.6%) doctors, 10 (35.7%) people with secondary and 2 (7.1%) with junior medical education, as well as 8 (28.6%) people of other medical institution personnel; group No. 2 consisted of 14 (31.1%) doctors, 26 (57.8%) nurses, 1 (2.2%) junior medical officer, as well as 4 (8.9%) specialist hospital support staff. In the first group there were 2 (7.1%) men in the second – 8 (17.8%). Median and interquartile age range of group No. 1 was 54.0 (45.8-62.3) years, group No. 2 – 44.5 (32.0-49.0) years. Anthropometric data (height, weight, body mass index) had no significant differences between the groups and fluctuated within the physiological norm.

Statistical processing of the obtained data was performed using the Windows 10 operating system (Microsoft Corporation, USA): STATISTICA v.12.5.192.5 statistical package (StatSoft, Inc., USA). The data are presented in the form of the number of cases, percentage of the total number of people in the group, median (Me) and interquartile range ($Q_{0.25}$-$Q_{0.75}$). The studied indicators had mainly

a categorical type of data. The normality of the distribution was checked using the Kolmogorov–Smirnov test, where the value of $p < 0.05$ indicated an abnormal distribution of the studied data. The differences between the groups were evaluated using Chi-Square test. The significance level (p-value) of the probability of rejection of the accepted statistical hypothesis was considered equal to 0.05. To assess the differences between the two study groups, the Wald–Wolfowitz Runs Test was also used, the differentiation was based on p-level values < 0.05. The third criterion was the Kruskal–Wallis test, which was used to assess the significance of the differences between four unrelated groups. Linear Discriminant Analysis was used in the work.

The expert opinion on the possibility of open publication of the obtained data was approved by the members of the expert commission of the Institute of Immunology and Physiology of the Ural Branch of the Russian Academy of Sciences before the transfer of information to the open press.

Results and discussion

Seventy-three people were examined, of whom 28 (38.4%) took cholecalciferol in order to prevent infection with the SARS-CoV-2 virus (group No. 1) and 45 people (61.6%) did not use it (group No. 2). The first stage of the study was a frequency comparative analysis of the data obtained at different stages of observation: before infection with the virus, during the disease and after two months of observation.

It was found that patients who took vitamin D before the disease were more likely (3.1 times) to have metabolic syndrome or type 2 diabetes: 21.4% versus 6.7% in group No. 2. Also, recipients who used cholecalciferol had hypertension more often (3.2 times) before the disease (50.0% vs. 15.6% in group No. 2). At the same time, the revealed differences were not statistically significant (based on Chi-Square test and Wald–Wolfowitz Runs Test). It is assumed that on the one hand, the reason for the discovered fact could be some age difference between the groups. On the other hand, the presence of concomitant pathology in respondents could cause a desire to reduce the risk of infection with the virus. And during the announcement of the coronavirus pandemic, patients used non-specific immunoprophylactic agents.

We studied data on the presence of changes in the cardiovascular system before COVID-19 (in particular, the presence of coronary heart disease, chronic heart failure, myocardial infarction, stroke, and others), the pulmonary system (chronic obstructive disease, emphysema, and others), the immune system (autoimmune and allergic reactions), the excretory system (kidney diseases). There were no differences between the groups. Groups No. 1 and No. 2 also did not differ in the number of annual previously tolerated acute respiratory viral infections. The frequency of hemocontact infections (HIV, hepatitis B, C), the presence of addictions (smoking) were also similar in both groups. The drug therapy available before the disease (hormones, sedatives, antidepressants, etc.)

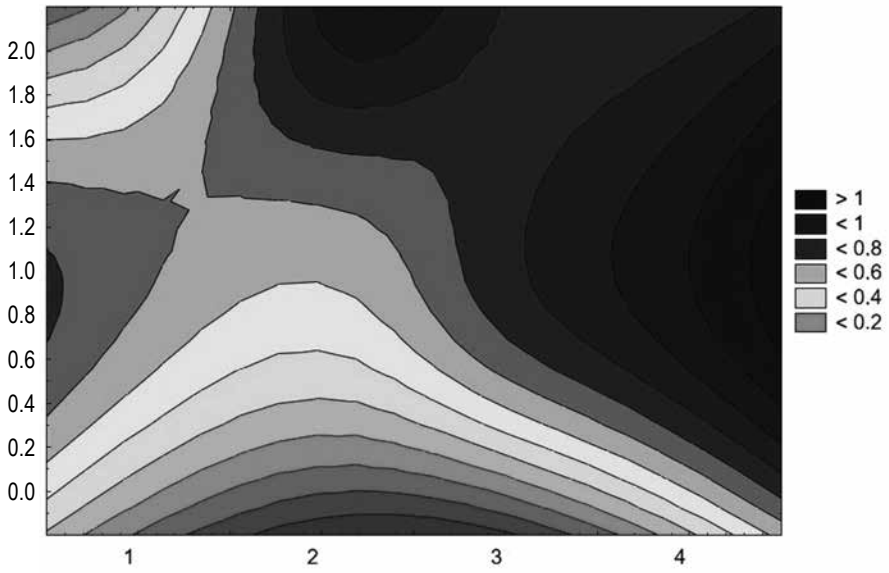

Figure 1. 3D Contour Plot of assessment of the severity of infection by study participants 2 months after the disease

Note. Horizontally, numbering of subgroups of participants: 1, did not use any immunoprophylactic agents (32 people); 2, did not take vitamin D, but took other immunoactive substances (13 people); 3, only vitamin D was taken to prevent infection (10 people); 4, vitamin D and other immunoactive substances were used (18 people); vertically, assessment of the severity of infection in points: 0, mild degree, 1, medium degree, 2, severe degree (darker color, heavier infection).

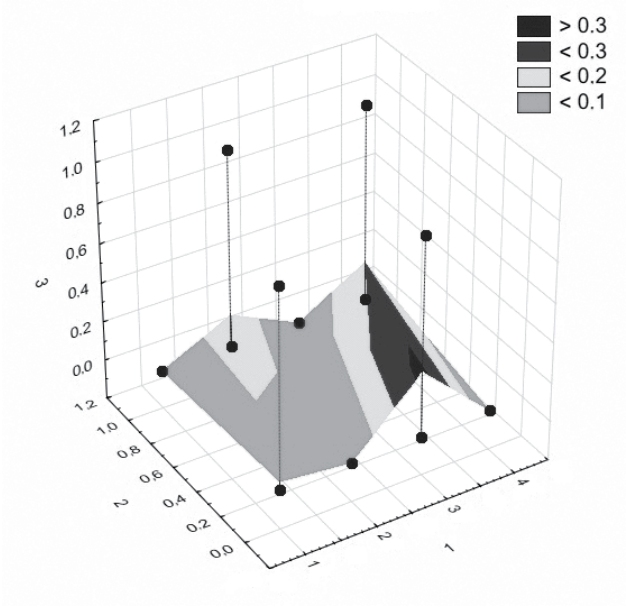

Figure 2. 3D Wafer Plot of assessment of the appearance of new diseases, as well as muscle and joint pain after Covid-19, depending on the use of immunoprophylactic agents

Note. On axis 1, numbering of subgroups of participants: 1, no immunoprophylactic agents were used; 2, did not take vitamin D, but took other immunoactive substances; 3, only vitamin D was taken to prevent infection; 4, vitamin D and other immunoactive substances were used; on axis 2, the appearance of joint and muscle pain (in points: 1, yes; 0, no); on axis 3, the appearance of new diseases after COVID-19 (in points: 1, yes; 0, no).

was the same. In general, the premorbid status of respondents in both groups was similar.

Analyzing the data on changes during COVID-19, it was found that the frequency of feverish conditions and the development of pneumonia in groups No. 1 and No. 2 was the same. None of the study participants were in the intensive care unit or on artificial ventilation. The median hospitalization time for groups No. 1 and No. 2 was 4 (0-11) and 0 (0-10) days, the duration of treatment was 21 (16-30) and 21 (14-28) days, respectively. Prophylactic administration of cholecalciferol had no effect on the development of pulmonary insufficiency and on the volume of lung tissue damage. The destruction was assessed by studying the results of computed tomography. Also, during the illness, there were no significant differences between the groups in the frequency of occurrence of neurological disorders, anosmia, dysgeusia and DIC syndrome. At the same time, there were differences in the frequency of development of respiratory distress syndrome. In particular, in patients taking cholecalciferol, the syndrome did not develop at all, in group No. 2 it was registered in 20.0% of cases (Chi-Square = 5.242, p = 0.02).

In this study, clinical and laboratory data after COVID-19 were evaluated. It was found that in patients of group No. 1, the concentration of IgG after 2 months was 3.8 times higher than the values in group No. 2 (Chi-Square = 9.268, p = 0.003) and amounted to 18.8 (18.0-21.7) BAU/mL (binding antibody units), whereas in patients who did not take vitamin D, IgG level was 5.0 (4.8-5.6) BAU/mL. The use of Linear Discriminant Analysis (Discriminant Function Analysis) allowed us to establish that in addition to the concentration of IgG in patients of the two groups, the level of class M immunoglobulins had statistically significant differences (Wilks' Lambda: 0.659 approx. F (7.32) = 2.367 p < 0.045) – after taking cholecalciferol, it was higher.

If we recall, in both groups (No. 1 and No. 2) there were respondents who used other immunoactive substances for preventive purposes, such as: riamilovir, umifenovir hydrochloride monohydrate, human recombinant interferon alpha-2b, zinc acetate, vitamin C. In the first group there were 18 such people (24.7% of all participants, 64.3% of those who took cholecalciferol). In the second group, there were 13 such respondents (17.8% of all participants, 28.9% of those who did not take cholecalciferol). Analysis of the data obtained using the Kruskal–Wallis test for four unrelated groups in assessing the differences in the severity of the infection showed the following. A graphical explanation of the data obtained is presented in Figure 1.

It was found that those who used other immunoactive substances and did not take vitamin D suffered the disease more easily than everyone else. The next in severity of infection were respondents who did not use any immunoprophylactic agents. Respondents who took cholecalciferol mainly assessed the severity of infection as average. The study participants who took both vitamin D and used other means of prevention suffered the most from COVID-19. An additional pairwise comparison of the data obtained showed that there were significant differences between those who did nothing and those who selectively took vitamin D (Chi-Square = 4.421, p = 0.004).

In this study, additional information was obtained that respondents who took cholecalciferol were more likely than others to report long-term fatigue (at least up to two months after the disease), as well as exacerbation of chronic and the appearance of new diseases (hypertension, cardialgia, bronchial asthma, allergies, decreased visual acuity), first-time muscle, joint and vertebral pains (Figure 2).

The phenomenon of arthralgia and other lesions of large joints in COVID-19 has already been described by us earlier [4]. Studies by other authors also report frequent complaints of increased fatigue, joint pain and myalgia, in general, musculoskeletal symptoms of

COVID-19 [6, 8]. At the same time, the role of vitamin D is considered exclusively from the standpoint of its insufficiency in a new coronavirus infection and its potential role in inhibiting hyperinflammatory reactions, as well as accelerating the healing process of affected areas, especially in lung tissue [2, 3].

Currently, the post-acute sequelae of COVID-19 is widely studied [11], which can manifest itself by the activation of chronic diseases and the appearance of new diseases due to infection [14]. As part of this, new information about the course of the distant period of COVID-19 is expected to appear in the near future. Also in this regard, do not forget that the effect of vitamin D can be not only phenotypic, but also determined by the polymorism of genes that regulate the transport and metabolism of the compound. The differences may also be related to insolation and other factors that deserve additional study [15].

Conclusion

In this study, the results of daily oral administration of cholecalciferol at a dose of 625-1250 IU were evaluated in order to prevent infection with the SARS-CoV-2 virus. It was found that vitamin D intake did not affect the incidence of fever, the incidence of lung pneumonia, as well as the volume of lung tissue damage (based on computed tomography data), the duration of hospitalization and the disease as a whole, and also did not prevent the development of anosmia and dysgeusia.

The use of vitamin D as a protective agent to prevent infection with the SARS-CoV-2 virus has had an impact on reducing the frequency / prevention of cases of respiratory distress syndrome during the disease. In particular, not a single case of this syndrome was detected in those taking cholecalciferol, whereas in the rest the syndrome was detected in 20% of cases. Also, those taking vitamin D recorded an increase in the formation of IgG to the SARS-CoV-2 virus 2 months after infection 3.8 times higher than the values recorded in respondents who did not take cholecalciferol.

Participants who took cholecalciferol suffered the infection more severely, especially if they used any other protective substances. Also, with the preventive intake of vitamin D after COVID-19, increased fatigue persisted longer, the appearance of new and activation of chronic diseases and muscle, joint and vertebral pains that appeared for the first time were reported more often, which corresponds to our data obtained earlier.

The limitation of the information obtained in this study may be a small sample of the study and, perhaps, as we now believe, a low dose of vitamin D taken, which was due to the lack of recommendations on the amount of vitamin intake at the time of the announcement of the COVID-19 pandemic.

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ЗНАЧЕНИЕ NOTCH-СИГНАЛИНГА В РЕГУЛЯЦИИ ДИФФЕРЕНЦИРОВКИ Treg-ЛИМФОЦИТОВ У БОЛЬНЫХ С ИНФИЛЬТРАТИВНЫМ ТУБЕРКУЛЕЗОМ ЛЕГКИХ

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Резюме. В современной литературе активно накапливаются данные о роли регуляторных Т-лимфоцитов (Treg) в иммунопатогенезе туберкулеза. Подавляющее действие Treg-клеток на пролиферацию, функциональную активность Th1-лимфоцитов и антигенпрезентирующих клеток позволяет рассматривать данную популяцию в качестве возможной мишени модуляции иммунного ответа у больных туберкулезом. Сигнальный путь Notch принимает участие в регуляции экспрессии транскрипционного фактора FoxP3 и, следовательно, способен поддерживать супрессорную активность Treg-лимфоцитов. Ключевая роль в функционировании сигнального каскада Notch принадлежит ферменту γ-секретазе, отщепляющему внутриклеточный домен рецептора (Notch ICD) с последующим образованием комплекса, регулирующего дифференцировку клеток. Активно изучаемым ингибитором γ-секретазы является DAPT – N-[N-(3,5-дифторфенацетил)-L-аланил]-S-фенилглицин трет-бутиловый эфир). Материалом для исследования служили мононуклеарные лейкоциты, выделенные из крови больных лекарственно-чувствительным и лекарственно-устойчивым туберкулезом легких методом градиентного центрифугирования до начала противотуберкулезной терапии. Клетки культивировали в условиях стимуляции антигенами микобактерий туберкулеза CFP10-ESAT6 или с добавлением в инкубационную среду ингибитора γ-секретазы (DAPT) в дозах 5 мкМ/л и 10 мкМ/л в комбинации с CFP10-ESAT6 при 37 °С и 5% CO_2 в течение 72 ч. Количество Treg-лимфоцитов оценивали методом проточной цитофлуориметрии путем определения экспрессии поверхностного рецептора CD4 (FITC) и внутриклеточного транскрипционного фактора FoxP3 (PE). В интактных культурах клеток больных туберкулезом легких относительное количество Treg-лимфоцитов статистически значимо ($p < 0,001$) превышало аналогичные показатели у здоровых доноров. Стимуляция клеток антигенами CFP10-ESAT6 сопровождалась увеличением доли $CD4^+FoxP3^+$-клеток в обеих группах больных туберкулезом. Добавление в инкубационную среду ингибитора γ-секретазы в концентрации 5 мкМ/л не приводило к статистически значимым изменениям количества Treg-лимфоцитов. Увеличение концентрации DAPT до 10 мкМ/л сопровождалось уменьшением количества Treg-лимфоцитов

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по сравнению с соответствующими показателями при стимуляции антигенами CFP10-ESAT6 во всех группах обследуемых. Вне зависимости от условий культивирования число $CD4^+FoxP3^+$-клеток у пациентов с лекарственной устойчивостью микобактерий превышало их количество у больных лекарственно-чувствительным туберкулезом легких. Угнетение сигнального пути Notch при помощи ингибитора γ-секретазы (DAPT) в концентрации 10 мкМ/л способствует снижению количества Treg-лимфоцитов у больных лекарственно-чувствительным и лекарственно-устойчивым туберкулезом легких. Уменьшение числа Treg-лимфоцитов при помощи ингибитора γ-секретазы подтверждает значение сигнального каскада Notch как потенциально возможной мишени для коррекции иммунносупрессорной активности Treg-лимфоцитов и патогенетической терапии туберкулеза.

Ключевые слова: Notch, Т-регуляторные клетки, дифференцировка, лекарственная устойчивость, туберкулез легких, гамма-секретаза, DAPT

SIGNIFICANCE OF NOTCH SIGNALING IN THE REGULATION OF Treg LYMPHOCYTE DIFFERENTIATION IN PATIENTS WITH INFILTRATIVE PULMONARY TUBERCULOSIS

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Abstract. Data on the role of regulatory T lymphocytes (Treg) in the immunopathogenesis of tuberculosis are actively accumulating in the current literature. The overwhelming effect of Treg cells on the proliferation, functional activity of Th1 lymphocytes and antigen-presenting cells allows to consider this population as a possible target of modulation of the immune response in patients with tuberculosis. The Notch signaling pathway participates in the regulation of FoxP3 transcription factor expression and, therefore, is capable of supporting suppressor activity of Treg lymphocytes. A key role in the functioning of the Notch signaling cascade belongs to the enzyme γ-secretase that cleaves the intracellular domain of the receptor (Notch ICD), with the subsequent formation of a complex that regulates cell differentiation. The actively studied inhibitor of γ-secretase is DAPT – N-[N-(3.5-difluorophenacetyl)-L-alanyl]-S-phenylglycine tert-butyl ester). Mononuclear leukocytes isolated from the blood of patients with drug-sensitive and drug-resistant pulmonary tuberculosis by gradient centrifugation before the start of anti-tuberculosis therapy were used as the material for the study. The cells were cultured under conditions of stimulation with *Mycobacterium tuberculosis* antigens CFP10-ESAT6 or with the addition of γ-secretase inhibitor (DAPT) at doses of 5 μM/L and 10 μM/L in combination with CFP10-ESAT6 at 37 °C and 5% CO_2 for 72 h to the incubation medium. The number of Treg lymphocytes was assessed by flow cytofluorimetry by determining the expression of the CD4 surface receptor (FITC) and the intracellular transcription factor FoxP3 (PE). In intact cell cultures of pulmonary tuberculosis patients, the relative number of Treg lymphocytes was statistically significantly ($p < 0.001$) higher than that of healthy donors. Stimulation of cells with CFP10-ESAT6 antigens was accompanied by an increase in the proportion of $CD4^+FoxP3^+$ cells in both groups of tuberculosis patients. Addition of γ-secretase inhibitor at a concentration of 5 μM/L to the incubation medium did not lead to statistically significant changes in the number of Treg lymphocytes. The increase in DAPT concentration up to 10 μM/L was accompanied by a decrease in the number of Treg lymphocytes in comparison with the corresponding indices upon stimulation with CFP10-ESAT6 antigens in all groups of the subjects. Regardless of cultivation conditions, the number of $CD4^+FoxP3^+$ cells in patients with drug-resistant mycobacteria exceeded their number in patients with drug-sensitive pulmonary tuberculosis. Inhibition of the Notch signaling pathway by a γ-secretase inhibitor (DAPT) at a concentration of 10 μM/L contributed to a decrease in the number of Treg lymphocytes in patients with drug-sensitive and drug-resistant pulmonary tuberculosis. Reduction of Treg lymphocyte number by γ-secretase inhibitor confirms the importance of Notch signaling cascade as a potential target for correction of Treg lymphocytes immunosuppressive activity and pathogenetic therapy of tuberculosis.

Keywords: Notch, T regulatory cells, differentiation, drug resistance, pulmonary tuberculosis, gamma-secretase, DAPT

Introduction

Protective control over the infectious process caused by *Mycobacterium tuberculosis* is ensured by the cooperative interaction of multiple immunocompetent cells, realized through juxtacrine and paracrine mechanisms [12]. Data on the role of regulatory T lymphocytes (Treg) in pathogenesis of immune response at pulmonary tuberculosis (PT) are actively accumulating in the modern literature [2, 3]. The immunosuppressive function of Treg cells is implemented through the secretion of cytokines (IL-10, TGF-β and IL-35), suppression of expression of costimulation molecules (CD80 and CD86) necessary for activation of helper T cells type 1 (Th1), and induction of granzyme-dependent apoptosis of target cells by dendritic cells [2]. Increased number of Treg lymphocytes in patients with PT correlates with multidrug resistance of the pathogen, bacillary load, and is also accompanied by chronicization and aggravation of the pathological process [8]. The overwhelming effect of Treg-cells on the proliferation and functional activity of Th1 lymphocytes allows us to consider this population as a possible target of modulation of the immune response in patients with tuberculosis [5]. The Notch signaling pathway has been shown to be an important mechanism of intercellular signaling that regulates innate and adaptive immune response responses [7, 10].

The literature presents data indicating the importance of the Notch molecular cascade in the pathogenesis of tuberculosis. It has been established that stimulation of mouse macrophages is accompanied by an increase in the expression of Notch receptors and its ligands, Jagged 1, Dll 1 and Dll 4. The progressive course of tuberculosis infection is associated with an increase in the number of monocytes/macrophages and dendritic cells expressing Notch-receptor ligand – Dll 4 [1, 9, 11]. The results of experiments on cell lines demonstrate the critical importance of Notch-1 signaling pathway in the regulation of transcription factor FoxP3 expression and, as a consequence, the maintenance of immunosuppressive function of Treg cells [1].

A key role in the functioning of the Notch signaling cascade belongs to γ-secretase, an enzyme that cleaves the intracellular domain of the receptor (Notch ICD), with the subsequent formation of a complex that regulates cell differentiation. A known inhibitor of γ-secretase, which is being actively studied, is DAPT – N-[N-(3.5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester [4, 6, 10, 13].

Materials and methods

The study involved 15 patients with newly diagnosed infiltrative pulmonary tuberculosis. The middle age of the patients was 45.4±6.58 years. Depending on the sensitivity of mycobacteria to anti- tuberculosis drugs, all patients were divided into two groups: 8 patients with drug-sensitive PT and 7 patients excreting mycobacteria resistant to at least isoniazid and rifampicin. The control group consisted of 8 healthy volunteers of comparable sex and age. The material for the study was whole peripheral venous blood taken before the start of anti-tuberculosis therapy. Mononuclear leukocytes were isolated from blood by gradient centrifugation (ρ = 1.077 g/mL). *Mycobacterium tuberculosis* antigens CFP10-ESAT6 (Diakintest, Generium, Russia) were added to the incubation medium at a dose of 10 μg/mL or γ-secretase inhibitor (DAPT, Tocris Bioscience, UK) at doses of 5 μM/L and 10 μM/L in combination with CFP10-ESAT6. Cells were cultured in complete RPMI-1640 medium with L-glutamine (BioloT LLC, Russia) at 37 °C and 5% CO_2 for 72 h. The number of Treg lymphocytes was estimated by flow cytofluorimetry by determining the expression of CD4 surface receptor (FITC, BD Biosciences, USA) and intracellular transcription factor FoxP3 (PE, BD Biosciences, USA). The results were processed using a statistical software package IBM SPSS Statistics 20. The Shapiro-Wilk test was used to check the correspondence of the data to the normal distribution law. Significance of differences in quantitative data was assessed using Mann–Whitney U nonparametric test. Wilcoxon test was used to assess the significance of differences in dependent data within the group. The results of statistical analysis were considered significant at the $p < 0.05$ level.

Results and discussion

The key role in the coordinated antigen-specific immune response to *Mycobacterium tuberculosis* belongs to homeostasis and dynamic interaction between dendritic cells, macrophages and various populations of T lymphocytes: Th1, Th2, Th17, Treg. The question about the molecular mechanisms of immunoregulatory imbalance remains open. One of the factors of pathological process progression in tuberculosis is excessive immunosuppressive activity of regulatory T cells. By inhibition of antigen-dependent differentiation and activation of apoptosis of Th1 lymphocytes, as well as suppressive influence on antigen-presenting cells, Treg population contributes to suppression of immune response and induces more severe course of disease with long-term persistence of pathogen.

The study showed that in patients with infiltrative drug-sensitive (DS) and drug-resistant (DR) PT in intact cultures the relative number of Treg lymphocytes ($CD4^+FoxP3^+$) was statistically

TABLE 1. RELATIVE CONTENT OF Treg LYMPHOCYTES IN PERIPHERAL BLOOD (% OF TOTAL LYMPHOCYTES) IN PATIENTS WITH INFILTRATIVE PULMONARY TUBERCULOSIS, Me ($Q_{0.25}$-$Q_{0.75}$)

Cultivation conditions in vitro	Healthy donors	Patients with infiltrative pulmonary tuberculosis	
		Drug-sensitive	Drug-resistant
Treg lymphocytes ($CD4^+FoxP3^+$)			
Intact culture	2.58 (2.37-3.15)	5.31 (5.24-5.39) $p_1 < 0.001$	4.88 (4.63-5.11) $p_1 < 0.001$ $p_4 = 0.022$
With added antigens (CFP10-ESAT6)	2.63 (2.43-3.21) $p_2 = 0.007$	5.7 (5.65-5.76) $p_1 < 0.001$ $p_2 = 0.012$	5.09 (4.78-5.22) $p_1 < 0.001$ $p_2 = 0.012$ $p_4 = 0.001$
With added antigens and DAPT (5 μM/L)	2.61 (2.41-3.2)	5.69 (5.62-5.73) $p_1 < 0.001$	5.09 (4.76-5.20) $p_1 < 0.001$ $p_4 = 0.001$
With added antigens and DAPT (10 μM/L)	2.39 (2.27-3.02) $p_3 = 0.008$	4.91 (4.86-5.00) $p_1 < 0.001$ $p_3 = 0.012$	4.15 (4.06-4.30) $p_1 < 0.001$ $p_3 = 0.012$ $p_4 = 0.001$

Note. p_1, level of statistical significance of differences compared with similar parameters in healthy donors; p_2, compared with baseline parameters (in intact cell culture); p_3, compared with parameters during antigen stimulation; p_4, compared with parameters in patients with drug-sensitive pulmonary tuberculosis.

significantly ($p < 0.001$) higher than that in healthy volunteers (Table 1). We may assume that increased number of $CD4^+FoxP3^+$ cells causes suppression of Th1-mediated immune response and may promote pathological process progression or, on the contrary, prevent formation of hyperergic immune reaction and lung tissue damage.

After stimulation of cell cultures with CFP10-ESAT6 antigens, we registered an increase in the proportion of $CD4^+FoxP3^+$ cells relative to initial values in both groups of patients and healthy donors (Table 1).

The addition of a γ-secretase inhibitor (DAPT) at a concentration of 5 μM/L to the cell suspension in combination with CFP10-ESAT6 antigens was not accompanied by statistically significant changes in the number of Treg lymphocytes both in PT patients and healthy donors (Table 1).

Increasing the DAPT dose up to 10 μM/L resulted in a decrease in the number of $CD4^+FoxP3^+$ cells compared to the corresponding indices upon stimulation with CFP10-ESAT6 antigens in all groups of subjects (Table 1).

It should be noted that regardless of cultivation conditions the number of Treg lymphocytes in patients with drug-resistant mycobacteria exceeded ($p_4 = 0.001$) their number in DS PT patients (Table 1). The obtained data may indicate a more pronounced inhibition of the functional activity of $CD4^+$Th1 lymphocytes, which provide cellular effector responses, in patients with DR PT.

Conclusion

Inhibition of the Notch signaling pathway by a γ-secretase inhibitor (DAPT) at a concentration of 10 μM/L contributes to a decrease in the number of Treg lymphocytes in patients with DS and DR PT. The reduction of Treg lymphocytes number by γ-secretase inhibitor confirms the importance of Notch signaling cascade as a potential target for correction of Treg lymphocytes immunosuppressive activity and pathogenetic therapy of tuberculosis.

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ФУНКЦИОНАЛЬНОЕ ИСТОЩЕНИЕ CD4$^+$Т-КЛЕТОК У ВИЧ/ВГС-КОИНФИЦИРОВАННЫХ ПАЦИЕНТОВ, ПОЛУЧАЮЩИХ ВЫСОКОАКТИВНУЮ АНТИРЕТРОВИРУСНУЮ ТЕРАПИЮ

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Резюме. ВГС-коинфекция широко распространена среди ВИЧ-инфицированных пациентов. В России до 50% ВИЧ-позитивных больных коинфицированы ВГС. Применение высокоактивной антиретровирусной терапии (ВААРТ), в большинстве случаев, приводит к подавлению репликации ВИЧ и восстановлению иммунной системы ВИЧ-инфицированных пациентов. Однако ВГС-коинфекция препятствует эффективному восстановлению CD4$^+$Т-клеток и повышает риск заболеваемости и смерти ВИЧ-инфицированных больных, получающих ВААРТ. Известно, что скорость прогрессии ВИЧ-инфекции и эффективность восстановления иммунной системы на фоне приема ВААРТ в значительной мере зависят от уровня активации иммунной системы и степени истощения CD4$^+$Т-клеток. Целью настоящей работы было определение уровня активации и истощения, а также цитокин-продуцирующей функции CD4$^+$Т-клеток, полученных из крови получающих ВААРТ ВИЧ/ВГС-коинфицированных и ВИЧ-моноинфицированных больных. В исследование были включены ВИЧ/ВГС-коинфицированные (n = 11) и ВИЧ-моноинфицированные (n = 10) пациенты, получающие ВААРТ более двух лет. В контрольную группу вошли 10 добровольцев без признаков ВИЧ- и ВГС-инфекций. Было установлено, что у ВИЧ/ВГС-коинфицированных пациентов, в сравнении со здоровыми людьми, повышены следующие показатели: доля активированных CD38$^+$HLA-DR$^+$ CD4$^+$Т-лимфоцитов ($p < 0,05$), уровень истощения CD4$^+$Т-клеток, определенный по плотности экспрессии TIGIT на поверхности каждой клетки ($p < 0,05$), и число CD4$^+$Т-лимфоцитов, способных производить интерферон-гамма (IFNγ) после активации ($p < 0,05$). Относительное число IFNγ-продуцирующих CD4$^+$Т-лимфоцитов в крови доноров позитивно коррелировало с долей активированных CD4$^+$Т-клеток ($R = 0,514$, $p < 0,01$). Важно отметить, что, несмотря на большое ко-

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личество IFNγ-продуцирующих CD4+Т-лимфоцитов, средняя продукция этого цитокина в CD4+Т-клетках ВИЧ/ВГС-коинфицированных больных была существенно ниже, чем у здоровых субъектов ($p < 0,05$). Продукция IFNγ в CD4+Т-лимфоцитах не зависела от степени их активации ($p > 0,05$). Негативная корреляционная связь была установлена между содержанием IFNγ и уровнем истощения CD4+Т-клеток ($R = -0,400$, $p < 0,05$). Показатели истощения CD4+Т-лимфоцитов также обратно коррелировали с содержанием CD4+Т-клеток в крови ($R = -0,598$, $p < 0,01$). Полученные сведения позволяют предположить, что ВГС-коинфекция приводит к выраженному функциональному истощению CD4+Т-клеток и тем самым может отягощать течение ВИЧ-инфекции у пациентов, принимающих ВААРТ.

Ключевые слова: ВИЧ-инфекция, гепатит С, CD4+Т-лимфоциты, иммунная активация, иммунное истощение, продукция цитокинов

FUNCTIONAL EXHAUSTION OF CD4+T CELLS IN HIV/HCV COINFECTED HAART-TREATED PATIENTS

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Abstract. Infection with hepatitis C virus (HCV) is common among HIV-positive patients, with up to 50% of them being coinfected in Russia. While highly active antiretroviral therapy (HAART) suppresses HIV replication and restores the immune system of HIV-infected subjects, HCV coinfection interferes with CD4+T cell regeneration and increases the risk of patients' morbidity and mortality. During HAART, HIV-infection progression and the immune system restoration efficiency largely depend on immune activation and CD4+T cell exhaustion. This study determined the level of activation, exhaustion, and cytokine production in CD4+T cells obtained from the peripheral blood of HAART-treated HIV/HCV coinfected and HIV monoinfected subjects. The study comprised 11 HIV/HCV coinfected individuals and 10 HIV monoinfected patients receiving HAART for more than two years, with a control group of 10 volunteers without the signs of HIV or HCV infections. Compared with healthy controls, HIV/HCV coinfected patients had an increased frequency of activated CD38+HLA-DR+ CD4+T lymphocytes ($p < 0.05$), a higher level of CD4+T cell exhaustion determined according to the TIGIT expression density per cell ($p < 0.05$), and a greater proportion of interferon-gamma (IFNγ)-producing CD4+T lymphocytes following activation ($p < 0.05$). The frequency of IFNγ-producing CD4+T cells in the donors' blood positively correlated with the proportion of activated CD4+T cells ($R = 0.514$, $p < 0.01$). Despite having a large number of IFNγ-producing CD4+T lymphocytes, the HIV/HCV coinfected patients' average production of IFNγ by CD4+T cells was significantly lower than that in healthy controls ($p < 0.05$). The IFNγ production in CD4+T lymphocytes did not depend on activation ($p > 0.05$). However, a negative correlation was established between the IFNγ production and the level of CD4+T cell exhaustion ($R = -0.400$, $p < 0.05$). The letter was also found to inversely correlate with the CD4+T cell counts in the donors' peripheral blood ($R = -0.598$, $p < 0.01$). These data suggest that HCV coinfection leads to pronounced functional exhaustion of CD4+T cells and may aggravate the course of HIV-infection in patients receiving HAART.

Keywords: HIV-infection, hepatitis C, CD4+T cells, immune activation, immune exhaustion, cytokine production

This work was carried out within the framework of the State assignment #121112500044-9.

Introduction

Due to shared transmission routes, hepatitis C virus (HCV) coinfection has become prevalent among HIV-positive individuals. Worldwide, approximately 25% of HIV-infected patients are coinfected with HCV [7], while in Russia the proportion of HIV/HCV coinfected subjects goes up to 50% [15]. The majority of HIV-positive people receive highly active antiretroviral therapy (HAART), which suppresses

HIV replication and leads to the immune system regeneration defined by the increase in the peripheral blood $CD4^+$T cell counts [6]. However, in HIV/HCV coinfected people receiving HAART, hepatitis C infection interferes with $CD4^+$T cell recovery [14]. Furthermore, compared with HAART-treated HIV-monoinfected subjects, HIV/HCV coinfected patients have a higher risk of developing non-AIDS-associated diseases [10], opportunistic infections [3], and death [1]. The mechanisms behind the negative effect of hepatitis C on the course of treated HIV-infection remain poorly understood.

Immune activation and $CD4^+$T cell exhaustion are critical for the natural history of HIV-infection, affecting both the pace of the disease progression and the immune restoration efficiency during HAART [9, 11]. In treated HIV-infected individuals, HCV coinfection has been shown to significantly increase the chronic immune activation and systemic inflammation levels [12]. However, it is unclear if it affects the $CD4^+$T cells' functional state. Therefore, the purpose of this study was to determine the level of $CD4^+$T cells' activation and exhaustion, as well as the cytokine-producing function of these lymphocytes obtained from the blood of HAART-treated HIV/HCV coinfected and HIV monoinfected patients.

Materials and methods

The work plan was approved by the ethics committee of the Perm Regional Center for the Prevention and Control of AIDS and Infectious Diseases (committee registration number IRB00008964). The study included HIV-infected patients receiving HAART for more than two years (viral load < 50 copies/mL): 1) HIV/HCV coinfected subjects (n = 11); and 2) HIV-monoinfected patients (n = 10). The healthy control group (HC) comprised 10 volunteers without HIV or HCV infections.

Blood was collected from the cubital vein into Vacutainer tubes containing ethylenediaminetetraacetic acid. $CD4^+$T lymphocytes count was assessed using the commercial BD Simultest™ IMK-Lymphocyte kit ("BD Biosciences", USA) with a CytoFLEX S flow cytometer ("Beckman Coulter", USA). The HIV viral load was determined using Versant HIV-1 RNA 3.0 assay b kits by means of a Versant 440 analyzer ("Siemens", Germany).

Mononuclear cells were isolated by density centrifugation using Diacoll (1.077 g/mL, "Diaem", Russia). The samples were stored in liquid nitrogen in a medium containing 90% fetal bovine serum (FBS, "Biowest", France) and 10% dimethyl sulfoxide ("AppliChem", Germany). In a day of the study, the cells were thawed at +37 °C, washed in 10 mL of complete culture medium (10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin ("Sigma", USA) in RPMI-1640), and then in 10 mL of Dulbecco's phosphate buffered saline (DPBS; "Gibco", USA). The cytokine-producing function of $CD4^+$T cells was assessed after 4 hours of incubation in complete culture medium containing phorbol 12-myristate-13-acetate (PMA), ionomycin, and brefeldin A ("BioLegend", USA).

Peripheral blood mononuclear cells were analyzed on CytoFLEX S flow cytometer ("Beckman Coulter", USA). Viable cells were identified by the absence of staining with the vital dye Zombie UV Fixable Viability Kit ("BioLegend", USA). Anti-CD3-BV605 and anti-CD4-PE antibodies ("BioLegend", USA) were used to identify $CD4^+$T lymphocytes. Expression of the TIGIT exhaustion marker was assessed using anti-TIGIT-AF488 antibodies ("BioLegend", USA). Anti-IFNγ-APC antibodies ("BioLegend", USA) were utilized to study the production of interferon-γ (IFNγ). Activated cells were identified using anti-CD38-PE/Fire700 ("BioLegend", USA) and anti-HLA-DR-APC-R700 ("BD Biosciences", USA) antibodies.

Statistical analysis and graph plotting were performed using the "Statistica 6" software. The data are presented as medians, interquartile ranges (25-75 percentile), and 10-90% intervals. Significance of differences between groups was established based on the Mann–Whitney U test. Correlation analysis was performed by the r-Pearson linear correlation test.

Results and discussion

All groups were similar according to age and gender (Table 1). The HIV-infection and HAART duration, HIV viral load, and $CD4^+$T cell counts did not differ between the groups comprised of HIV-infected subjects. However, $CD4^+$T cell counts in these groups were significantly reduced compared to HCs ($p < 0.05$).

In HIV/HCV coinfected patients compared with healthy individuals, the proportion of activated ($CD38^+HLA\text{-}DR^+$) $CD4^+$T lymphocytes was increased (Figure 1A; $p < 0.05$). Meanwhile, the difference between HIV monoinfected patients and healthy controls was not statistically significant ($p > 0.05$).

The level of exhaustion in $CD4^+$T cells of HIV/HCV coinfected subjects was also increased, as evidenced by the higher frequency of $TIGIT^+CD4^+$T lymphocytes ($p < 0.05$; data not shown) and the elevated expression of the inhibitory receptor TIGIT on the surface of these cells (Figure 1B; $p < 0.05$).

TABLE 1. CLINICAL CHARACTERISTICS OF HIV-INFECTED AND HEALTHY INDIVIDUALS

Characteristics	HIV/HCV coinfected patients	HIV monoinfected patients	Healthy Controls (HC)
Number of enrollments	11	10	10
Age (years)	40.0* (37.5-42.0)	43.5 (37.5-44.8)	43.0 (37.0-45.8)
Females (%)	45	50	50
HIV-infection duration (years)	16.0 (10.0-19.0)	9.5 (5.0-16.0)	–
HAART duration (years)	8.0 (3.8-13.0)	5.0 (2.5-6.2)	–
HIV viral load (copies/mL)	< 50**	< 50	–
CD4$^+$T cell counts (μL^{-1})	542 (405-663) pHC < 0.05	616 (480-763) pHC < 0.05	707 (646-880)

Note. *, the data presented as medians and interquartile ranges. **, the test-system sensitivity limit. HAART, highly active antiretroviral therapy. Significance of differences between groups was established based on the Mann–Whitney U test.

The number of CD4$^+$T cells that produced IFNγ upon activation with PMA and ionomycin was significantly higher in HIV/HCV-positive individuals as compared with healthy donors (Figure 2A; $p < 0.05$). Across all study groups, the number of IFNγ-producing CD4$^+$T lymphocytes positively correlated with the relative number of activated (CD38$^+$HLA-DR$^+$) CD4$^+$T cells (Figure 2B).

Importantly, although the number of IFNγ-producing CD4$^+$T lymphocytes in HIV/HCV coinfected individuals was increased, the average production of IFNγ in CD4$^+$T cells of these patients was significantly lower than that of healthy subjects (Figure 3A; $p < 0.05$). The IFNγ production in CD4$^+$T lymphocytes did not depend on the percent of activated cells. However, a negative correlation was established between IFNγ production and the level of CD4$^+$T cell exhaustion (Figure 3B).

Both phenotypic and functional indexes of CD4$^+$T lymphocyte exhaustion correlated with

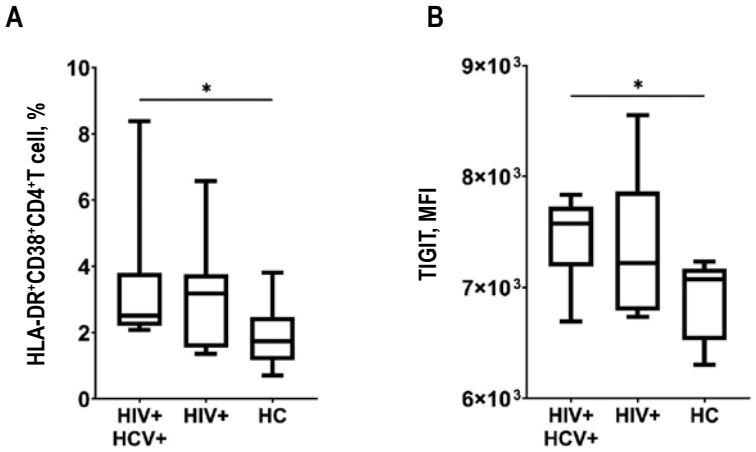

Figure 1. Level of CD4$^+$T cell activation (A) and exhaustion (B) in HIV/HCV coinfected people (HIV+HCV+), HIV monoinfected patients (HIV+) and healthy control (HC) subjects

Note. Medians (horizontal lines within rectangles), interquartile ranges (rectangles), and 10-90% intervals (vertical lines) are shown. *, $p < 0.05$ (Mann–Whitney U test). MFI, mean fluorescence intensity.

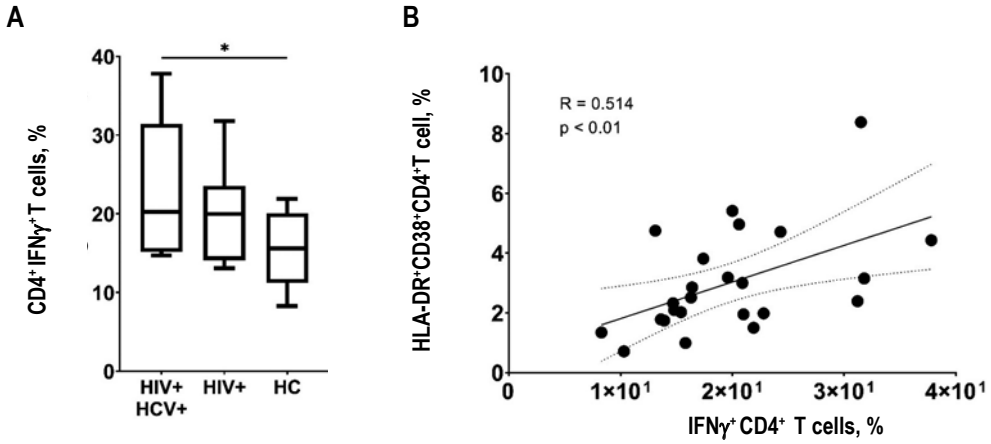

Figure 2. Frequency of IFNγ producing CD4+T lymphocytes (A) and its correlation with the relative number of activated CD4+T cells (B)

Note. (A) Medians (horizontal lines within rectangles), interquartile ranges (rectangles), and 10-90% intervals (vertical lines) are shown. *, $p < 0.05$ (Mann–Whitney U test). (B) Individual donor values, regression line and 95% confidence intervals are shown (r-Pearson correlation test). "HIV+HCV+", HIV/HCV coinfected patients; "HIV+", HIV monoinfected patients; "HC", healthy controls.

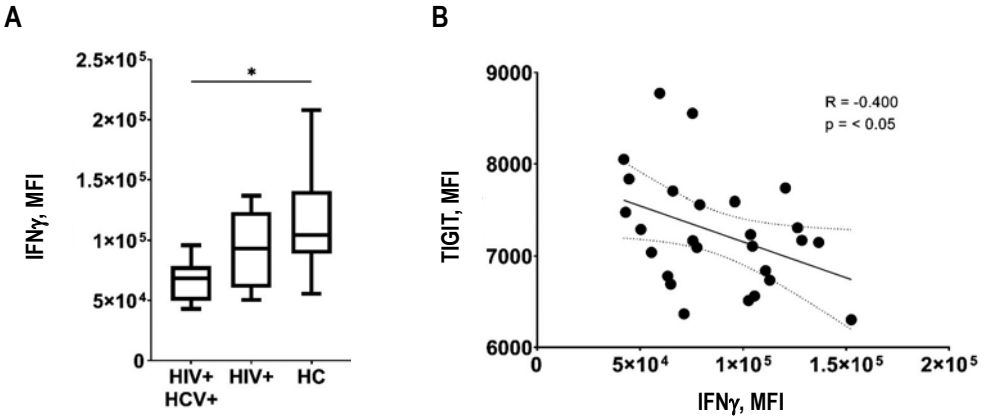

Figure 3. Level of IFNγ production (A) and its correlation with the degree of exhaustion (B) in CD4+T cells

Note. As for Figure 2.

CD4+T cell counts in donors' blood. Specifically, CD4+T lymphocytes count was directly related to the production of IFNγ ($R = 0.455$, $p < 0.05$) and inversely related to the proportion of exhausted CD4+T cells ($R = -0.598$, $p < 0.01$).

These data demonstrate that in HIV-infected patients, HCV coinfection is associated with a high level of CD4+T cells' activation and exhaustion. CD4+T lymphocytes in HIV/HCV coinfected patients show both phenotypic (expression of the inhibitory receptor – TIGIT) and functional (decrease in IFNγ production) signs of exhaustion. The frequency of exhausted CD4+T cells inversely correlate with the total number of CD4+T lymphocytes in donors' blood. These findings suggest that the negative impact of HCV on the course of HIV infection controlled by HAART is due to the functional exhaustion of CD4+T cells.

Previously, researchers assessed the level of CD4+T cell exhaustion in HIV/HCV coinfected patients by analyzing the relative number of lymphocytes expressing inhibitory receptor PD-1 [4]. The authors found that the frequency of PD1+CD4+T lymphocytes in HIV/HCV coinfected patients was higher than that in HIV monoinfected subjects or healthy individuals. A noteworthy detail is that the surface expression of inhibitory receptors alone does not allow identifying truly exhausted CD4+T cells, as these molecules are also expressed by CD4+T lymphocytes that have received an activation signal [13]. However, exhausted and activated CD4+T cells differ significantly in terms of the

inhibitory receptors' expression level [5]. The amount of inhibitory receptors expressed on the surface of $CD4^+$T cells in HIV/HCV coinfected patients have not been reported in the literature yet. To account for this limitation, we assessed the level of $CD4^+$T cells exhaustion by measuring the surface expression of the inhibitory receptor TIGIT, rather than by identifying $TIGIT^+CD4^+$T lymphocytes alone. Our findings show that the level of TIGIT expression on the surface of $CD4^+$T lymphocytes is significantly higher in HIV/HCV coinfected patients than in healthy individuals.

Determining the state of exhaustion in T cells requires assessing both their phenotype and function. TIGIT that was shown to be abundant on $CD4^+$T cells of HIV/HCV coinfected subjects inhibits the interaction between the costimulatory CD226 molecule and their common ligand, CD155 [8], leading to a decrease in T lymphocyte proliferation and cytokine production [5]. Previous studies have shown that in HIV-infected patients exhausted $TIGIT^+CD8^+$T cells are characterized by the reduced production of IFNγ, TNF , and IL-2 [2]. However in HIV/HCV coinfected patients, the cytokine-producing ability of the exhausted $CD4^+$T cell pool has not been evaluated. In this study, we have demonstrated for the first time that the IFNγ production is reduced in $CD4^+$T lymphocytes of HIV/HCV coinfected patients receiving HAART. These results strongly suggest a high level of $CD4^+$T cell exhaustion in patients coinfected with HIV and HCV.

Conclusion

An important finding of this study was the establishment of a link between the frequency of $CD4^+$T lymphocytes expressing TIGIT, the level of IFNγ production, and the amount of $CD4^+$T cells in the patients' blood. $CD4^+$T cell counts are a significant clinical indicator in HIV-infection as it determines the likelihood of developing non-AIDS-related diseases and death. Therefore, it can be assumed that the $CD4^+$T cell exhaustion caused by HCV coinfection may adversely impact the course of HIV-infection in patients adherent to HAART.

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ПРОДУКТЫ КИШЕЧНЫХ БАКТЕРИЙ ВИЧ-ИНФИЦИРОВАННЫХ ПАЦИЕНТОВ ПРЕПЯТСТВУЮТ РЕГЕНЕРАЦИИ CD4$^+$Т-ЛИМФОЦИТОВ

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Резюме. У части ВИЧ-инфицированных больных, несмотря на подавление репликации вируса на фоне приема антиретровирусных препаратов, не происходит эффективного прироста числа периферических CD4$^+$Т-лимфоцитов (иммунный неответ на терапию). Одним из значимых факторов в развитии иммунодефицита считается иммунная активация, причиной которой, среди прочих, является поступление в кровоток бактериальных продуктов в результате нарушения целостности кишечного барьера. Кроме того, микрофлора кишечника продуцирует различные растворенные вещества, которые могут накапливаться в крови и проявлять токсические свойства. Целью настоящей работы была оценка влияния микробных продуктов кишечного происхождения – паракрезол сульфата и индоксил сульфата – на число CD4$^+$Т-лимфоцитов ВИЧ-зараженных пациентов, получающих антиретровирусную терапию. Объектом исследования служила периферическая кровь ВИЧ-инфицированных субъектов с различной эффективностью восстановления иммунной системы на фоне проводимой терапии и неинфицированных доноров. Концентрация IL-6 ($p = 0,012$), IP-10 ($p = 0,0004$) и sCD14 ($p = 0,003$) в плазме крови ВИЧ-зараженных иммунных неответчиков была повышена по сравнению с соответствующими показателями лиц с эффективным восстановлением численности CD4$^+$Т-клеток (иммунные ответчики). Хотя обе группы ВИЧ-позитивных субъектов не различались по уровню липополисахарида и I-FABP в плазме крови, содержание паракрезол сульфата ($p = 0,001$) и индоксил сульфата ($p = 0,042$) у иммунных неответчиков было увеличено. В экспериментах *in vitro* было установлено негативное дозозависимое влияние паракрезол сульфата и индоксил сульфата на жизнеспособность и митотическую активность CD4$^+$Т-лимфоцитов. Таким образом, у ВИЧ-инфицированных пациентов с нарушенной регенерацией CD4$^+$Т-лимфоцитов на фоне проводимой антиретровирусной терапии отмечается более высокий уровень системного воспаления, чем у субъектов, отвечающих на лечение приростом численности CD4$^+$Т-клеток. Выраженность повреждения кишечного барьера и нагрузка бактериальными компонентами, выходящими в кровоток, у ВИЧ-зараженных лиц с различной эффективностью восстановления иммунитета в ответ на лечение примерно одинаковая. При этом плазма крови иммунных неответчиков значительно обогащена микробными продуктами ки-

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шечного происхождения: паракрезол сульфатом и индоксил сульфатом. Выявленное в присутствие данных токсинов существенное снижение пролиферативной способности CD4+Т-клеток, стимулированных в условиях *in vitro*, и индукция их гибели могут быть одной из причин неэффективного восстановления численности CD4+Т-лимфоцитов у ВИЧ-инфицированных лиц, получающих антиретровирусную терапию.

Ключевые слова: ВИЧ-инфекция, бактериальные токсины, кишечник, CD4+Т-лимфоциты, антиретровирусная терапия, регенерация иммунитета

IN HIV-INFECTED PATIENTS, INTESTINAL BACTERIA-DERIVED PRODUCTS INTERFERE WITH CD4+T CELL REGENERATION

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Abstract. Despite successful suppression of viral replication by antiretroviral drugs there is no significant increase in the number of peripheral CD4+T lymphocytes in some HIV-infected patients (immune non-response to therapy). One of the crucial factors for immunodeficiency aggravation is immune activation developing in response to the bacterial products entry into the bloodstream through the damaged intestinal barrier. Additionally, the intestinal microflora produces various solutes that accumulate in the blood and exhibit toxic properties. This work aimed to evaluate the effect of intestinal microbial products (para-cresol sulfate and indoxyl sulfate) on the number of CD4+T lymphocytes in HIV-infected patients receiving antiretroviral therapy. The object of the study was the peripheral blood of HIV-infected subjects with different immune system restoration efficiency during the therapy. Uninfected donors were enrolled as healthy controls. Plasma concentrations of IL-6 ($p = 0.012$), IP-10 ($p = 0.0004$), and sCD14 ($p = 0.003$) in HIV-infected immune non-responders were increased compared with those in individuals with effective restoration of CD4+T cells (immune responders). Although both groups of HIV-positive subjects did not differ in plasma lipopolysaccharide and I-FABP levels, para-cresol sulfate ($p = 0.001$) and indoxyl sulfate ($p = 0.042$) concentrations were increased in immune non-responders. *In vitro* experiments showed a negative dose-dependent effect of para-cresol sulfate and indoxyl sulfate on the viability and mitotic activity of CD4+T lymphocytes. Thus, in HIV-infected patients with impaired regeneration of CD4+T lymphocytes during antiretroviral therapy, a higher level of systemic inflammation is noted than in subjects responding to treatment with an increase in the number of CD4+T cells. The severity of the intestinal barrier damage and the load of bacterial components released into the bloodstream are approximately the same in HIV-infected individuals with different efficiency of immune recovery in response to treatment. Simultaneously, the blood plasma of immune non-responders is significantly enriched with microbial products of intestinal origin: para-cresol sulfate and indoxyl sulfate. The significant decrease in the proliferative capacity of CD4+T cells stimulated *in vitro* and the induction of their death in the presence of these toxins may be a reason for the ineffective restoration of the number of CD4+T lymphocytes in HIV-infected individuals receiving antiretroviral therapy.

Keywords: HIV infection, bacterial toxins, intestine, CD4+T lymphocytes, antiretroviral therapy, immune regeneration

The work was carried out within the framework of the state assignment No. AAAA-A19-119112290007-7.

Introduction

In HIV-infected patients, the use of antiretroviral therapy (ART) leads to suppression of viral replication and a subsequent increase in the peripheral CD4+T lymphocyte count (standard response) [2]. However, up to 30% of patients referred to as "immune non-responders" (INRs), develop a discordant response to treatment, in which, despite an effective viral load suppression, the number of blood CD4+T cells remains low [10]. INRs compared with subjects giving a standard response to ART have a significantly increased risk of morbidity and mortality from both AIDS-associated and non-AIDS-associated diseases [9].

Immune activation is considered one of the most significant factors in the development of immunodeficiency during HIV-infection. The generally accepted concept of immune activation is based on the idea that intestinal barrier integrity is disrupted and the microbial products enter the bloodstream, maintaining high chronic systemic inflammation level [3, 4]. HIV-infected individuals develop intestinal dysbiosis, which manifests in a reduction in bacterial community diversity [6, 11]. Additionally, the intestinal flora produces gut

bacteria-derived solutes, including the so-called "uremic toxins", the accumulation of which in patients with chronic kidney disease is associated with clinical progression and cardiovascular complication development [8, 15]. The most studied uremic toxins are para-cresol sulfate (PCS) and indoxyl sulfate (IS). Their precursors, para-cresol and indole, are formed as a result of amino acid fermentation by intestinal bacteria. After sulfation, these products enter the bloodstream in the form of PCS and IS. Although PCS and IS have pro-inflammatory effect on a number of cell populations [1, 5], data on their effects on T lymphocytes are limited.

This work **aimed** to evaluate the effect of para-cresol sulfate and indoxyl sulfate on $CD4^+$T lymphocytes in HIV-infected patients receiving ART.

Materials and methods

The study was approved by the ethics committee of the Perm Regional Center for Protection against AIDS and Infectious Diseases (No. IRB00008964). Each subject signed informed consent. The study enrolled HIV-infected patients with a suppressed viral load (< 50 copies/ml of blood) who were adherent to ART for more than two years. The immune system regeneration was assessed by the number of blood $CD4^+$T lymphocytes and was compared to the established threshold value of 350/μL [10]. Patients were divided into groups: 1) immune non-responders (INRs; n = 16) with $CD4^+$T cell count < 350/μL; and 2) immune responders (IRs; n = 21) with $CD4^+$T cell count > 350/μL. The third group enrolled relatively healthy controls without HIV-infection (HC; n = 20). All the examined subjects had no clinical signs of kidney dysfunction.

Blood was sampled into vials containing ethylenediaminetetraacetic acid (Weihai Hongyu Medical Devices Co Ltd, China). The peripheral blood $CD4^+$T lymphocyte number was assessed with a CytoFLEX S flow cytometer (Beckman Coulter, USA) using a commercial Immunocytometry Systems (BDIS) Simultest™ kit (Becton Dickinson, USA). The blood plasma concentrations of interleukin-6 (IL-6), interferon-gamma induced protein 10 (IP-10), soluble CD14 (sCD14), and fatty acid-binding intestinal protein (I-FABP) were determined using R&D Systems ELISA kits (USA). The content of bacterial lipopolysaccharide (LPS) in blood plasma was assessed with the LAL assay QCL-1000 chromogenic kit (Lonza, USA). The studies were conducted according to the manufacturers' instructions. Analysis of PCS and IS in plasma was performed using high-performance liquid chromatography. The effect of PCS and IS on the T lymphocytes proliferative capacity and their resistance to apoptosis was assessed *in vitro* using mononuclear leukocytes obtained from healthy volunteers. The isolated cells were stained with CellTrace Violet (Invitrogen, USA) according to the manufacturer's instructions.

T lymphocyte proliferation was induced by stimulation of the CD3/CD28 complex using a polymer nanomatrix conjugated with CD3 and CD28 agonists (T Cell TransAct; Miltenyi Biotec, USA). Unstimulated cells served as control. Samples were incubated for 72 h in the presence of PCS (ApexBio, USA) or IS (Sigma-Aldrich, USA) at final concentrations of 1 μM, 10 μM, 25 μM, 50 μM or 0.1 μM, 1 μM, 10 μM, respectively. Control samples with stimulated and unstimulated cells did not contain these reagents. After incubation, the cells were harvested and stained with anti-CD3-PE and anti-CD4-V450 antibodies (Becton Dickinson, USA). The proliferation of T lymphocytes was assessed with flow cytometry according to the dilution of the tracking dye in daughter cell generations. The level of $CD4^+$T cell apoptosis was measured by flow cytometry using a commercial FITC AnnexinV Apoptosis Detection Kit I (BD, USA).

Statistical analysis of the obtained data was carried out using nonparametric methods. The median and interquartile ranges (25-75th percentiles) were calculated. The significance of differences between groups was determined using the Mann–Whitney U test or the Kruskal–Wallis test.

Results and discussion

All three groups had no differences in age or gender composition. The INR and IR groups did not differ in HIV-infection or ART duration. In accordance with the selection criteria, the absolute number of $CD4^+$T lymphocytes in INRs was significantly lower than that in the comparison groups ($p < 0.001$). The number of $CD4^+$T cells in IRs was still reduced compared with the corresponding rate in HC ($p < 0.001$).

The study of the inflammation, bacterial translocation, and intestinal epithelium damage indices in the blood plasma of HIV-infected subjects revealed the following (Figure 1).

Compared with IRs, INRs had significantly increased content of IL-6 ($p = 0.012$) and IP-10 ($p = 0.0004$), which indicates a higher level of systemic inflammation. Although the sCD14 concentration in INRs exceeded that in IRs ($p = 0.003$), the LPS level did not differ between the two groups. It is known that the CD14 molecule on the membrane of monocytes/macrophages is a co-receptor for LPS, and its presence in the bloodstream in a soluble form (sCD14) serves as an indicator of bacterial translocation [4]. Based on the fact that CD14 is shed from the cell surface during cell stimulation [14], the presence of sCD14 in blood plasma can also be regarded as a result of monocyte activation. According to some [4], activation, and inflammation in HIV-infection may be due to the release of bacterial products into the bloodstream

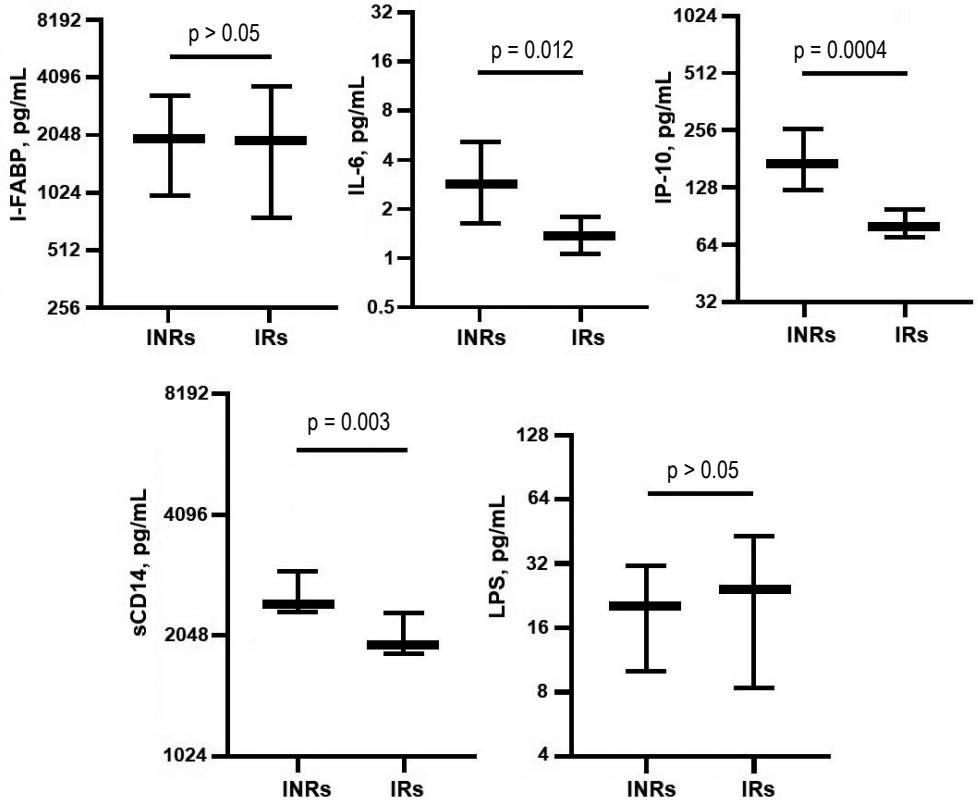

Figure 1. Systemic inflammation, bacterial translocation, and intestinal epithelium damage indices in the blood plasma of HIV-infected patients

Note. INR, immune non-responders; IR, immune responders. Medians (horizontal lines) and interquartile ranges (vertical bars) are shown. Statistical calculations were made using the Mann–Whitney method.

following increased intestinal permeability. When assessing the intestinal epithelium destruction index (I-FABP) we did not reveal any differences between the INR and IR groups. Thus, one can assume that intestinal permeability is not a major reason for the increased systemic activation in HIV-infected individuals with impaired $CD4^+$T lymphocyte regeneration. The same was confirmed by the level of plasma LPS. In addition to bacterial components, various waste products of the intestinal flora can enter the bloodstream [7, 12]. Their entry into the circulation does not require an increase in intestinal permeability since it occurs in a healthy body during the fermentation of food compounds.

Analysis of the gut-derived bacterial products in the blood plasma revealed the following. Median PCS concentrations in INRs, IRs, and HCs were 65.4 μM, 31.7 μM, and 28.5 μM, respectively. Statistically significant differences were found only between the INR and IR groups ($p = 0.001$). Compared with PCS, the content of IS in the blood plasma of all examined subjects was significantly lower. The median concentrations of this microbial toxin in INRs, IRs, and HCs were 8.1 μM, 6.1 μM, and 4.2 μM, respectively. These indices in INRs were significantly higher than the corresponding values in IRs ($p = 0.042$) and HCs ($p = 0.015$). It is known that uremic toxin accumulation in blood occurs due to the kidney function impairment [5, 15]. As there were no signs of kidney dysfunction in any subject, the obtained data indicate the enrichment of the blood plasma of INRs with uremic toxins: compared with IRs in INRs the content of PCS was almost 2 times higher, and the level of IS was 1.3 times higher. Since both substances are products of the intestinal microflora metabolism, then, apparently, the bacterial composition in IRs and INRs differ and is characterized by the predominance of PCS and IS producers in the latter. These toxins may additionally contribute to increased inflammation in INRs and affect $CD4^+$T cells.

The impact of PCS and IS on the viability and proliferative capacity of $CD4^+$T lymphocytes was assessed *in vitro*. The concentrations of PCS and IS (see Materials and Methods) were applied on the basis of literature data on their physiological values in serum/plasma [7, 13]. An assessment of $CD4^+$T lymphocytes binding AnnexinV revealed the following (Figure 2).

In control samples incubated without the PCS or IS, the frequency of cells prone to apoptosis was relatively low. But, in cell samples containing PCS, the level of $CD4^+$T lymphocytes that bound AnnexinV increased significantly (Figure 2A). The effect was

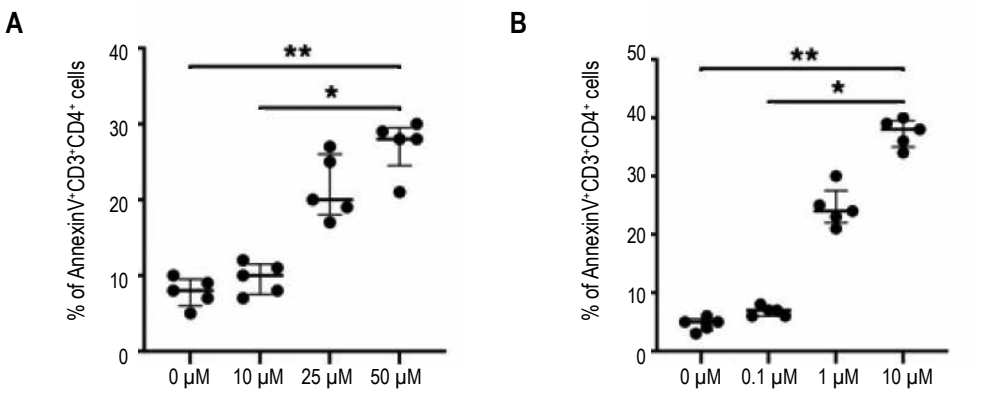

Figure 2. Effect of para-cresol sulfate (A) and indoxyl sulfate (B) on the CD4⁺T lymphocyte viability

Note. The abscissa shows the concentrations of the uremic toxins in the sample. *, $p < 0.05$; **, $p < 0.01$. Statistical calculations were performed using the Kruskal–Wallis method.

dose-dependent. At a PCS concentration of 50 μM, the frequency of cells prone to apoptosis exceeded 25%. Similar results were revealed when assessing the effect of IS on the CD4⁺T lymphocyte viability (Figure 2B).

Furthermore, PCS and IS had a negative impact on the stimulated CD4⁺T cells proliferation (Figure 3).

The number of stimulated CD4⁺T lymphocytes that underwent more than one cycle of division while being incubated in the presence of toxins for 72 h was significantly reduced. The effect of PCS and IS was dose-dependent. At the maximum concentrations used (50 μM of PCS and 10 μM of IS), almost all stimulated cells were unable to proceed to the second round of mitosis. Therefore, both PCS and IS cause CD4⁺T lymphocyte death and disrupt their mitotic activity. The effect of these gut-derived microbial products on CD4⁺T cells is strongly dose-dependent.

Conclusion

Thus, in contrast to HIV-infected subjects with a standard response to treatment, patients with a discordant response to ART are characterized by higher levels of systemic inflammation. Simultaneously, in subjects with a standard and discordant response to ART, the severity of intestinal barrier destruction and the amount of bacterial components entering the bloodstream are approximately the same. However, the content of gut-derived microbial products, such as para-cresol sulfate and indoxyl sulfate, is increased in the blood plasma of HIV-infected immune non-responders. Apparently, in patients with a discordant response to ART, this may be the result of the unique intestinal microflora composition, enriched in the taxa-producers of para-cresol sulfate and indoxyl sulfate. We have shown for the first time the negative impact of these gut-derived microbial products on CD4⁺T lymphocytes, namely, a significant decrease in the proliferative capacity of cells stimulated *in vitro* andthe induction of their death. This may be a reason for the ineffective restoration of the CD4⁺T lymphocyte counts in HIV-infected patients receiving ART.

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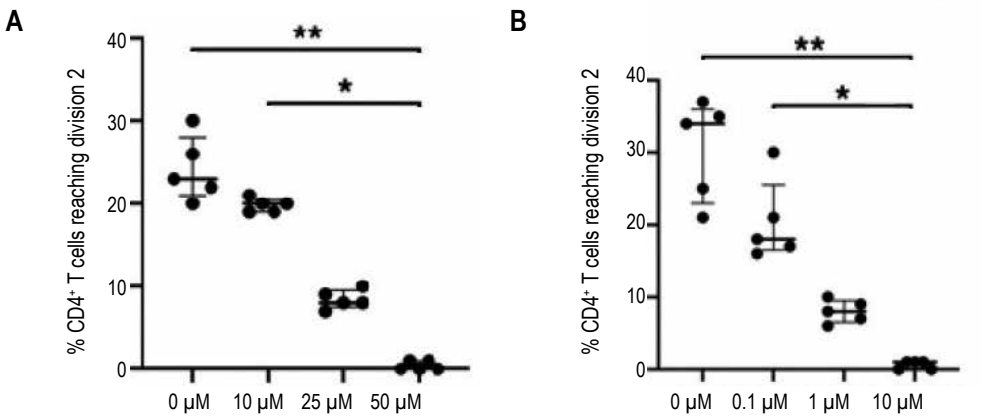

Figure 3. Effect of para-cresol sulfate (A) and indoxyl sulfate (B) on the CD4⁺T lymphocyte proliferative capacity

Note. As for Figure 2.

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СРАВНИТЕЛЬНАЯ ОЦЕНКА ЭФФЕКТИВНОСТИ ПЕПТИДСОДЕРЖАЩЕГО ПРЕПАРАТА И ПОЛИОКСИДОНИЯ В ЛЕЧЕНИИ ХРОНИЧЕСКОГО ПАРОДОНТИТА

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Резюме. В настоящее время существующие методики лечения хронического пародонтита не способны оказывать комплексное влияние на проблему. Сложность этиопатогенетических взаимодействий в развитии данного заболевания, а также высокая распространенность инфекционных патологий полости рта обусловливают склонность данного заболевания к хронизации. Пародонтит, развивающийся на фоне изменений локального и общего иммунного статуса, оказывает значительное влияние на качество жизни пациентов, а также затрудняет последующее восстановительное лечение. Таким образом, подход к лечению данной патологии должен быть направлен не только на устранение этиологического фактора, но и на коррекцию иммунологического фона. В соответствии с этим, в последние годы проводятся активные исследования и разработка новых методик лечения и препаратов, которые бы оказывали комплексный этиопатогенетический эффект на данное заболевание.

В данной статье приводится сравнительная оценка классического и экспериментального методов лечения хронического пародонтита. Основываясь на воссоздании экспериментальной модели хронического воспаления тканей пародонта на крысах линии Wistar, были сравнены методы топической терапии композицией «кремнийорганический глицерогидрогель – пептид» и «Полиоксидоний», проведена сравнительная оценка активности данных препаратов с контрольными группами, лечение которых осуществлялось «кремнийорганический глицерогидрогель» и «Метрогил Дента».

Ранее нами были проведены отдельные исследования эффективности применения композиции «кремнийорганический глицерогидрогель – пептид», а также способа лечения пародонтита путем инъекций препарата «Полиоксидоний». Их сравнивали с классическим методом лечения данного заболевания для получения соответствующих данных и результатов.

На наш взгляд, полученные данные представляют значительный интерес. Проведена оценка и сравнение клинических и гистологических данных, которые показали, что все препараты оказывают положительное воздействие на процессы регенерации тканей, однако композиция «кремнийорганический глицерогидрогель – пептид», за счет особенности гидрогеля действовать в качестве транску-

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танного проводника, показала более быстрое противомикробное и патогенетическое действие, что позволяет комплексно подойти к решению данной проблемы. По сравнению с группами глицерогидрогель кремния и «Полиоксидоний» сроки клинического улучшения в группе глицерогидрогель-пептид увеличились на 57%, а с группой «Метрогил Дента» показатели улучшились примерно на 15%.

Ключевые слова: кремнийорганический глицерогидрогель, антимикробные пептиды, хронический пародонтит, Полиоксидоний, Метрогил Дента, воспаление

COMPARATIVE EVALUATION OF THE EFFECTIVENESS OF A PEPTIDE-CONTAINING DRUG AND POLYOXYDONIUM IN THE TREATMENT OF CHRONIC PARODONTITIS

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Abstract. Currently, the available methods of treating parodontitis are not able to have a complex effect. Therefore, in recent years, there has been an active search and development of new methods of treatment and new drugs that have a complex etiopathogenetic effect on this disease.

This article provides a comparative evaluation of the classical and experimental methods of treating chronic periodontitis. Based on the reconstruction of an experimental model of chronic inflammation of periodontal tissues of the Wistar rat line, we compared methods of topical therapy by "Organosilicon Glycerohydrogel – Peptide" and "Polyoxidonium" compositions. A comparative assessment of the activity of these drugs with control groups, which were treated with "Organosilicon Glycerohydrogel" and "Metrogyl Denta", was carried out.

Previously, we carried out separate studies of the effectiveness of the use of the composition "organosilicon glycerohydrogel – peptide", as well as the method of treatment of periodontitis, by injecting the drug "Polyoxidonium". They have been compared with the classic treatment for this disease to obtain relevant data and results.

In our opinion, the data obtained are of considerable interest. The assessment and comparison of clinical and histological data have been carried out, which showed that all drugs had a positive effect on the processes of tissue regeneration. However, the composition "Organosilicon Glycerohydrogel-peptide", due to the characteristics of the hydrogel, which is acting as a transcutaneous conductor, showed a faster antimicrobial and pathogenetic effect, which allows a comprehensive approach to solving this problem. In comparison with the groups of "Organosilicon Glycerohydrogel" and "Polyoxidonium", the period of clinical improvement increased by 57% in the group of "Glycerohydrogel-Peptide", and, in the "Metrogyl Denta" group, the indicators improved by 15% approximately.

Keywords: silicon glycerohydrogel, antimicrobial peptides, chronic parodontitis, Polyoxidonium, Metrogyl Denta, inflammatory

Introduction

The peculiarities of the development and course of periodontitis, as well as the imperfection of comprehensive programs for the prevention and treatment of this disease, contribute to the high prevalence of this pathology. According to the World Health Organization, the prevalence of this disease among the adult population is about 90-95%.

The polymorphism of etiological factors, the complexity of pathogenetic processes and the high resistance of microorganisms to a wide range of antibacterial drugs [2] causes the necessity of improvement of methods of treating periodontitis, as well as the need to search for modern and universal drugs that would be able to have a multi-link effect [14].

Periodontitis is an inflammatory disease of the tissue complex of the dentoalveolar segments, including the supporting-retaining ligamentous apparatus of the tooth (periodontium) [1], bone tissue and the complex of soft tissues of the dentoalveolar segment (periosteum, gums), leading to atrophic processes in these tissues and, as a result, to loss of teeth. From the point of view of modern concepts of the development of this disease, atrophic processes are explained by the development of cellular and humoral reactions of the immune system in the tissues of the mucous membrane.

In chronic parodontitis, microorganisms colonize in the gingival sulcus, which activates the mechanisms of innate immunity in the periodontal tissues. Toll-like receptors located on epithelial cells recognize pathogens and activate local immune responses, induce the production of cytokines, chemokines, and antimicrobial peptides. The lack of production of antimicrobial peptides is one of the most important factors that determine the chronicity of pathological processes in the mucous membrane.

The available drugs for the treatment of periodontitis are capable of either exerting an effect only on pathogenesis, by suppressing inflammatory reactions, or only on the etiological factor, due to the presence of antimicrobial agents, such as chlorhexidine, in the composition. Accordingly, they are not capable of having a complex effect.

In addition to that, with the lack of a proper approach to the treatment of periodontitis in the early stages, the pathogenic microbiome is activated [5] and the immune responses are destabilized, which leads to the chronization of the process and the lack of a proper response to the following treatment [7]. Thus, it is needed to base the treatment on using drugs that support the activation of immune responses and influence the etiological factor of the disease.

Conservative therapy is of great importance in the treatment of chronic parodontitis. Compared to other methods, such as surgical and orthopedic treatment, conservative therapy allows us to approach the problem of this disease in the least invasive, but at the same time, no less effective approach. The possibility of using it in the early stages of the disease, as well as using it as a supportive therapy, allows us to achieve the elimination of foci of inflammation, long-term stabilization of the periodontal condition, and also to prevent the transition of the inflammatory process to deep-lying tissues.

The most perspective drugs with these characteristics are "Polyoxidonium" and the "Organosilicon Glycerohydrogel – peptide" composition. Composition with peptides, in contrast to "Polyoxidonium" [3, 6, 13], is capable of both activating the immune response and directly affecting the etiological factor of periodontitis: microorganisms. Also, in combination with "Organosilicon Glycerohydrogel", which has transcutaneous activity, this composition has the great potential in the treatment.

The presence of transcutaneous activity of the gel allows one to minimize the dose of the administered drug and shorten the duration of treatment, while not reducing the effectiveness of treatment.

Thus, **the aim of the study** was a comparative assessment of new methods of treatment of chronic periodontitis, with the classical treatment regimen with the antimicrobial agent 'Metrogyl Denta'.

Materials and methods

Based in the Federal State Institute of Immunology and Physiology, Ural Branch of the Russian Academy of Sciences, Russian Federation, Yekaterinburg, a study was carried out on 4 groups of laboratory rats of the "Wistar" line. Each group included 10 rats. The study included recreating a model of chronic periodontitis by inserting a 12 mm needle into the periodontal space and its following extraction on the 26th day (RF patent No. 2545923) [4, 12]. All painful procedures were realized in accordance with The WMA Declaration of Helsinki.

In the first and second groups, treatment was carried out by application of "Glycerohydrogel-Peptide" and "Organosilicon Hydrogel" (IOS UB of the RAS, Yekaterinburg) compositions to the area of the inflammatory focus, with the following clinical assessment of the effectiveness of treatment [9].

In the third group, treatment was carried out by injecting the 'Polyoxidonium' (RPU Petrovax pharm, Russia), drug into the gums. In the fourth group, by application of 'Metrogyl-Denta' (Unique pharmaceutical Lab, India) gel to the area of the inflammatory focus [10].

Data about the control groups, which did not get the treatment, has been described in previous studies and used for comparison [8, 11].

Results and discussion

As a comparative analysis of the studies, the changes in clinical parameters were assessed, as well as the analysis of the histological picture. For comparison of clinical indicators, the following symptoms were taken into account: hyperemia and swelling of the gums, discharge of pus from the periodontal space, change in features. In comparison with the groups of "Organosilicon Glycerohydrogel" and "Polyoxidonium", the period of clinical improvement increased by 57% in the group of "Glycerohydrogel-Peptide", and, in the "Metrogyl Denta" group, the indicators improved by 15% approximately.

In addition, comparative histological analysis was carried out to assess the nature of morphological changes.

According to the results of the histological study, it was noted that in the group of "Organosilicon Glycerohydrogel", the histological picture had a dynamic improvement: plasmacytic infiltration gradually decreased, and the processes of tissue repair tended to a slower restoration of the normal structure. In this group, the regeneration process took 21 to 30 days. While in the "Glycerohydrogel-Peptide" group, a more saccadic improvement was observed: extensive plasmacytic infiltration was observed on days 3-15 after treatment, and the histological picture improved dramatically on days 16-20 (Figures 1, 2, 3, see 3rd page of cover).

The composition of "Glycerohydrogel-Peptide" showed a better therapeutic effect than the groups of "Organosilicon Glycerohydrogel", "Polyoxidonium" and "Metrogyl Denta", since the change in the clinical and histological picture was more intense. An expansive change in the structure of tissues and histological signs of inflammation allows us to assume that the composition of "Glycerohydrogel-Peptide" affects several pathogenetic links of the disease, in particular, the cellular and humoral links of immunity.

In addition, the nature of the change in the clinical picture after treatment with the "Glycerohydrogel-Peptide" composition allows us to suggest that the time frame for clinical improvement is an indicator of good antimicrobial activity of the composition. Acceleration of the healing time and a different clinical course of the inflammatory process when using the "Glycerohydrogel-Peptide" composition, in comparison with the other groups, showed the effectiveness of topical therapy of chronic periodontitis with using peptides in the studied animals.

Conclusions

1. Histological examination confirmed the normalization of tissue structure in all groups. However, the period of regeneration in the "Glycerohydrogel-Peptide" group was 1.25 times shorter (16 to 20 days) than in other groups (from 20 days).

2. Due to the presence of "Glycerohydrogel" and peptide in the composition, both the pathogenetic and etiological links of the disease are influenced.

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ДИАГНОСТИЧЕСКИ ЗНАЧИМЫЕ ИЗМЕНЕНИЯ СУБПОПУЛЯЦИЙ $CD11b^+CD64^-CD32^+CD16^+$, $CD11b^+CD64^+CD32^+CD16^+$ НЕЙТРОФИЛЬНЫХ ГРАНУЛОЦИТОВ ИММУНОКОМПРОМЕТИРОВАННЫХ ЖЕНЩИН С ХРОНИЧЕСКИМИ ИНФЕКЦИОННО-ВОСПАЛИТЕЛЬНЫМИ ЗАБОЛЕВАНИЯМИ ГЕНИТАЛЬНОГО ТРАКТА РАЗЛИЧНОЙ ЭТИОЛОГИИ

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Резюме. Хронические воспалительные заболевания органов малого таза (ХВЗОМТ) у женщин остаются основной проблемой в структуре гинекологических заболеваний ввиду значимости медицинских и социально-экономических последствий. Течение и исход ХВЗОМТ зависят от состояния иммунной системы. Актуальным представляется изучение рецепторного аппарата нейтрофильных гранулоцитов (НГ), участвующих в противоинфекционной защите при заболеваниях различной этиологии. Цель: уточнить особенности вариантов количественных и фенотипических изменений субпопуляций НГ $CD11b^+CD64^-CD32^+CD16^+$, $CD11b^+CD64^+CD32^+CD16^+$ иммунокомпрометированных женщин в период обострения ХВЗОМТ различной этиологии. Тестировали НГ периферической крови 70 женщин 20-40 лет: группа исследования 1 (ГИ1) – 20 иммунокомпрометированных женщин в период обострения ХВЗОМТ с моно- или микст-латентными или рецидивирующими различными вирусными инфекциями (хронические герпес-вирусные инфекции, папилломавирусная инфекция, рекуррентные ОРВИ); группа исследования 2 (ГИ2) – 30 иммунокомпрометированных женщин с

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ХВЗОМТ бактериальной этиологии; группа сравнения – 20 условно-здоровых женщин. Методом проточной цитометрии (CYTOMICS FC500, США) определяли количество НГ и уровень экспрессии рецепторов субпопуляций $CD11b^+CD64^-CD32^+CD16^+$НГ (мажорной) и $CD11b^+CD64^+CD32^+CD16^+$НГ (минорной). Установлено, что у иммунокомпрометированных женщин с ХВЗОМТ в период обострения выявлены диагностически значимые различия в субпопуляционном составе НГ. При ХВЗОМТ бактериальной этиологии (ГИ2) снижение субпопуляции $CD11b^+CD64^-CD32^+CD16^+$НГ и увеличение в 7,6 раз субпопуляции $CD11b^+CD64^+CD32^+CD16^+$НГ в отличие от ХВЗОМТ, протекающих в сочетании с рецидивирующей или персистирующей вирусной инфекцией (ГИ1). Негативная трансформация субпопуляций НГ связана с преимущественным снижением уровня экспрессии активационного CD16. Выявлено отсутствие адекватного ответа на воспаление – отсутствие повышения экспрессии активационного CD11b в мажорной субпопуляции в ГИ1, а также в минорной субпопуляции в ГИ1 и ГИ2. В мажорной субпопуляции ГИ2 выявлено нарушение – снижение экспрессии активационного маркера CD11b в период обострения ХВЗОМТ. Также при наличии различной вирусной инфекции и ХВЗОМТ (ГИ1) в негативно измененной минорной субпопуляции выявлено снижение уровня экспрессии CD16, повышение уровня экспрессии CD64 и CD32. Определение субпопуляционного состава $CD11b^+CD64^-CD32^+CD16^+$ и $CD11b^+CD64^+CD32^+CD16^+$ НГ и их фенотипа можно использовать как в качестве диагностических маркеров для дифференциальной диагностики ХВЗОМТ вирусной и бактериальной этиологии, так и для последующей разработки новых методов таргетной иммуномодулирующей терапии.

Ключевые слова: нейтрофильные гранулоциты, субпопуляции, фенотип, диагностический маркер, хронические воспалительные заболевания органов малого таза, иммунокомпрометированность

DIAGNOSTICALLY SIGNIFICANT CHANGES IN SUBSETS $CD11b^+CD64^-CD32^+CD16^+$, $CD11b^+CD64^+CD32^+CD16^+$ NEUTROPHILIC GRANULOCYTES OF IMMUNOCOMPROMISED WOMEN WITH CHRONIC INFECTIOUS AND INFLAMMATORY DISEASES OF THE GENITAL TRACT OF VARIOUS ETIOLOGIES

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Abstract. Chronic pelvic inflammatory disease (PID) in women remains a problem due to the importance of medical consequences. The study of the receptor apparatus of neutrophilic granulocytes (NG) involved in anti-infective protection in diseases of various etiologies seems to be relevant. Aim: to clarify the features of variants of quantitative and phenotypic changes in subsets of NG $CD11b^+CD64^-CD32^+CD16^+$, $CD11b^+CD64^+CD32^+CD16^+$ of immunocompromised women during exacerbation of chronic PID of various etiologies. We were tested women 20-40 years: Study Group 1 (SG1, n = 20) – chronic PID during the exacerbation with mono- or mixed latent/recurrent various viral infections (chronic herpes-virus infections, papillomavirus infection, recurrent ARVI); Study Group 2 (SG2, n = 30) – chronic PID of bacterial etiologies; Comparison Group (CG) – 20 healthy women. The number of subsets $CD11b^+CD64^-CD32^+CD16^+$NG (major) and $CD11b^+CD64^+CD32^+CD16^+$NG (minor), receptor expression density (MFI) was determined (FC500, USA). It was found that in PID during the period of exacerbation, diagnostically significant differences in the

subset composition of NG were revealed. We got a decrease in the $CD11b^+CD64^-CD32^+CD16^+$NG subset and in 7,6 times increase in the $CD11b^+CD64^+CD32^+CD16^+$NG subset in SG2 with chronic PID of bacterial etiology, in contrast to chronic PID occurring in combination with recurrent/persistent viral infection SG1. Negative transformation of NG subsets is associated with a predominant decrease in the level of expression of the activation CD16. The absence of an adequate response to the infectious and inflammatory process was revealed – the absence of an increase in the expression of the activation CD11b in the major subset in SG1, as well as in the minor subset in groups SG1 and SG2. In the major subset of NG in groups SG2 a decrease in the expression of the activation marker CD11b. In the various viral infections and PID (SG1), in the negatively altered minor subset of NG we got a decrease of expression of CD16, an increase of expression of CD64 and CD32. Determination of subsets of $CD11b^+CD64^-CD32^+CD16^+$, $CD11b^+CD64^+CD32^+CD16^+$NG and their phenotype can be used as diagnostic markers for the differential diagnosis of PID of viral and bacterial etiology, and for the development of new methods of targeted immunomodulatory therapy.

Keywords: neutrophilic granulocytes, subsets, phenotype, diagnostic marker, chronic pelvic inflammatory disease, immunocompromised

Introduction

Chronic inflammatory diseases of the pelvic organs (pelvic inflammatory disease, PID) are one of the main problems among gynecological diseases due to their wide distribution, and they can be represented both as separate nosological forms and in various combinations. Despite the improvement of methods of diagnosis and treatment according to the World Health Organization (WHO), 448 million new cases of chronic inflammatory diseases of the pelvic organs are registered annually in the world (up to 60% of the total number of gynecological diseases) [10, 12]. According to modern scientific data, the etiology of chronic PID is polymicrobial, associated not only with obligate pathogens (Neisseria gonorrhoeae, Chlamydia trachomatis, etc.), but also with opportunistic microflora that is part of the normal vaginal microbiota. The main features are the variety of the clinical picture, with a short interrecurrent period or a sluggish protracted course [2, 3, 9]. Cases with a severe and complicated course have become more frequent; persistent recurrence of the inflammatory process, the absence of a positive effect on the ongoing etiotropic and pathogenetic therapy, as a result of which the formation of an adhesive process and impaired reproductive functions is possible.

The immune system plays a significant role in the pathogenesis of chronic PID, and it is from its adequate or defective functioning that the characteristics of the course and outcome of the disease depend [8]. Thus, impaired functioning of local or systemic immunity contributes to the emergence of atypically occurring acute or chronic infectious and inflammatory diseases of the reproductive tract causes the onset and chronicity of infectious and inflammatory processes of extragenital localization of various etiologies, which are resistant to therapy – antiviral, antibacterial, antifungal and anti-inflammatory [7], which also indicates clinical signs of immunocompromised being. The particular interest is the study of the functioning of neutrophilic granulocytes (NG), one of the most important cells of innate immunity, which perform a number of effector and regulatory functions during the infectious process. They are the first cells and ready to respond to the invasion of pathogens and the increase in the growth of opportunistic microorganisms in the microbiome. At the same time, the symbiotic microflora (lacto- and bifidobacteria), being in close relationship with NG, stimulates the formation of NET, thereby exerting a regulatory effect on the state of the microbiocenosis of the genital tract [11].

According to modern scientific data, the antimicrobial activity of NG is associated with certain surface membrane receptors. Thus, the surface membrane molecules of NG receptors CD64, CD32, CD16, CD11b form different subsets of NG with different phenotypes, performing both effector and regulatory functions: immunomodulating, immunostimulating, immunosuppressive [1]. Overexpression or defects in the expression of one or more receptors cause disturbances in the effector and/or regulatory functions of various NG subsets. Thus, in case of dysfunctions of the NG receptor apparatus caused by impaired expression of CD16, CD11b molecules, which are activation markers of the NG effector functions, dysregulatory disorders of the anti-infective immune defense are observed. Because of this there are acute severe infectious and inflammatory diseases, prolonged sluggish or recurrent chronic infectious and inflammatory processes that do not respond to etiotropic therapy. At the same time, studies devoted to the study of the quantitative characteristics and phenotypic features of the

$CD11b^+CD64^-CD32^+CD16^+$, $CD11b^+CD64^+CD32^+CD16^+$NG subsets of immunocompromised women with chronic PID have not been conducted.

Taking into account the above, it seems promising to study the quantitative characteristics and phenotypic features of subsets of $CD11b^+CD64^-CD32^+CD16^+$, $CD11b^+CD64^+CD32^+CD16^+$ neutrophilic granulocytes of women with chronic PID. Clarification of variants of the defective phenotype of NG subsets and their quantitative imbalance can contribute to the development the methods for diagnosing the defective functioning of NG subsets. The obtained new data can be the basis for the creation of new targeted methods of immunomodulatory therapy, organically included in the complex treatment of chronic PID.

Aim: to clarify the features of variants of quantitative and phenotypic changes in subsets of $CD11b^+CD64^-CD32^+CD16^+$, $CD11b^+CD64^+CD32^+CD16^+$ neutrophilic granulocytes of immunocompromised women during the exacerbation of chronic PID of various etiologies.

Materials and methods

Under our supervision were 50 women from 20 to 40 years old in the period of exacerbation of chronic PID (chronic metritis, chronic salpingo-oophoritis), with clinical signs of immunocompromised. Groups were formed depending on the clinical and anamnestic data: Study Group 1 (SG1) and Study Group 2 (SG2). Women of both groups were characterized by a long history of chronic PID (more than 5 years), frequent exacerbations (3 or more times a year) or a sluggish protracted course of exacerbations, the absence of a stable clinical effect when using systemic and local anti-inflammatory therapy. SG1 included 20 immunocompromised women with chronic PID (40% of all cases). When studying the history data according to the "immunological history" program (Nesterova I.V., 1992), criterial signs of immunocompromise were revealed, indicating that SG1 patients suffered from mono- or mixed latent or recurrent various viral infections in 100% of cases. They had chronic herpes virus infections caused by herpes simplex viruses types I and II, genital and orofacial localization with a frequency of exacerbations up to 5-6 times a year, human papillomavirus infection (anogenital warts), recurrent acute respiratory viral infections with a frequency of episodes up to 7-8 times a year. SG2 included 30 immunocompromised women (60%) with chronic PID of various bacterial etiologies. The comparison group (CG) consisted of 20 conditionally healthy women of reproductive age from 20 to 40 years old who applied to the clinic to choose a contraceptive method. An immunological study of the "major" and "minor" subsets, $CD11b^+CD64^-CD32^+CD16^+$, $CD11b^+CD64^+CD32^+CD16^+$NG, was carried out with the determination of the percentage of each NG subset (%NG) and assessment of their phenotype, taking into account the density of expression of each receptor by the value of the mean of fluorescence index (MFI) by flow cytometry (CYTOMICS FC500, USA). Appropriate monoclonal antibodies to CD11b, CD64, CD32, CD16 molecules were used. For statistical processing of the obtained data, Microsoft Excel 2016, StatPlus 2010 software packages and non-parametric tests – the Wilcoxon and Mann–Whitney criteria were used. The results were expressed as a median (upper and lower quartile) – Me ($Q_{0.25}$-$Q_{0.75}$). Differences were considered statistically significant at $p < 0.05$.

Results and discussion

It was revealed that in the peripheral blood samples of the comparison group (CG) (conditionally healthy women), the major subset of NG $CD11b^+CD64^-CD32^+CD16^+$ occurs in 94.90 (93.98-97.40) %, and the minor subset of NG $CD11b^+CD64^+CD32^+CD16^+$ – in 1.24 (0.29-5.41) %.

In women with SG1 during the exacerbation of chronic PID, the quantitative composition of the major and minor subsets did not statistically significantly differ from the indicators of the comparison group ($p > 0.05$). At the same time, a negative transformation of the phenotype of both subsets was revealed. Thus, in the major subset $CD11b^+CD64^-CD32^+CD16^+$NG, the CD16 expression level was in 1,3 times lower, and the CD32 expression level was in 1.2 times higher relative to the CG ($p_{1,2} < 0.05$). At the same time, in the minor subset of $CD11b^+CD64^+CD32^+CD16^+$NG, there was a statistically significant increase in the expression density of the CD64 molecule according to MFI – in 3 times, and the CD32 molecule – in 1.9 times relative to CG ($p_{1,2} < 0.05$). A significant decrease in the level of expression of the CD16 molecule was found by 1.6 times relative to CG ($p < 0.05$). The revealed fact attracted attention that the expression of the CD11b molecule in the major and minor subsets did not change and remained at the CG level ($p > 0.05$) (Table 1).

In SG2 during the exacerbation of chronic PID, a statistically significant decrease in the proportion of the major subset $CD11b^+CD64^-CD32^+CD16^+$NG – up to 87.35 (84.69-88.38) and a significant increase by 7.6 times in the content of the minor subsets of $CD11b^+CD64^+CD32^+CD16^+$NG relative to CG

TABLE 1. CHANGES IN SUBSETS OF $CD11b^+CD64^-CD32^+CD16^+$ AND $CD11b^+CD64^+CD32^+CD16^+$ NEUTROPHIL GRANULOCYTES OF IMMUNOCOMPROMISED WOMEN DURING EXACERBATION CHRONIC PELVIC INFLAMMATORY DISEASES, Me ($Q_{0.25}$-$Q_{0.75}$)

Indicator	Comparison Group (CG)	Study Group 1 (SG1)	Study Group 2 (SG2)
$CD11b^+CD64^-CD32^+CD16^+$			
%NG (%NG)	94.90 (93.98-97.40)	93.98 (89.84-95.71)	87.35 (84.69-88.38)* ^
MFI CD16	118.5 (108.00-146.25)	90.5 (79.37-106.50)*	93.90 (81.80-107.00)*
MFI CD32	3.79 (3.50-4.13)	4.73 (4.36-6.42)*	4.04 (3.00-4.85)
MFI CD11b	25.10 (21.60-27.15)	22.10 (19.05-26.77)	15.50 (11.06-20.70)*
$CD11b^+CD64^+CD32^+CD16^+$			
%NG (%NG)	1.24 (0.29-5.41)	2.32 (1.42-6.97)	9.42 (7.53-11.40)* ^
MFI CD64	2.40 (2.07-5.06)	7.34 (6.88-15.35)*	1.69 (1.51-1.97)* ^
MFI CD16	149.00 (128.00-157.00)	94.80 (55.20-126.00)*	109.00 (89.90-121.00)*
MFI CD32	5.84 (4.24-6.50)	11.10 (7.27-18.55)*	5.21 (3.68-5.84)^
MFI CD11b	33.70 (24.10-37.40)	30.75 (23.80-46.75)	23.20 (16.92-29.35)

Note. *, the reliability of differences in indicators from the values of the comparison group, $p < 0.05$; ^, the reliability of differences in indicators in relation study group 1 (SG1), $p < 0.05$.

($p_{1,2} < 0.05$). The expression density level of the CD11b molecule in SG2 was statistically significantly reduced by 1,6 times in the minor subset of $CD11b^+CD64^+CD32^+CD16^+$NG ($p < 0.05$), while in the major subset of $CD11b^+CD64^-CD32^+CD16^+$NG it remained at the level of CG indicators ($p > 0.05$). The expression density level of the CD32 molecule in SG2 did not change statistically, both in the major and minor NG subsets relative to the CG ($p_{1,2} > 0.05$) (Table 1).

Comparing the results of NG phenotyping in women during the exacerbation of chronic PID in SG1 and SG2, statistically significant changes were revealed. In SG2 the relative content of the major subset $CD11b^+CD64^-CD32^+CD16^+$NG was reduced by 1.1 times ($p < 0.05$), and the subset of the minor $CD11b^+CD64^+CD32^+CD16^+$NG was increased by 4 times compared to SG1 ($p < 0.05$). Changes in the phenotype were found only in the minor subset. Thus, in SG1, compared with SG2, the expression level of the CD64 receptor increased by 4.3 times, and the expression level of CD32 increased by 2.1 times ($p_{1,2} < 0.05$) (Table 1).

Taking into account the data obtained, it is evident that in immunocompromised women during the exacerbation of chronic PID in the 2 groups of SG1 and SG2, there are quantitative changes and various options for negative restructuring of the phenotype of the NG subsets as a "watchdog" of the predominant major subset $CD11b^+CD64^-CD32^+CD16^+$NG and a minor subset $CD11b^+CD64^+CD32^+CD16^+$NG.

In SG1 a transformation of the phenotypic profile in the subsets $CD11b^+CD64^-CD32^+CD16^+$NG and $CD11b^+CD64^+CD32^+CD16^+$NG was revealed due to a decrease in the density of CD16 expression and an increase in the density of CD32 expression. However, the level of CD64 expression density in the minor subset increased statistically significantly, which may indirectly indicate the stimulation of its expression by the cytokine environment associated with the presence of recurrent or persistent viral infection.

In SG2, in the subset $CD11b^+CD64^-CD32^+CD16^+$NG, the phenotypic profile is characterized by a quantitative decrease in CD16 expression relative to the Comparison Group. An increase in the number of the $CD11b^+CD64^+CD32^+CD16^+$NG subset, which is diagnostically significant in the presence of a bacterial infection was revealed. We have previously shown that the content of the $CD11b^+CD64^+CD32^+CD16^+$NG subset increases many times with the progression of

the severity of the inflammatory process [4, 6]. Also in the SG2 there was a decrease in the expression density of CD16 in the NG subsets and CD64 in the minor NG subset.

It is shown lack of receptor expression CD11b in subsets of NG women in both SG during an exacerbation of chronic PID. It is known that the CD11b receptor is a signaling partner for other receptors, in particular Fcγ receptors, which regulate chemotaxis of NG to the inflammation, adhesion, phagocytosis, respiratory burst, and degranulation [5]. A defect in the activation of the CD11b receptor and Fcγ receptors indirectly indicates a violation of the effector functions of NG.

Changes in the subset composition of NG and their phenotype do not contribute to the full implementation of the effector functions of NG in immunocompromised women with chronic PID and may be the reason for the maintenance of a chronic inflammatory process and the absence of a stable positive effect from etiopathogenetic therapy.

Conclusion

As a result of this study, it was found that in immunocompromised women suffering from chronic PID for more than 5 years, diagnostically significant differences in the subset composition of NG were revealed during the exacerbation period. There is a decrease in the $CD11b^+CD64^-CD32^+CD16^+NG$ subset and in 7,6 times increase in the $CD11b^+CD64^+CD32^+CD16^+NG$ subset in chronic PID of bacterial etiology (SG2), in contrast to chronic PID occurring in combination with recurrent or persistent viral infection (SG1). Negative transformation of NG subsets is associated with a predominant decrease in the level of expression of the activation molecule CD16. The absence of an adequate response to the infectious and inflammatory process was revealed: the absence of an increase in the expression of the activation molecule CD11b in the major subset in SG1, as well as in the minor subset in groups SG1 and SG2. At the same time, in the major subset of SG2, a significant impairment: a defect characterized by a decrease in the expression of the activation marker CD11b in the major subset during the exacerbation of chronic PID. In SG1 there is a negative change in the minor subset of $CD11b^+CD64^+CD32^+CD16^+NG$, a decrease in CD16 expression, an increase in the expression level of CD64 and CD32.

Taking into account the data obtained it can be assumed that the determination of the subset composition of $CD11b^+CD64^-CD32^+CD16^+$ and $CD11b^+CD64^+CD32^+CD16^+NG$ and the clarification of their phenotypic characteristics can be used as diagnostic markers for the differential diagnosis of chronic PID of viral and bacterial etiology, however this issue requires further research.

The obtained data on the negative quantitative and phenotypic transformation of NG subsets – major $CD11b^+CD64^-CD32^+CD16^+$ and minor $CD11b^+CD64^+CD32^+CD16^+$ in women suffering from chronic PID of various etiologies, can serve as the basis for creating new integrated approaches to the immunodiagnosis of multivariate changes in major and minor subsets of NG. In addition, prospects are opening up for the development of new methods of targeted immunomodulatory therapy aimed at reprogramming negatively transformed subsets of NG in chronic PID, which should help to restore the full effector functions of NG, improve the implementation of both antiviral and antibacterial protection, and, as a result, achieve positive results, including clinical and immunological effects.

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ОЦЕНКА ВЛИЯНИЯ INOSINE PRANOBEX НА СИСТЕМУ МАТРИКСНЫХ БЕЛКОВ У ПАЦИЕНТОК С ХРОНИЧЕСКИМИ ВИРУСНЫМИ ЦЕРВИЦИТАМИ

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Резюме. Репродуктивный потенциал как женщин, так и мужчин ежегодно снижается. Нарушению репродуктивной функции способствуют множество факторов – химические, физические, механические, психогенные, однако наиболее выраженное влияние на репродукцию оказывают биологические факторы. Хронический вирусный цервицит может являться не только причиной бесплодия и репродуктивных потерь, но и развитием интраэпителиальных дисплазий, а также рака шейки матки. ПВИ, как моноинфекция, встречается достаточно редко, наряду с ВПЧ, общими путями передачи и входными воротами, выступают другие урогенитальные инфекции. Наиболее частной ассоциацией с ПВИ является герпесвирусная инфекция. Увеличение ММР как системно, так и на локальном уровне может свидетельствовать о нарушении процессов клеточного моделирования, что способствует развитию аутоиммунного воспаление с дальнейшей деструкцией тканей репродуктивного тракта. Активация ММР способствует выходу ВПГ из нервных ганглиев и реактивации инфекции. Терапия ВПЧ и ГВИ носит дискутабельный характер. Единого стандарта лечения нет, но существует ряд препаратов, которые обладают противовирусным и иммуномодулирующим эффектами. В настоящее время отсутствуют исследования динамики влияния ВПЧ и ВПГ инфекции на состояние ММР и TIMP при терапии Inosine pranobex. Цель исследования – оценить изменения матриксных металлопротеиназ 2 и 9 и их тканевых ингибиторов 1-го и 2-го типа у пациенток с папилломавирусной и герпетической инфекциями после терапии Inosine pranobex.

Проведено обследование 76 пациенток с папилломавирусной и герпетической инфекциями, получавших терапию препаратами с действующим веществом Inosine pranobex. Определение уровней ММР-2, ММР-9 и TIMP-1, TIMP-2 в сыворотке крови проводили с помощью специфических реактивов фирмы R&D Diagnostics Inc. (США).

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Динамика показателей в сыворотке крови пациенток с ПВИ показала снижение уровня MMP-2, MMP-9, TIMP-1 с одновременным повышением TIMP-2 относительно показателей до терапии. У пациенток с ПВИ и ГВИ терапия Inosine pranobex показала снижение показателей MMP-2 и MMP-9, отсутствие изменений в содержании TIMP-1, но повышение сывороточного содержания TIMP-2. До применения терапии было установлено повышение коэффициента в основных группах в сравнении с группой контроля, однако наибольшее увеличение установлено в группе с ассоциацией инфекций. После терапии установлена положительная динамика в основных группах. Так, коэффициент в I группе снизился и стал равен контрольным значениям. Во II группе пациенток коэффициент, несмотря на снижение, остался выше контрольных величин и выше в сравнении с I группой женщин.

Ключевые слова: вирус папилломы человека, герпетическая инфекция, матриксные металлопротеиназы, тканевые ингибиторы, противовирусная терапия, Inosine pranobex

EVALUATION OF THE INFLUENCE OF INOSINE PRANOBEX ON THE MATRIX PROTEIN SYSTEM IN PATIENTS WITH CHRONIC VIRAL CERVICITIS

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Abstract. The reproductive potential of both women and men is declining every year. Many factors contribute to the violation of the reproductive function – chemical, physical, mechanical, psychogenic, however, biological factors have the most pronounced effect on reproduction. Chronic viral cervicitis can be not only the cause of infertility and reproductive losses, but also the development of intraepithelial dysplasia, as well as cervical cancer. PVI, as a monoinfection, is quite rare along with HPV. Other UGIs (urogenital infections) act as common routes of transmission and entry gates. The most common association with PVI is herpesvirus infection. An increase in MMP, both systemically and at the local level, may indicate a violation of cell modeling processes, which contributes to the development of autoimmune inflammation with further destruction of the tissues of the reproductive tract. Activation of MMP promotes the release of HSV from the nerve ganglia and reactivation of the infection. Therapy for HPV and HVI (herpes virus infections) are debatable. There is no single standard of treatment, but there are a number of drugs that have antiviral and immunomodulatory effects. Currently, there are no studies on the dynamics of the effect of HPV and HSV infection on the state of MMPs and TIMPs during Inosine pranobex therapy. Objective: to evaluate changes in matrix metalloproteinases 2 and 9 and their tissue inhibitors types 1 and 2 in patients with human papillomavirus and herpes infections after Inosine pranobex therapy.

6 patients with papillomavirus and herpetic infections were examined and treated with drugs containing the active ingredient Inosine pranobex. The levels of MMP-2, MMP-9 and TIMP-1, TIMP-2 in blood serum were determined using specific reagents from R&D Diagnostics Inc. (USA).

The dynamics of indicators in the blood serum of patients with PVI showed a decrease in the level of MMP-2, MMP-9, TIMP-1 with a simultaneous increase in TIMP-2 relative to the values before therapy. In patients with PVI and HVI, Inosine pranobex therapy showed a decrease in MMP-2 and MMP-9 levels, no changes in the content of TIMP-1, but an increase in the serum content of TIMP-2. Prior to the use of therapy, an increase in the ratio in the main groups in comparison with the control group was found, however, the largest increase was found in the group with the association of infections. After therapy, positive dynamics was established in the main groups. Thus, the ratio in group I decreased and became equal to the control values. In the II group of patients, the ratio, despite the decrease, remained higher than the control values and higher in comparison with the I group of women.

Keywords: human papillomavirus, herpes infection, matrix metalloproteinases, tissue inhibitors, antiviral therapy, Inosine pranobex

Introduction

Currently, the demographic situation is one of the most important issues all over the world, both from a social and economic point of view. Reproductive potential of both women and men is declining every year. Many factors contribute to the violation of the reproductive function – chemical, physical, mechanical, psychogenic, however, biological factors have the most pronounced effect on reproduction. Inflammatory diseases of the female reproductive system play important role in the opposition, adhesion and invasion of the ovum to the endometrium, as well as in the further prolongation of pregnancy. Up to 20% of spontaneous abortions and 6-7% of non-developing pregnancies in the 1st trimester are registered annually [1, 13].

According to data for 2021, the frequency of infertility in the Russian Federation ranges from 17.2 to 24% [15]. According to the literature, one of the main clinical markers of infection is cervicitis. The high prevalence of cervicitis is associated with an asymptomatic course of the disease, especially with the viral nature of the pathogen. Chronic viral cervicitis (CVC) can be not only the cause of infertility and reproductive losses, but also the development of intraepithelial dysplasia, as well as cervical cancer [1]. The greatest etiological role in the development of CVC is attributed to human papillomavirus and herpesvirus infections.

According to statistics, after the first 2 years of the onset of sexual activity, up to 82-84% of women are considered infected with HPV. Voznesenskaya N.V. (2013) found that having even one permanent sexual partner, the risk of infection is 20% [13]. This may affect the vulva, cervix and/or vagina. On the vulva, PVI appears as genital warts. Most often, the urogenital tract is affected by HPV types -6, -11, -16, -18, -31, -35, -40, and -52 [2, 6].

Papillomaviruses are the only group of viruses for which it has been proven that they induce the formation of tumors in humans under natural conditions. They are most often induced by HPV type 16, which is found in 50-80% of samples of moderate and severe dysplasia of the squamous epithelium of the cervix and in 90% of cervical cancer. Paying great attention to HPV oncogenesis, only a few researchers have studied the effect of PVI on fertility, and therefore this issue is still controversial. Recent evidence suggests that PVI can affect a woman's reproductive function and significantly reduce the effectiveness of assisted reproductive technologies [5].

A systematic literature review from 1994-2014 showed that the effect of PVI on a woman's reproductive potential is possibly associated with the genotype of the virus, high or low oncogenicity, at least in idiopathic cases of infertility. Thus, Souho et al. (2015) in their study found that trophoblasts transfected with plasmids carrying the HPV type 16 genome undergo apoptosis 3-6 times higher than trophoblasts transfected with empty plasmids. This process of apoptosis may be responsible for placental insufficiency and failure of trophoblast invasion of the uterine wall, which may eventually lead to early miscarriage or premature rupture of the membranes. The same opinion is shared by other authors [14].

PVI, as a monoinfection, is quite rare along with HPV. PVH and other UGIs (urogenital infections) act as common routes of transmission and entry gates. Moreover, it has been proven that the presence of other UGIs optimizes the conditions for HPV persistence [4]. There is a whole range of infectious agents, the presence of which leads to metabolic and morphological changes in the endometrial tissue, and disruption of its receptor apparatus, which leads to infertility or early pregnancy loss [11, 13]. However, the most common association with PVI is herpes virus infection [11]. Herpesvirus infection is a predisposing factor to spontaneous miscarriages or intrauterine growth retardation; it also causes pathology of the fetus and newborn, because cytopathic, teratogenic and mutagenic effects [15]. There is also a hypothesis that a herpes infection can stimulate HPV replication and help integrate its genome into the host cell genome [4, 12], which is an important condition for tumor transformation.

This hypothesis is confirmed by other authors, saying that the combination of HVI and PVI increases the risk of manifestation of cervical neoplasia. Based on the 2006 data presented by the World Health Organization, it was believed that the main etiological factor in the development of CC was HSV, but now scientists have identified the true etiological factor in CC – human papillomavirus, and HSV is considered a cofactor [4]. Most people are carriers of the HSV virus. The primary infection of a person can be either one or several types of HSV that can circulate in the human body throughout life. Many types of herpes can persist for a long time, however, depending on the state of innate and adaptive immunity, a recurrent form of herpes infection may develop [4].

Currently, in the study of the morphology of the tissues of the reproductive tract, much attention is paid to the study of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). MMPs are involved in many morphological processes of the tissues of the female reproductive tract – proliferation, migration, and apoptosis. MMPs play a major role in the protein metabolism of connective tissue, the processes of normal development of the extracellular matrix, and in oncogenesis and angiogenesis. An increase in MMP, both systemically and at the local level, may indicate a violation of cell modeling processes, which contributes to the development of autoimmune inflammation with further destruction of the tissues of

the reproductive tract. MMP activation contributes to the release of HSV from the nerve ganglia and reactivation of the infection [7, 11, 14].

Studies have shown that an imbalance of MMP-2 and MMP-9 interferes with trophoblast invasion and normal remodeling of the spiral arteries in early pregnancy, which can lead to reproductive disorders. In prolonged pregnancy, in the late stages, MMP-2 and MMP-9 are involved in the implementation of endothelial dysfunction leading to antenatal disorders [9, 12, 13].

Today, HPV and HVI therapy is debatable. There is no single standard of treatment, but there are a number of drugs that have antiviral and immunomodulatory effects. The main goal is not only to eliminate clinical symptoms, but also to reduce the likelihood of recurrence of the infection [2], as well as the maximum preparation of a woman for a safe pregnancy and childbirth without complications for her and the fetus. Scientists around the world agree that the highest efficiency of HPV eradication is observed with combined treatment, i.e., surgical and therapeutic, with the help of immunomodulators. However, in their studies with antiviral drugs of various origins, they show high rates of cure, remission, elimination of the virus from the body, but do not specify the most effective antiviral and immunomodulatory drug, which is important for the treatment of PVI [6].

One of the main medications used to achieve an antiviral and immunomodulatory effect is a pharmaceutical of synthetic origin, a purine derivative "Inosinum pranobexum" [8]. Immunomodulating manifestations are manifested by activation of innate immunity, increased production of interleukins, as well as chemotactic and phagocytic activity of monocytes, macrophages and polymorphonuclear cells, and increased antibody synthesis.

The potential of Inosin pranobex against viral infections is also confirmed by an increase in the natural killer (NK) population and increased NK activity [3, 10]. Chemotaxis and phagocytosis of neutrophils, monocytes, and macrophages are also potentiated by Inosin pranobex, while the NK activity of eosinophils is enhanced by an increase in the amount of lgG and complement surface markers triggered by the administration of Inosin pranobex [10]. The humoral immune response is mainly enhanced by stimulating the differentiation of B-lymphocytes into plasma cells and increasing the production of antibodies [10].

Various hypotheses have been put forward in an attempt to explain the antiviral properties of Inosin pranobex. It is believed that the antiviral activity of Inosin pranobex is the result of an increase in the host's immune response due to the drug [10]. From this point of view, the drug enhances the biochemical processes in lymphocytes as soon as they are triggered by viral antigens, since it is not able to independently stimulate lymphocytes at rest [3, 10].

Currently, there are no studies on the dynamics of the effect of HPV and HSV infection on the state of MMPs and TIMPs during Inosine pranobex therapy. Purpose of the study: To assess the dynamics of the level of matrix metalloproteinases 2 and 9 and their tissue inhibitors of types 1 and 2 in patients with human papillomavirus and herpes infections before and after Inosine pranobex therapy.

Materials and methods

We examined 76 female patients. The average age of the patients was 33±1.7 years. The patients were divided into a main group depending on the etiological factor and a control group of practically healthy women (n = 30). The main group: I – with papillomavirus infection in a monovariant (n = 22), and II – association of papillomavirus and herpes infection (n = 24). At the first stage of the research, the levels of MMP and TIMP were determined before Inosine pranobex therapy. At the second stage, all patients of the main group were prescribed Inosine pranobex therapy and in the second group, with the association of PVI and HVI, Valacyclovir was included in the scheme in combination with the main therapy.

The therapy was carried out on the basis of clinical protocols and taking into account the inclusion of drugs with an immunomodulatory effect with according to the instructions for their use.

Inosine pranobex was prescribed at a dose of 50 mg/kg/day, divided into 3 doses, per os after meals, 28 days in the form of mono- or combination therapy. Valaciclovir was prescribed per os at a dose of 500 mg 2 times a day for 10 days, then 500 mg 1 time a day for 20 days.

A comprehensive clinical and laboratory study was performed twice, initially before therapy and one month after therapy, on an outpatient basis according to a single program including clinical, functional, biochemical and immunological examination in order to detail the immunological mechanisms of chronic inflammation associated with the viral factor of the urogenital tract of women.

Determination of the level of matrix metalloproteinases (MMP-2, MMP-9) and their tissue inhibitors (TIMP-1, TIMP-2) in blood serum was carried out by solid-phase ELISA using specific reagents from R&D Diagnostic Inc. (USA).

Statistical data processing was carried out using IBM SPSS® v. 22 programs. Intra- and inter-group differences were assessed using the Mann–Whitney criterion. To check the relationship or independence between the values, the Spearman correlation coefficient was determined. The associative relationship of indicators with signs was assessed using the odds ratio

and their 95% confidence intervals. The significance level $p < 0.05$ was considered statistically significant.

Results and discussion

In the study of the level of MMP and TIMP in the blood serum of patients before therapy, multifaceted results were obtained (Table 1). The levels of MMP-2 and 9, before the use of Inosine pranobex, increased in all major groups compared to the control group. MMP-2 increased by 1.3 times in comparison with reference values ($p < 0.01$). The concentration of MMP-9 in the blood serum was increased by 1.3 times compared to the control ($p < 0.01$). Thus, the increase in MMP-9 was registered in the main groups, regardless of the nature of the pathogen, but the content of MMP-9 in the group with PVI ($p_{1-2} = 0.03$) was increased in comparison with the group with HVI + PVI.

The content of TIMP-1 in the blood serum of the patients was high in comparison with the control group ($p < 0.01$). At the same time, the content of TIMP-1 in the II group of patients was higher in comparison with the I ($p_{1-2} = 0.02$) group of women. TIMP-2 indicators, on the contrary, were reduced in all groups relative to the reference values ($p < 0.05$). It was found that the content of TIMP-2 in the blood serum in the group with PVI + HVI was 1.4 times lower compared to group I ($p_{1-2} = 0.05$).

Before the use of Inosine pranobex therapy, MMP levels were high, which may indicate that, being the key enzymes of basement membrane degradation, this may contribute to successful trophoblast invasion, which is regulated by MMP and TIMP, while MMP-2 regulates the earliest stage of interaction between the embryo and endometrium. It is also worth noting that, unlike other types of MMPs (MMP-1, MMP-3, MMP-9), whose expression may depend on cytokines, growth factors, and hormonal status, MMP-2 expression does not depend on these factors [12]. In turn, TIMP plays the role of a limiting factor in the degree of invasion [12].

The mechanism of action of TIMP is based on the inhibition of MMP activity by binding to the Zn site of MMP and, thus, controlling the physiological processes in the body. However, with an increase in MMP-2, excessive remodeling of endometrial tissues can occur, which can contribute to impaired implantation and invasion of the trophoblast, and with a decrease in TIMP-2, the regulation of this ratio is disturbed, which can lead to reproductive disorders [12].

TABLE 1. LEVELS OF MATRIX METALLOPROTEASES AND THEIR TISSUE INHIBITORS IN PATIENT BLOOD SERUM, Me ($Q_{0.25}$-$Q_{0.75}$), ng/mL

Indicator	Control group (n = 30)	Group I (n = 22) 1	Group II (n = 22) 2	Confidence level (p)
MMP-2	167.0 (145-182)	237.39** (213.88-243.29) $p_{Before-I} = 0.007$	206.89** (198.63-275.63) $p_{Before-II} = 0.007$	
Inosine pranobex		144.28** (129.41-168.16)	159.39 (144.62-184.12)	$p_{1-2} < 0.05$
MMP-9	291.28 (168.44-305.10)	394.20** (308.81-425.72) $p_{Before-I} = 0.004$	299.91* (255.92-401.39) $p_{Before-II} = 0.05$	$p_{1-2} = 0.03$
Inosine pranobex		298.98* (208.83-349.64)	232.75* (227.83-241.25)	$p_{1-2} < 0.01$
TIMP-1	205.08 (180.21-222.10)	270.09** (260.66-285.54) $p_{Before-I} = 0.004$	306.32** (288.84-311.65) $p_{Before-II} = 0.09$	$p_{1-2} = 0.02$
Inosine pranobex		222.14* (201.62-223.81)	303.61** (243.12-337.10)	$p_{1-2} < 0.05$
TIMP-2	169.04 (73.06-227.66)	135.62* (124.34-136.97) $p_{Before-I} = 0.040$	98.81* (91.21-125.06) $p_{Before-II} = 0.001$	$p_{1-2} = 0.05$
Inosine pranobex		160.42 (156.40-164.44)	143.58** (123.07-164.62)	$p_{1-2} < 0.05$

Note. Statistical significance of differences with the control group: *, $p < 0.05$; **, $p < 0.01$. Statistical significance between groups: p_{1-2}, groups I and II – with HPV and HPV + HSV. Statistical significance between groups after therapy: $P_{Before-I}$, before therapy and group I after therapy Inosine pranobex (I) – with PVI; $P_{Before-II}$, before therapy and group I after Inosine pranobex (II) therapy – with PVI + BVI.

TABLE 2. RATIO OF MATRIX METALLOPROTEINASE 2 AND TISSUE INHIBITOR 2 IN BLOOD SERUM IN PATIENTS OF THE MAIN GROUPS AND IN THE CONTROL GROUP

Indicator	Control group (n = 30)	Group-I (n = 22) 1	Group-II (n = 24) 2	Statistical validity (p)
MMP-2:TIMP-2 Before therapy	0.98±0.05	1.75±0.06* $p_{Before-I} < 0.001$	2.09±0.07** $p_{Before-II} = 0.01$	$p_{1-2} < 0.002$
Inosine pranobex		0.91±0.09	1.13±0.02*	$p_{1-2} < 0.01$

Note. As for Table 1.

Taking into account the statistically significant changes in MMP-2 and TIMP-2 in blood serum, and taking into account that TIMP-2 predominantly binds MMP-2, the indicator of MMP-2 to TIMP-2 was calculated (Table 2), where we obtained agreement with data of other authors on the enhancement of the invasive potential of the viral agent [7, 9, 12]. The concentration of matrix metalloproteinase-2 is more affected by herpes virus infection, as a reflection of the interaction of the system of proteolytic enzymes – matrix metalloproteinases with the immune system, cytokines and cellular elements.

In group I, against the background of the use of Inosine pranobex, there was a positive trend. The dynamics of indicators in the blood serum of patients showed a decrease in the level of MMP-2 by an average of 1.6 times, MMP-9 – by 1.3 times, TIMP-1 – by 1.2 times with a simultaneous increase in TIMP-2 by 1.2 times relative to pre-treatment scores. Comparing the changes in the subgroups, it was found that there were significant differences between the content of metalloproteinases and their inhibitors in the blood serum of patients. A significant decrease in MMP-2 was also found relative to the parameters of the control group in patients after therapy.

In patients of group II, Inosine pranobex therapy showed a slightly different picture. A decrease in MMP-2 and MMP-9 levels was demonstrated by 1.2 times, no changes in the content of TIMP-1, but an increase in the serum content of TIMP-2 by an average of 1.4 times. The achievement of control values of MMP-2 after therapy with Inosine pranobex was revealed.

Before therapy, an increase in the ratio was noted in the major groups compared to the control group, however, the largest increase was found in the group with the association of infections ($p_{1-2} < 0.002$).

An increase in the ratio in all groups may indicate increased damage to the extracellular matrix, which is associated with protein degradation and leads to the formation of an unstable connective tissue framework of organs and tissues, including the reproductive tract, contributing to the development of prenatal complications in the future. Also, an increased ratio may indicate the development of immune inflammation with an increase in tissue vascular permeability, activation of angiogenesis, the progression of oncogenesis. Matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) can play an important role in the invasion and metastatic spread of malignant neoplasms connected with uncontrolled degradation of the extracellular matrix [7, 9, 12].

After therapy, positive dynamics was established in the main groups. Thus, the ratio in group I decreased and became equal to the control values. In the II group of patients, the ratio t, despite the decrease, remained higher than the control values ($p_{1-2} < 0.05$) and higher in comparison with the I group of women ($p_{1-2} < 0.002$).

The data after therapy showed positive dynamics both in terms of mono-indicators and the ratio. Analyzing the results obtained, it was found that IP contributes to pronounced positive changes, in groups with associations of infections. This indicates a more pronounced effect of the use of IP on reducing the activity of matrix metalloproteinases and their tissue inhibitors in women with mixed infections.

Conclusion

As a result of our study, a positive dynamic of MMPs and TIMPs in the blood serum of patients after Inosine pranobex therapy was revealed, which may be a predictor in the regression of the inflammatory process and inhibition of viral infection activation. However, further research is required to develop new therapeutic regimens for the treatment of papillomavirus and herpetic infections.

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ИММУНОЛОГИЧЕСКИЕ МАРКЕРЫ ПРИ ОСЛОЖНЕНИЯХ ЭНДОПРОТЕЗИРОВАНИЯ СУСТАВОВ

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Резюме. Перипротезная инфекция суставов до сих пор остается сложной клинической проблемой, поскольку точное определение этого состояния и надежные лабораторные маркеры пока отсутствуют. Данное исследование было направлено на оценку информативности определения некоторых субпопуляций лимфоцитов и моноцитов у пациентов с перипротезной инфекцией суставов и неинфекционными осложнениями эндопротезирования. В данное исследование было включено 34 пациента с хронической перипротезной инфекцией, 12 – с неинфекционными осложнениями и 30 практически здоровых лиц. Количество $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+HLA\text{-}DR^+$, $CD4^+CD45RA^-CD45RO^+$, $CD4^+CD45RA^+CD45RO^-$ и $CD14^+HLA\text{-}DR^+$ субпопуляций лимфоцитов и моноцитов в периферической крови определяли методом проточной цитометрии. Оценку экспрессии мембранных антигенов проводили по средней интенсивности флуоресценции. У пациентов с перипротезной инфекцией суставов было выявлено достоверное увеличение субпопуляций $CD3^+CD4^+$ ($p < 0,01$) и достоверное снижение субпопуляций $CD3^-CD16^+CD56^+$ ($p < 0,005$) при сравнению с контрольной группой. Содержание $CD19^+$ лимфоцитов у этих больных было достоверно выше, чем у лиц с неинфекционными осложнениями ($p < 0,005$), последняя группа также характеризовалась более высоким содержанием активированных Т-лимфоцитов ($CD3^+HLA\text{-}DR^+$) по сравнению с контрольной ($p < 0,001$). Количество «наивных» Т-лимфоцитов ($CD4^+CD45RA^+CD45RO^-$) было ниже у больных с перипротезной инфекцией суставов, чем у больных с неинфекционными осложнениями ($p < 0,05$), и в обеих группах этот показатель был достоверно ниже, чем в контрольной ($p < 0,001$). Содержание Т-клеток памяти ($CD4^+CD45RA^-CD45RO^+$), напротив, было достоверно повышено в обеих сравниваемых группах ($p < 0,05$). В группе больных с перипротезной инфекцией суставов количество активированных моноцитов ($CD14^+HLA\text{-}DR^+$), а также показатель экспрессии данного активационного маркера были существенно ниже, чем в двух остальных группах ($p < 0,05$ и $p < 0,001$ соответственно). Таким образом, оценку субпопуляций лимфоцитов и моноцитов периферической крови, в том числе изучение интенсивности экспрессии активационных маркеров, можно, вместе с другими общепринятыми клинико-лабораторными показателями, дополнительно использовать для проведения дифференциального диагноза между перипротезной инфекцией суставов и неинфекционными осложнениями эндопротезирования.

Ключевые слова: эндопротезирование суставов, осложнения, перипротезная инфекция, проточная цитометрия, лимфоциты, моноцит

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IMMUNOLOGICAL MARKERS OF ARTHROPLASTY FAILURE

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Abstract. Periprosthetic joint infection still remains a clinical challenge since accurate definition of this condition and reliable laboratory markers have not been established yet. This study aimed to evaluate the benefit of some lymphocyte and monocyte subset determination in patients with periprosthetic joint infection and non-infectious arthroplasty failure. Thirty-four patients with chronic periprosthetic joint infection, 12 patients with non-infectious arthroplasty and 30 healthy persons were included in the study. The counts of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+$HLA-DR^+, $CD4^+CD45RA^-CD45RO^+$, $CD4^+CD45RA^+$ $CD45RO^-$ and $CD14^+$ HLA-DR^+ subsets in peripheral blood were assessed by flow cytometry. The assessment of the intensity of antigen expression was carried out according to mean fluorescence intensity. A significant increase in $CD3^+CD4^+$ subsets ($p < 0,01$) and a significant decrease in CD3-$CD16^+CD56^+$ subsets ($p < 0,005$) were revealed in patients with periprosthetic joint infection compared to the healthy controls. The content of $CD19^+$ lymphocytes in these patients was significantly higher than in aseptic ones ($p < 0,005$); the latter group was also characterized by more pronounced increase in the number of activated T lymphocytes ($CD3^+$HLA-DR^+) compared to controls ($p < 0,001$). Patients with periprosthetic joint infection showed decreased "na ve" T lymphocytes ($CD4^+CD45RA^+CD45RO^-$) count compared to aseptic ones ($p < 0,05$), and both groups showed a decrease counts compared to controls ($p < 0,001$). On the contrary, memory T lymphocyte ($CD4^+CD45RA^-CD45RO^+$) count was significantly increased in both compared groups ($p < 0,05$). Patients with periprosthetic joint infection compared with other two groups demonstrated a significant decrease in the number of activated monocytes ($CD14^+$HLA-DR^+) and pronounced decrease in the expression intensity of this marker on cell membrane ($p < 0,05$ and $p < 0,001$, respectively). Thus, evaluation of lymphocyte and monocyte subsets, including expression of cell activation antigens could be useful as additional laboratory test in combination with other conventional methods for differentiation between periprosthetic joint infection and aseptic arthroplasty failure.

Keywords: joint arthroplasty, complications, periprosthetic joint infection, flow cytometry, lymphocytes, monocytes.

Introduction

Arthroplasty failure is a serios complication of joint replacement surgery and may be caused by periprosthetic joint infection (PJI) or non-infectious factors. This condition often requires revision surgery and implant replacement. PJI occurs in 1 to 3% of patients after total joint arthroplasty and accounts for 20% to 50% cases of implant failures [6, 9, 12]. A combination of clinical signs, intraoperative findings and pre- and intraoperative laboratory tests (peripheral blood counts, serum inflammatory markers, synovial fluid examination, microbiological culture, tissue histology) is used for PJI diagnosis [8].

Non-infectious arthroplasty failure (NIAF) includes aseptic inflammation, implant instability, periprosthetic fracture, osteolysis/adverse tissue reaction and other reasons and occurs in 50 to 80% of arthroplasty failures [6, 9, 12]. As with arthroplasty failure due to PJI, surgical intervention is usually need to treat NIAF. Though mechanical-related failures are typically diagnosed by X-ray, there are no perfect assays for non-mechanical failures that often difficult to differentiate from PJI due to inflammatory responses at affected areas [4].

The actual incidence of PJI may be significantly higher, since a significant proportion of these cases in patients with instability endoprosthesis or isolated pain syndrome has so far been erroneously regarded as aseptic cases. In routine practice, physicians face challenges in diagnosis of PJI and its differentiation from NIAF. Biofilms, low-grade infection or culture-negative microorganisms have been reported to significantly reduce the sensitivity and specificity of laboratory tests [5, 7, 13]. Underdiagnosing PJI is followed by inappropriate treatment with severe consequences.

Recently, several new serum and synovial fluid biomarkers (α-definsine, calprotectin, interleukin-1, interleukin-6, interleukin-17, leukocyte esterase, lipocalin, procalcitonin) were proposed to confirm PJI [2, 3, 5, 7]. Despite promising data, many authors note, that underlying immune disorders or other inflammatory diseases, as well as co-morbidities, may affect test results [1, 4, 10, 11]. The purpose of the study was to evaluate the benefit of some lymphocyte and monocyte subsets determination as well as expression of cell activation antigens in patients with PJI and NIAF.

Materials and methods

Forty-six patients after total large joint replacement were included in the study. According to

International Consensus Criteria on PJI (2018) after revision arthroplasties 34 patients were classified as chronic PJI (19 males, 15 females, mean age 51±8 years) and 12 patients as NIAF (4 males, 8 females, mean age 47±6 years), namely implant instability or aseptic inflammation. Complications developed 4,4±2,6 years after the main operation. Thirty healthy persons (12 males, 18 females, mean age 43±11 years) were recruited in control group. All patients signed informed consent forms prior to being enrolled.

Standard laboratory evaluation was performed for all patients: peripheral blood cell count, erythrocyte sedimentation rate, C-reactive protein. Synovial fluid and periprosthetic tissue samples obtained intraoperatively were sent for microbiological and histological examination. If there were any signs of infection, a two-stage revision with the installation of a cement spacer impregnated with antibiotics or resection arthroplasty were performed. The counts of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+$HLA-DR^+, $CD4^+CD45RA^-CD45RO^+$, $CD4^+CD45RA^+$ $CD45RO^-$ and $CD14^+$HLA-DR^+ subsets in peripheral blood were assessed by flow cytometry (FACSCalibur, Becton Diskinson, USA). The assessment of the intensity of antigen expression was carried out according to median fluorescence intensity (MFI).

Statistical analysis was performed in Statistica 10.0 Software for Windows. Normally distributed continuous data were shown as mean ± standard deviation (SD) and compared using Student's t-test. A p value < 0.05 was considered statistically significant.

Results and discussion

As shown in Table 1, a significant increase in $CD3^+CD4^+$ subsets ($p < 0.01$) and a significant decrease in $CD3^-CD16^+CD56^+$ subsets ($p < 0,005$) were revealed in patients with PJI compared to the controls. The content of $CD19^+$ lymphocytes in patients with chronic PJI was significantly higher than in aseptic ones ($p < 0.005$); the latter group was also characterized by more pronounced increase in the number of activated T lymphocytes ($CD3^+$HLA-DR^+) ($p < 0.001$). Patients with PJR showed decreased "naïve" T lymphocytes ($CD4^+CD45RA^+CD45RO^-$) count compared to aseptic ones ($p < 0.05$), and both groups showed a decrease counts compared to controls ($p < 0.001$). On the contrary, memory T lymphocyte ($CD4^+CD45RA^-CD45RO^+$) count was significantly increased in both compared groups ($p < 0.05$).

Quite often, a violation of the functional activity of monocytes is detected in critical conditions (sepsis and other serios infections). Monocytes in healthy persons express molecules of HLA-DR with high density and easy determined by flow cytometry. Patients with PJI compared with patients with NIAF and controls showed not only a significant decrease in the number of monocytes ($CD14^+$) expressing HLA-DR antigen

TABLE 1. LYMPHOCYTE AND MONOCYTE SUBSETS AND EXPRESSION OF CELL ACTIVATION ANTIGENS IN PATIENTS WITH PERIPROSTHETIC JOINT INFECTION AND NON-INFECTIOUS ARTHROPLASTY FAILURE

Rate	PJI (n = 34)	NIAF (n = 12)	Controls (n = 30)	p
$CD3^+$, %	76.5±1.3	69.4±4.3	73.9±1.5	
$CD3^+$, abs	1760±45	1688±112	1038±47	
$CD3^+CD4^+$, %	51.8±1.8	48.0±3.4	45.9±1.4	*< 0.01
$CD3^+CD4^+$, abs	903±32	810±57	644±58	*< 0.01
$CD3^+CD8^+$, %	25.3±2.8	24.4±3.9	30.7±3.6	
$CD3^+CD8^+$, abs	445±26	361±49	425±9	
$CD19^+$, %	13.6±3.2	6.8±1.3	10.2±0.7	**< 0.005
$CD19^+$, abs	466±110	248±32	143±17	**< 0.005
$CD3^-CD16/56^+$, %	9.1±1.6	16.8±4.3	15.4±1.2	*< 0.005
$CD3^-CD16/56^+$, abs	289±53	409±21	456±19	*< 0.005
$CD4^+/CD8^+$	2.0±0.8	2.2±0.9	1.5±0.7	
$CD3^+$HLA-DR^+, %	8.9±2.3	11.8±1.2	6.5±0.4	*< 0.001
$CD4^+/45RO^+45RA^-$, %	40.4±6.1	44.7±4.4	14.6±9.2	*p < 0.001
$CD4^+/45RA^+45RO^-$, %	6.6±4.5	12.8±1.8	30.1±8.9	*p < 0.05. **p < 0.001
$CD14^+$HLA-DR^+, %	78.8±2.6	90.7±2.4	88.7±1.1	* **p < 0.05
MFI $CD14^+$ HLA-DR^+ (units)	57.0±2.9	186.7±15.4	184.6±9.9	* **p < 0.001

Note. *, statistically significant differences with parameters of the control group; **, statistically significant differences with parameters of the comparison groups.

($p < 0.05$), but also more pronounced decrease in the expression intensity of this marker according to the MFI parameter ($p < 0.001$).

Conclusion

As already mentioned above, many serum and synovial biomarkers may help differentiation of PJI and NIAF, but there remain cases that are clinically challenging to classify. The profile of different cell subpopulations during PJI and NIAF is still being investigated [1, 4]. The data obtained demonstrated a pronounced decrease in the number monocytes expressing the HLA-DR antigen, as well as a decrease in its density expression on the surface of monocytes and T lymphocytes may indicate low functional activity of these cells, especially antigen presentation and regulation of intercellular interactions. The present study showed that evaluation of lymphocyte and monocyte subset and expression of cell activation antigens could be useful, especially in combination with conventional methods, for diagnosing of PJR and differentiation between PJI and NIAF. New data concerning host immune reactions during arthroplasty failure may potentially identify cell subsets involved in inflammation related to surgical procedures or underlying inflammatory disorders. That may provide insights into future diagnostic and possibly treatment opportunities. The search for the most diagnostically accurate combinations of clinical and laboratory criteria should be continued.

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ВЛИЯНИЕ ЭЛЕКТРОСТИМУЛЯЦИИ МЫШЦ БЕДРА НА УРОВЕНЬ ИНТЕРЛЕЙКИНА-6 ПРИ ТРАВМАТИЧЕСКОМ ПОВРЕЖДЕНИИ ПЕРЕДНЕЙ КРЕСТООБРАЗНОЙ СВЯЗКИ КОЛЕННОГО СУСТАВА

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Резюме. Травматическое повреждение передней крестообразной связки приводит к нарушению опороспособности и механической нестабильности конечности. Одним из частых осложнений после травмы, является артрогенное мышечное торможение, вследствие ингибирования четырехглавой мышцы и развития функциональной контрактуры. Напротив, одним из показателей высокой мышечной активности является достаточный уровень в крови функциональных мышечных белков-миокинов, в частности интерлейкина-6, экспрессирующихся и высвобождающихся мышечными волокнами. Целью исследования явилось изучение уровня интерлейкина-6 у мужчин с повреждением передней крестообразной связки в динамике электромиостимуляции четырехглавой мышцы бедра.

В исследовании принимали участие 23 мужчины, средний возраст 34,8±2,2 года, с травматическим повреждением передней крестообразной связки, которым за 10 дней до оперативного вмешательства проводилась электромиостимуляция четырехглавой мышцы бедра на аппарате INTELECT® Advanced (Chattanooga (DJO), США). Контрольную группу составили 12 здоровых мужчин, средний возраст 32,2±2,4 года. Уровень IL-6 определяли в сыворотке крови до электромиостимуляции, и в динамике с помощью набора для иммуноферментного анализа («Вектор-Бест», г. Новосибирск). Обработку полученных данных проводили с применением пакета лицензионных программ Statistica. vers. 10.0.

Базальный уровень IL-6 в основной группе составил 1,28 (0,87-1,72) пг/мл, что значимо ниже в сравнении с показателем здоровых лиц 5,2 (3,8-6,1) пг/мл и обусловлено низким уровнем физической активности, вследствие функциональной контрактуры четырехглавой мышцы. В динамике электромиостимуляции на 5-е сутки уровень IL-6 значимо увеличился в 3,2 раза от базального уровня, на 10-е сутки в 4,6 раз, при этом не превышая показателя группы здоровых лиц. При сокращении мио-

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цитов, в цитоплазме клеток повышалась концентрация миокина – интерлейкина-6, способствующая накоплению в мышечной клетке макроэргов, вследствие миокин-зависимой активации гликогенолиза. Репаративные и противовоспалительные свойства IL-6 реализуются в стимулируемых поперечно-полосатых мышцах по механизму классического сигналинга, способного блокировать активацию универсального внутриклеточного фактора транскрипции NF-κB, в отношении продукции провоспалительных цитокинов. Таким образом, электромиостимуляция до начала оперативного лечения приводит к повышению концентрации в крови миокина – IL-6, что способствует увеличению противовоспалительного и репаративного потенциала поврежденных тканей.

Ключевые слова: мужчины, повреждение передней крестообразной связки, электромиостимуляция, миокины, IL-6, репарация

EFFECT OF ELECTRICAL STIMULATION OF THE THIGH MUSCLES ON THE LEVEL OF INTERLEUKIN-6 IN TRAUMATIC INJURIES OF THE ANTERIOR CRUCIATE LIGAMENT OF THE KNEE JOINT

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Abstract. Traumatic damage to the anterior cruciate ligament leads to impaired support and mechanical instability of the limb. One of the frequent complications after injury is arthrogenic muscle inhibition due to inhibition of the quadriceps muscle and the development of functional contracture. On the contrary, one of the indicators of high muscle activity is a sufficient level in the blood of functional muscle proteins – myokines, in particular interleukin-6, which are expressed and released by muscle fibers. The aim of the study was to study the level of interleukin-6 in men with damage to the anterior cruciate ligament in the dynamics of electromyostimulation of the quadriceps femoris.

The study involved 23 men, mean age 34.8±2.2 years, with traumatic injury of the anterior cruciate ligament, who, 10 days before surgery, underwent electromyostimulation of the quadriceps femoris using the INTELECT® Advanced device (Chattanooga (DJO), USA). The control group consisted of 12 healthy men, mean age 32.2±2.4 years. The level of IL-6 was determined in the blood serum before electromyostimulation, and in dynamics using a kit for enzyme immunoassay (Vector-Best, Novosibirsk). The obtained data were processed using the Statistica licensed software package v. 10.0.

The basal level of IL-6 in the main group was 1.28 (0.87-1.72) pg/mL, which is significantly lower than in healthy individuals 5.2 (3.8-6.1) pg/mL and is due to a low level of physical activity due to functional contracture of the quadriceps muscle. In the dynamics of electromyostimulation on the 5th day, the level of IL-6 significantly increased by 3.2 times from the basal level, on the 10th day by 4.6 times, while not exceeding that of the group of healthy individuals. With the reduction of myocytes, the concentration of myokine interleukin-6 increased in the cytoplasm of cells, which contributes to the accumulation of macroergs in the muscle cell, due to myokine-dependent activation of glycogenolysis. The reparative and anti-inflammatory properties of IL-6 are realized in stimulated striated muscles by the classical signaling mechanism that can block the activation of the universal intracellular transcription factor NF-κB in relation to the production of pro-inflammatory cytokines. Thus, electromyostimulation before the start of surgical treatment leads to an increase in the concentration of myokine IL-6 in the blood, which contributes to an increase in the anti-inflammatory and reparative potential of damaged tissues.

Keywords: men, anterior cruciate ligament injury, electromyostimulation, myokines, IL-6, repair

Introduction

Traumatic injuries of the anterior cruciate ligament (ACL) are leading with an incidence of 4 cases per 1000 people [1]. Traumatic ACL injury occurs 20-30 times more often than ruptures of the posterior cruciate ligament, more often in women than in men [7], presumably due to the peculiarities of the hormonal background [2]. ACL is the most important component of knee joint stabilization, which has an extensive proprioceptive field, which is the primary link of the kinematic apparatus. The impulses coming from the proprioceptors limit and mechanically stabilize the muscle response. Rupture of the ACL leads to impaired support ability and mechanical instability of the limb [4]. Most often, the ACL is damaged by a sharp deviation of the lower leg outward and torsion of the thigh inward. One of the classic complications that occurs after a knee injury is arthrogenic muscular inhibition (AMT), diagnosed by difficulty or absence of limb extension due to inhibition of the quadriceps muscle and the development of functional contracture of the hamstring. The prolonged course of the AMT phenomenon leads to atrophy of the quadriceps muscle, constant pain in the knee, dynamic instability of the knee joint and has a negative impact on the outcome of surgical treatment [11].

At the same time, one of the indicators of high muscle activity during intensive motor mode is a sufficient level in the blood of functional muscle proteins – myokines, which are expressed and released by muscle fibers and have a polymodal effect on target organs and tissues [10]. In recent years, numerous data have been accumulated on the effect of myokines on the main homeostatic systems: nervous, immune, endocrine. By exerting a local paracrine effect, myokines, in particular IL-6, can affect the signaling pathways involved in muscle metabolism [10].

In connection with the above, the aim of our work was to study the dynamics of the level of myokine IL-6 in men with ACL damage in the dynamics of static electrical myostimulation (EMS) of the quadriceps femoris muscle.

Materials and methods

The main study group consisted of 23 men, whose average age was 34.8±2.2 years, with a verified diagnosis according to ICD-10 S83.5. Stretching, rupture and overstrain of the anterior cruciate ligament of the knee joint with an injury less than 4 months old, admitted for reconstructive surgery. The main complaints of patients: a feeling of instability in the damaged knee joint (87%), moderate pain (64%), muscle hypotrophy of the limb (43%). To verify the diagnosis, an orthopedic examination and X-ray examination were performed. All patients 10 days before surgery underwent EMS of the quadriceps femoris muscle daily, working cycle 10 s stimulation/10 s relaxation, for 20 min on the INTELECT® Advanced combination therapy device (Chattanooga (DJO), USA). The current strength was increased every 2-3 cycles after the start of exposure, until noticeable muscle contractions appeared.

The control group consisted of 12 healthy men with an average age of 32.2±2.4 years. The level of IL-6 was detected in the venous blood serum of healthy individuals and patients with ACL damage in dynamics before EMS, on the 5th and 10th days using a set of reagents for enzyme immunoassay (Vector-Best, Russia) in the concentration range 0-300 pg/

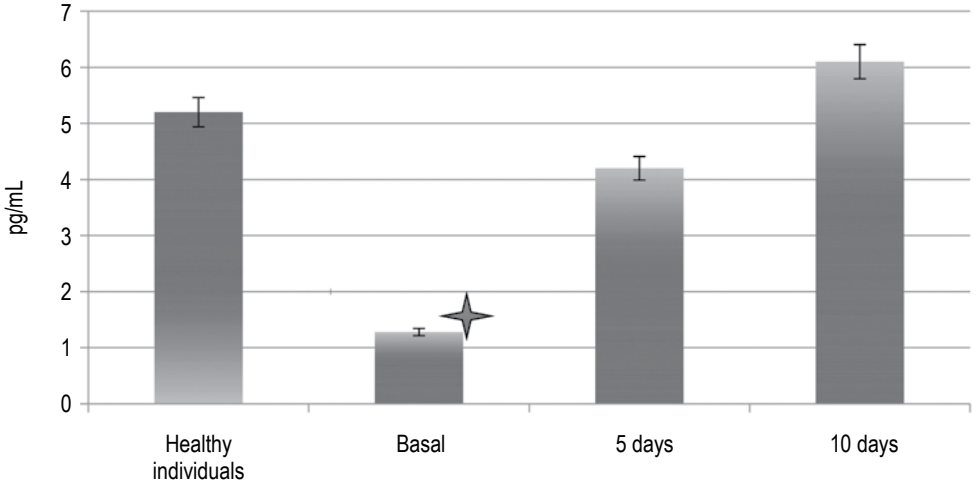

Figure 1. Level of IL-6 in the blood of healthy individuals and in the dynamics of electromyostimulation

mL, at a wavelength of 450 nm. The obtained data were processed using the Statistica licensed software package v. 10.0 (StatSoft Inc., USA). When comparing data, the nonparametric Mann-Whitney test was used, in the dynamics of EMS using the Wilcoxon test. Differences were considered significant at $p \leq 0.05$.

Results and discussion

Prior to the EMS of the quadriceps femoris, the basal level of IL-6 in the main group was 1.28 (0.87-1.72) pg/mL, which is significantly lower than in healthy individuals 5.2 (3.8-6.1) pg/mL (Figure 1). In the dynamics of EMS on the 5th day, the level of IL-6 reached a value of 4.2 (3.4-5.1), on the 10th value 6.1 (4.9-6.8), significantly different from the basal concentration. At the same time, the values of IL-6 on the 5th and 10th days did not exceed the values obtained in the group of healthy individuals.

When exposed to and propagated by an electrical impulse with a frequency of more than 10 Hz, depolarization of the sarcolemma occurs, associated with a change in the potential difference. Control over the electromechanical conjugation of contraction processes during EMS of the striated fibers of the quadriceps muscle is designed to be carried out by the intracellular T-system and the sarcoplasmic reticulum, causing a directed current of calcium ions in the sarcoplasm. The maximum intensity of the contractile act occurs with the synergy of the frequency ranges of nerve impulses and electrical stimulation. An impulse arriving at a frequency of more than 50 Hz forms and prolongs the excitation of the nerve fiber and induces passive muscle contraction. It is known that pulsed currents of low frequency, acting on the system of blood and lymph capillaries, contribute to the redistribution of the tissue component of the fluid.

With the reduction of myocytes, in the cytoplasm of cells, in addition to an increase in the level and enzymatic activity of macroergs (creatine phosphate, adenosine triphosphate), synthetic activity increases in relation to the production of myokines. Our studies have shown that the baseline level of interleukin-6 before the EMS procedure was significantly reduced in comparison with healthy men, probably due to a low level of physical activity associated with injury and conscious limitation of muscle loads. On the 5th day and then on the 10th day, there was an exponential increase in the level of myokine IL-6 in the blood, however, its level at the end of the EMS course did not exceed the levels of healthy individuals, which suggests the presence of other, in particular, anti-inflammatory and reparative properties. myokine in relation to muscle tissue. Some authors believe that an increased level of IL-6 contributes to the accumulation of macroergs in the muscle cell.

Literature sources state that the role of IL-6 is not limited to participation in the acute-phase systemic inflammatory response, but the upregulation of its concentration in the acceptable physiological range contributes to the supply of actively functioning muscles with a set of energy substrates [3, 6]. A striking example is the myokine-dependent activation of glycogenolysis in hepatocytes with a parallel increase in the expression of type 4 glucose transporter on hepatocytes and myocytes [9]. There is information about the stimulation of lipolysis processes in adipocytes and the oxidation of free fatty acids by subthreshold concentrations of IL-6, which are utilized by actively functioning myocytes [8].

The pleotropic biological properties of IL-6 are realized through the coordinated work of the receptor complex, consisting of the IL-6R monomer, which directly binds the myokine itself, and the active gp130 subunit, which mediates a cascade of proteolytic transformations leading to the launch of the JAK/STAT (janus kinase/signal transducer and activator) signaling cascade. of transcription protein) or MAPK (mitogen-activated protein kinase) [12]. The reparative and anti-inflammatory properties of IL-6 are realized in stimulated striated muscles by the classical signaling mechanism that can block the activation of the universal intracellular transcription factor NF-κB, an inducer of the signaling cascade responsible for generating an inflammatory response [13, 14].

Relatively recently, it was shown that IL-6 promotes the formation of an alternative phenotypic transformation of macrophages into the M2 form, which, through the production of a number of anti-inflammatory cytokines, IL-10 and TGF-β, is involved in the processes of repair and remodeling, including in damaged muscle tissue. It has also been shown that IL-6 exhibits the properties of an inducer of the expression of the alpha chain of the IL-4 receptor (IL-4R) in M2 macrophages, sensitizing these cells to IL-4-mediated activation [5].

Conclusion

Thus, EMS before the start of surgical treatment of traumatic ACL injury leads to an increase in the blood concentration of myokine IL-6, within the range of healthy individuals, which contributes to an increase in the anti-inflammatory and reparative potential of damaged tissues.

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ДИНАМИКА СУБПОПУЛЯЦИЙ Т-ХЕЛПЕРОВ В КРИТИЧЕСКОМ ПЕРИОДЕ ТЯЖЕЛОЙ МЕХАНИЧЕСКОЙ ТРАВМЫ У ДЕТЕЙ

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Резюме. Тяжелая механическая травма является одной из основных причин детской инвалидизации и смертности. Развивающийся дисбаланс гипервоспаления и иммуносупрессии в критическом периоде тяжелой травмы повышает риск развития инфекционных осложнений и/или полиорганной недостаточности. Целью работы было определение информативных иммунологических критериев тяжести повреждения, оценки риска развития осложнений и прогноза исхода травматической болезни у детей при тяжелой механической травме (ТМТ, ISS ≥ 16, n = 87) в группах с благоприятным (n = 47) и неблагоприятным исходом (n = 40), а также в зависимости от развития гнойно-септических осложнений (ГСО, n = 16) и синдрома полиорганной недостаточности (СПОН, n = 11). Методом проточной цитометрии проведена оценка содержания Т-хелперов (Th) и их субпопуляций: регуляторные Т-лимфоциты – $CD4^+CD127^{low}CD25^{high}$ (Treg), Th17-го типа – $CD4^+CD161^+$ и $CD4^+CD127^{higt}CD25^{high}$Т-клетки (Т127hi) в 1-е, 3-и, 5-е, 7-е, 14-е сутки с момента получения травмы. Группу сравнения составили 34 ребенка с травмой легкой и средней степени тяжести (ЛТ, ISS < 16). Контрольную группу в исследовании составили 80 условно здоровых детей, сопоставимых по возрасту и полу. В исследовании выявлена обратная зависимость тяжести травмы, степени кровопотери и исхода с абсолютным числом всех субпопуляций, однако для Th и Treg отмечена наиболее сильная связь (R Спирмана ≤ -0,70, p < 0,00001). Для группы ТМТ обнаружено выраженное снижение абсолютного количества клеток Th, Treg, Т127hi и Th17 в раннем посттравматическом периоде с тенденцией к повышению к 14-м суткам. Значения в первые сутки для показателей пациентов с ЛТ соответствовали значениям контрольной группы и значимо отличались от группы ТМТ. Были выявлены различия в динамике процентного содержания субпопуляций Th в остром посттравматическом периоде. Процентное содержание Th17 и Т127hi достоверно повышено в 1-3-й и 3-7-й дни после травмы соответственно в сравнении с кон-

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трольной группой и ЛТ. Для процентного содержания Treg достоверных различий в первую неделю после травмы выявлено не было. Низкий уровень абсолютного количества клеток Treg у пациентов с ТМТ на первые сутки после травмы в значительной степени связан с развитием ГСО и неблагоприятным исходом. Динамика абсолютного количества клеток Th17 в остром посттравматическом периоде значимо отличалась у детей с ТМТ при развитии СПОН. Абсолютное количество Th17 достоверно снижено в 3-7-й дни после травмы в группе ТМТ с СПОН. Результаты исследования свидетельствуют о том, что у детей уровни Treg, T127hi и Th17 достоверно связаны с тяжестью травмы и могут быть использованы для прогнозирования исхода травматического заболевания и оценки риска развития ГСО и СПОН.

Ключевые слова: дети, тяжелая травма, политравма, субпопуляции Т-хелперов, синдром полиорганной недостаточности, инфекционные осложнения

DYNAMICS OF T HELPER SUBPOPULATIONS IN THE CRITICAL PERIOD OF SEVERE INJURY IN CHILDREN

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Abstract. Severe mechanical injury is one of the main reasons behind children's disability and mortality. Severe injure induces a complex host immune response to tissue injury, a parallel pro- and anti-inflammatory state, bearing an elevated risk for infectious complications (IC) and/or multiple organ failure (MOF). This study aimed to determine the informative immunological criteria of traumatic injury severity and prognosis outcome in children (severe injury group (SInj, ISS ≥ 16), n = 87; mild/moderate injury group (MInj, ISS < 16), n = 34) based on the assessment of absolute cell count (abs) and percentage of such T helper subpopulations as regulatory T lymphocytes – $CD4^+CD127^{low}CD25^{high}$(Treg), Th17 lymphocytes – $CD4^+CD161^+$ and $CD4^+CD127^{higt}CD25^{high}$ T cells(T127hi) in severe injury cases grouped by the outcome (favorable, n = 47; unfavorable, n = 40) and depending on IC (n = 16) and the development of MOF (n = 11) on the 1^{st}, 3^d, 5^{th}, 7^{th}, 14^{th} day after injury. The control group was comprised of 80 apparently healthy children comparable in age and sex. An inverse relationship between severity of injury, degree of blood loss and outcome of injury was revealed with the abs of all Th populations, but for Th abs and Treg abs the most significant correlation was found (Spearman's R ≤ -0,70, p < 0.00001). For SInj group, a pronounced decrease of Th abs, Treg abs, T127hi abs and Th17 abs, in the acute post-traumatic period with an increase to 14 days was revealed. The values of in the first day for indicators of patients with MInj group correspond to the values of control group and significantly differ from SInj group. There are different kinetics of percentage Th subpopulations in peripheral blood of children with severe injuries. The Th17%$CD4^+$ and T127hi%$CD4^+$ significant increase in 1^{st}-3^d and 3^d-7^{th} days after injury respectively in comparation with control and MInj groups. There were no differences between groups in terms of Treg%$CD4^+$. The lower-level Treg abs in trauma patients admitted to the ICU is significantly associated with develop the infectious complications and outcome of trauma. The Th17 abs is significantly reduced in 3-7^{th} days after the injury in the SInj group with MOF. The results of the study indicate that in children levels of Treg, T127hi and Th17 is significantly associated with severity of injury and may be used to predict outcome of trauma and assess the risk of IC and MOF.

Keywords: children, severe injury, polytrauma, T helper subpopulations, multiple organ failure, infectious complications

Introduction

Severe mechanical injury is one of the main reasons behind children's disability and mortality [3]. Severe injury induces a complex host immune response to tissue injury, a parallel pro- and anti-inflammatory state, bearing an elevated risk for infectious complications (IC) and/or multiple organ failure (MOF) [1, 5]. There is usually a period of prominent immunosuppression the pathogenesis of which is largely shaped by the decreasing level of

T lymphocytes (Th) in severe injury [7]. Activity, absolute cell number and frequency of such T helper subpopulations as regulatory T lymphocytes (Treg) – $CD4^+CD127^{low}CD25^{high}$ and Th17 lymphocytes (Th17) can be a significant marker in determining the severity of the pathological process and predicting its outcome. Regulatory T cells play an important role in the orchestration of self-tolerance and immune reaction. It has been demonstrated that Tregs were activated in response to injury which driven trauma-induced suppression of Th1 responses and T cell anergy [4, 8]. Moreover, they have an important but not yet profoundly characterized role during the post-traumatic inflammation; and may, therefore, be an important determinant of the extent and/or severity of the post-traumatic immunosuppression as well [9]. Th17 cell is a novel subset of $CD4^+$T cells characterized by the production of IL-17 and other abundant cytokines such as IL-22, and IL-26 and TNFα with a week and proinflammatory response [12]. Trauma patients admitted to the intensive care unit (ICU) ward have identified increased Th17 cells during the first week after admission, which are correlated with development of early poor outcomes [2]. Therefore, we have characterized and analyzed the dynamics of some subsets of Th, which may play an important role in the post-traumatic immunosuppression, and thereby the recovery from trauma in children.

The purpose of this study was to identify informative immunological criteria for the traumatic disease severity and as applicable to children. The identification relies on the assessment of absolute and relative number of T helper subpopulations – Th17, Treg and $CD4^+CD127^{higt}CD25^{high}$ lymphocytes.

Materials and methods

The study involved 87 patients (58 boys (66.6%), 35 girls (33.4%); 331 observation sessions) with severe injury (SInj), treated at the Department of Anesthesiology and Resuscitation of the Research Institute of Emergency Pediatric Surgery and Traumatology. We used the laboratory of the National Medical Research Center for Children's Health for laboratory studies, which were prescribed 1 to 5 times, depending on the length of stay of a given child at the ICU. The median age of the children was 12.0 (5.75-15.0) years (Me ($Q_{0.25}$-$Q_{0.75}$)). The time options for laboratory studies were the first, third, fifth, seventh and 14th days from the day of injury. The comparison group was comprised of 34 patients (15 boys (44.1%), 19 girls (55.9%); 34 observation sessions) with mild/moderate injury (MInj) treated at the Department of Neurotrauma. The control group was comprised of 80 apparently healthy children, all of them underwent medical examination at the National Medical Research Center for Children's Health. The children were comparable in age and sex: age – 12.41 (4.4-16.2) years; 47 boys (58.7%), 33 girls (41.3%).

Assessing the injury, we relied on the Injury Severity Score (ISS) and the Glasgow Coma Scale (GCS). The outcome of an SInj was assessed with the help of the Glasgow Outcome Scale (GOS) and the Severe Injury Outcomes Scale (OISS) [11]. These scales were applied to assess the condition of the patient at discharge from the hospital.

The patients in our study met the following criteria: severe injury (ISS ≥ 16), aged 1-18 years, admittance to the ICU within 72 hours. Concomitant acute inflammatory and chronic diseases were a reason for exclusion.

At the first stage, we analyzed the results from the control group, the MInj (ISS 4.0 (4.0-9.0)) and the SInj group (ISS 26.0 (21.0-29.0)). At the second stage, we analyzed the two groups from SInj formed with the help of GOS and OISS, the favorable outcome group (SInjfav, n = 47) and the unfavorable outcome group (SInjunfav, n = 40). The distribution into these groups was based on the scores: patients were allocated to the SInjfav group if they scored 4-5 points on the GOS scale and 1-2 points on the OISS scale, and to the SInjunfav group if they scored 1-3 points on the GOS scale and 3-5 points on the OISS scale.

Clinical and laboratory indicators of systemic inflammatory response syndrome and organ failure were evaluated in all patients with severe injury. Organ functioning was assessed daily after admission to the ICU using MODS (Multiple Organ Dysfunction Score) [6]. Patients with severe injury were divided into groups depending on infectious complications (IC n = 16) and the development of MOF (MOF n = 11).

We assessed the quantity of Th – $CD4^+$ cells, T127hi – $CD4^+CD127^{higt}CD25^{high}$, Th17 – $CD4^+CD161^+$, Treg – $CD4^+CD127^{low}CD25^{high}$ in the patients. Two-platform technology enabled assessment of the quantitative indicators of the subpopulation composition of peripheral blood T helpers. The absolute number of lymphocytes was calculated with the help of a Sysmex XT-2000i hematology analyzer (Sysmex Corporation; Japan). The preparation of samples for cytofluorimetric analysis included incubation of 100 μL of whole blood with 10 μL of monoclonal antibodies tagged with fluorochromes for 20 min in a dark place. The erythrocytes were lysed with BD FACS™ Lysing Solution (BD Biosciences; USA); the duration of incubation therewith in the dark at room temperature did not exceed 10-12 minutes. The resulting samples were analyzed in a Novocyte flow cytometer (ACEA Biosciences; USA). The surface markers used to determine lymphocyte subpopulations were as follows: CD45, IgG1, IgG2a, CD3, CD4, CD25, CD127, CD161 (Beckman

Coulter, USA; BD Biosciences, USA; SONY corp., Japan).

Analysis of quantitative indicators of the population composition of blood lymphocytes in children is particularly difficult due to the existence of age characteristics. To unify the indicators, we previously carried out a transformation according to the formula, considering the normative values for different age groups of the parameters of the main and small populations of peripheral blood lymphocytes [10]:

Xn = (Xmin-X) / 0,01*(Xmax – Xmin)

X – the value of the studied indicator, Xn – the value of the indicator normalized by the age norm, Xmax is the upper limit of the age norm, Xmin is the lower limit of the age norm.

After the transformation, an array of data was obtained on all the main indicators of the population composition of peripheral blood lymphocytes in children, in which the values of the studied indicators are presented in uniform conventional units. If the obtained value of the studied indicator falls into the range from 0 to 100, then it corresponds to the age norm. Thus, this allows you to analyze the data without considering the age characteristics of the dynamics of the quantitative indicators of the population composition of blood lymphocytes in children.

We used MS Excel 365 (Microsoft corp.; USA), Statistica 10 (StatSoft, Inc.; USA) to process the data obtained. The results are presented as a median (Me) and quartiles ($Q_{0.25}$-$Q_{0.75}$). Mann–Whitney U test and Kruskal–Wallis test with Bonferroni correction enabled comparison of differences in the attributes. Spearman's rank correlation coefficient (R) was used to assess relations between the attributes. The conclusions were considered significant at $p < 0.05$ (*).

Results and discussion

The absolut cell count and relative amount Th, T127hi, Treg and Th17 obtained in children on the 1st day of injury, underwent a correlation analysis with clinical parameters (Table 1). An inverse relationship between the severity of the injury, the degree of blood loss and the outcome of the injury according to OISS was revealed with the absolute number of all analyzed lymphocyte populations, but for Th and Treg absolut cell count the most significant correlation was found (Spearman's $R \leq -0.70$, $p < 0.00001$) (Table 1, Figure 1B, C). The similar data have been obtained

TABLE 1. SPEARMAN'S R CORRELATION COEFFICIENT FOR Th, T127hi, Treg AND Th17 ABSOLUTE CELL COUNT AND PERCENTAGE OF T127hi, Treg AND Th17 AND CLINICAL INDICATORS IN THE 1ST DAY OF INJURY IN CHILDREN

Index	Factor	N	Spearman R	t(N-2)	p-level	p-level
ISS	Th abs	102	-0.70	-9.76	0.00000	< 0.00001
	Treg abs	102	-0.71	-10.08	0.00000	< 0.00001
	Treg %CD4	102	-0.05	-0.52	0.60616	
	T127hi abs	102	-0.50	-5.80	0.00000	< 0.00001
	T127hi %CD4	102	0.27	2.85	0.00524	< 0.01
	Th17 abs	102	-0.59	-7.25	0.00000	< 0.00001
	Th17 %CD4	102	0.34	3.57	0.00055	< 0.01
OISS	Th abs	102	-0.52	-6.12	0.00000	< 0.00001
	Treg abs	102	-0.52	-6.06	0.00000	< 0.00001
	Treg %CD4	102	-0.03	-0.30	0.76363	
	T127hi abs	102	-0.47	-5.34	0.00000	< 0.00001
	T127hi %CD4	102	0.07	0.73	0.47008	
	Th17 abs	102	-0.51	-5.92	0.00000	< 0.00001
	Th17 %CD4	102	0.18	1.86	0.06590	
Blood loss degree	Th abs	102	-0.54	-6.47	0.00000	< 0.00001
	Treg abs	102	-0.52	-6.16	0.00000	< 0.00001
	Treg %CD4	102	0.09	0.94	0.35031	
	T127hi abs	102	-0.39	-4.30	0.00004	< 0.00001
	T127hi %CD4	102	0.25	2.56	0.01186	< 0.05
	Th17 abs	102	-0.48	-5.46	0.00000	< 0.00001
	Th17 %CD4	102	0.26	2.66	0.00914	< 0.01

in other studies. It has been shown that persistence of lymphopenia shaped by the decreasing level of Th in severe injury following trauma is correlated with severity and is associated with poorer prognosis [7]. The lymphocyte counts decrease immediately after trauma in patients compared to the control group [5].

Using the nonparametric Kruscal-Wallis test with Bonferroni correction, we compared the differences in Th, T127hi, Treg and Th17 absolut cell count and the frequency of T127hi, Treg and Th17 in children with injury over time in different groups: Control, MInj and SInj groups (Table 2, Figure 1A, D-I).

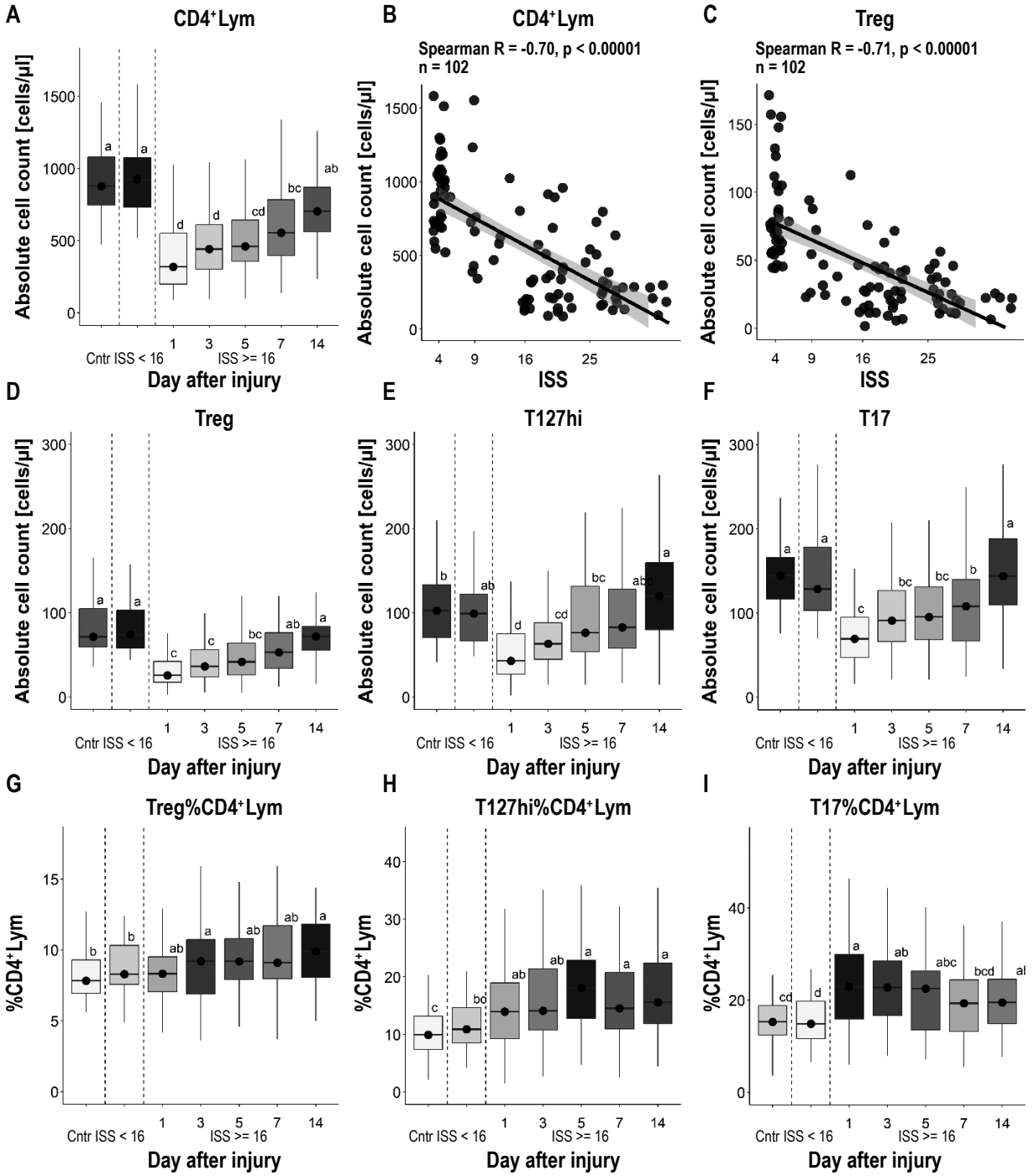

Figure 1. Dynamics of Th absolute cell count in the critical period of injury in children (A). Relationship between the Th and Treg absolute cell count and Injury Severity Score in the 1st day of injury in children (B, C). Absolute cell count dynamics of T127hi, Treg and Th17 cells in the critical period of injury in children (D-F). The frequency dynamics of T127hi, Treg and Th17 cells in the critical period of injury in children (G-I)

Note. Me ($Q_{0.25}$-$Q_{0.75}$) Min-Max; the significance is represented by letters according to pairwise comparation through the Kruskal–Wallis test adjusted with Bonferroni correction; comparison groups: Cntr, control group; ISS < 16, MInj group; SInj group by day after severe injury.

TABLE 2. DYNAMICS OF Th, T127hi, Treg AND Th17 ABSOLUTE CELL COUNT, PERCENTAGE AND NORMALIZED ABSOLUTE CELL COUNT IN THE CRITICAL PERIOD OF SEVERE INJURY IN CHILDREN IN COMPARATION WITH CONTROL GROUP AND MINJ GROUP AND ALSO IN SINJ GROUP DEPENDING DEVELOPMENT OF INFECTIOUS COMPLICATION, MULTIPLE ORGAN FAILURE DEVELOPMENT AND OUTCOME OF SEVERE INJURY, Me ($Q_{0.25}$-$Q_{0.75}$)

Factor		Control	MInj	SInj, day after injury				
				1	3	5	7	14
n		80	34	68	87	35	74	67
Th	abs	886.57 (752.75-1094.20)	923.50 (729.6-1076.2)	319.22 (199.63-550.65)	446.18 (302.98-615.83)	460.40 (356.36-642.75)	565.28 (398.96-804.30)	720.83 (562.76-942.50)
Treg	abs	71.67 (59.37-105.07)	74.02 (58.06-103.31)	25.90 (17.49-42.23)	36.50 (24.08-56.48)	41.76 (26.30-64.30)	53.18 (34.01-76.50)	72.00 (55.90-84.00)
	% Th	7.83 (6.95-9.29)	8.30 (7.58-10.33)	8.33 (7.06-9.50)	9.20 (6.88-10.72)	9.20 (7.92-10.80)	9.10 (7.98-11.70)	9.90 (8.05-11.80)
	abs n, c. u.		39.50 (11.21-101.87)	-112.64 (-139.44--78.08)	-75.26 (-118.09--29.15)	-60.96 (-106.17--3.86)	-28.33 (-76.38-56.26)	39.04 (-25.23-89.81)
MOF Treg abs n, c. u.	N			-111.55 (-148.04--80.69)	-73.33 (-117.56--24.42)	-60.03 (-93.94--10.00)	-23.33 (-69.52-58.58)	40.98 (-25.23-89.81)
	Y			-125.74 (-131.87--111.79)	-98.83 (-132.77--66.92)	-136.17 (-173.36-45.49)	-73.33 (-114.58-29.48)	25.82 (-15.69-67.30)
IC Treg abs n, c. u.	N			-102.25 (-133.34--72.87)**	-72.42 (-118.62--22.39)	-49.03 (-93.94-22.38)*	-28.33 (-76.38-56.26)	50.00 (-33.37-89.82)
	Y			-144.00 (-154.27--130.84)**	-90.15 (-119.48--74.18)	-125.58 (-156.43--90.00)*	-26.59 (-73.52-32.33)	13.77 (-10.80-53.54)
Outcome Treg abs n, c. u.	F			-96.74 (-134.04--60.20)*	-72.42 (-117.56--20.00)	-58.35 (-92.95--23.53)	-11.11 (-64.96-61.14)	44.52 (-35.56-93.02)
	UF			-128.24 (-141.98--102.64)*	-83.03 (-119.19--48.81)	-78.19 (-128.23-48.82)	-47.30 (-90.79-38.83)	36.67 (-8.69-89.51)
T127hi	abs	102.65 (70.62-133.63)	99.23 (66.79-122.26)	43.04 (27.03-75.31)	63.18 (44.72-88.23)	76.31 (53.92-131.67)	82.85 (58.26-127.99)	120.74 (81.00-161.54)
	% Th	9.91 (7.38-13.16)	10.90 (8.53-14.62)	13.95 (9.25-18.95)	14.10 (10.78-21.42)	18.10 (12.75-22.90)	14.50 (10.95-20.75)	15.60 (11.85-22.40)
	abs n, c. u.		50.94 (9.10-110.44)	-48.26 (-82.90-0.61)	-17.18 (-50.30-32.94)	19.50 (-17.53-99.38)	26.33 (-31.68-110.29)	104.30 (3.77-168.58)
MOF T127hi abs n, c. u.	N			-50.91 (-87.06--0.40)	-13.60 (-49.51-50.94)	21.07 (-10.94-96.17)	33.14 (-14.01-113.31)**	109.43 (3.77-168.58)
	Y			-62.91 (-76.57--41.56)	-29.20 (-71.00--1.14)	-68.42 (-81.58-99.39)	-32.92 (-84.10--24.51)**	88.55 (2.59-174.04)

Таблица 2 (окончание)
Table 2 (continued)

Factor		Control	MInj	SInj, day after injury				
				1	3	5	7	14
n		80	34	68	87	35	74	67
IC T127hi abs n, c. u.	N			-51.42 (-81.05- -0.96)	-12.46 (-48.45- 35.73)	24.21 (-7.08- 99.39)*	32.94 (-17.76- 120.00)	109.43 (2.69- 168.58)
	Y			-70.16 (-111.08- -25.12)	-32.68 (-84.91- 0.45)	-49.77 (-96.77- -10.33)*	-26.75 (-40.80- 11.57)	41.75 (22.13- 195.53)
Outcome T127hi abs n, c. u.	F			-37.16 (-85.65- 26.00)	-24.67 (-58.74- 15.63)	16.54 (-10.94- 80.44)	33.14 (-0.81- 131.02)*	114.22 (17.55- 168.66)
	UF			-60.70 (-87.85- -24.76)	-1.89 (-47.12- 43.47)	19.50 (-68.42- 99.39)	-4.26 (-44.96- 58.67)*	82.76 (-10.91- 166.05)
Th17	abs	144.36 (116.90- 166.04)	128.61 (103.20- 178.35)	69.31 (47.03- 95.12)	91.15 (66.29- 126.83)	95.62 (70.50- 133.57)	109.37 (67.36- 141.61)	144.92 (111.13- 196.72)
	% Th	15.30 (12.40- 18.80)	14.90 (11.65- 19.78)	22.90 (15.93- 29.90)	22.75 (16.68- 28.52)	22.50 (13.60- 26.35)	19.30 (13.20- 24.38)	19.50 (14.90- 24.55)
	abs n, c. u.		17.20 (-60.85- 99.92)	-160.14 (-211.16- -101.13)	-93.31 (-149.53- -23.86)	-88.23 (-152.06- -11.65)	-53.08 (-146.56- 21.43)	28.32 (-53.72- 128.90)
MOF Th17 abs n, c. u.	N			-160.38 (-211.41- -101.89)	-81.08 (-144.59- -18.00)*	-60.26 (-121.19- -4.21)**	-45.45 (-139.56- 22.21)*	38.74 (-43.19- 147.54)
	Y			-156.14 (-181.26- -136.85)	-170.82 (-232.67- -101.43)*	-277.79 (-325.00- -215.66)**	-137.74 (-237.73- -67.58)*	-6.65 (-58.13- 44.37)
IC Th17 abs n, c. u.	N			-153.46 (-197.67- -99.62)	-90.47 (-148.16- -18.62)	-64.95 (-121.43- -1.95)*	-46.31 (-132.29- 21.43)	19.50 (-60.09- 126.42)
	Y			-202.25 (-259.88- -167.77)	-128.30 (-158.49- -38.85)	-152.06 (-204.08- -124.06)*	-141.99 (-153.13- -66.07)	43.14 (-16.14- 137.67)
Outcome Th17 abs n, c. u.	F			-163.97 (-213.25- -80.74)	-96.94 (-149.53- -25.18)	-45.71 (-119.09- 6.48)	-24.06 (-113.70- 23.74)	24.61 (-45.15- 119.70)
	UF			-157.40 (-204.81- -139.29)	-81.08 (-148.16- -22.73)	-116.70 (-219.45- -45.28)	-112.23 (-153.72- -7.99)	28.32 (-56.18- 147.17)

Note. Mann–Whitney U test; *, p < 0,05; **, p < 0,01; comparison groups: MOF group (N – no, Y – yes); IC, infectious complication (N – no, Y – yes); Outcome (F – favorable outcome, UF – unfavorable outcome); c. u., conventional units.

For SInj group, a pronounced decrease of Th, Treg, T127hi and Th17 absolute cell count, in the acute post-traumatic period with an increase to 14 days was revealed. The values of in the first day for indicators of patients with MInj correspond to the values of control group and significantly differ from patients with SInj in the 1-7th day for Th17 and Treg, for T127hi in the 1st-3d day after the injury (Figure 1A, D-F).

There are different kinetics of the percentage Th subpopulations in peripheral blood of children with severe injuries. The percentage of Th17 and T127hi significant increse in 1st-3d and 3d-7th days after injury respectively in comparation with Control and MInj group (Figure 1H, I). Zhang et al. demonstrated that the level of Th17 showed increased initially and then decreased in patients with thoracic trauma.

The frequency of Th17 was significantly increased in traumatic patients compared to healthy controls on the day after admission [12]. At the same time, in our study there were no differences between groups in terms of the percentage of Treg cells (Figure 1G). In contrast, it has been demonsrated the percentage of Treg cells in the peripheral blood was higher in patients with thoracic trauma compared with that of healthy group [12].

Using the nonparametric Mann–Whitney test, we compared the differences in normalized absolut cell count Treg (Tregn), T127hi (T127hin) and Th17 (Th17n) in children with severe injury over time in different groups: with and without MOF, with and without IC and outcome groups (Table 2). Patients with MOF had significantly lower median values of analised parmetrs within 1st-7th days after admission to the ICU than patients without MOF. Significant differences in Th17n and T127hin level were found in MOF groups from 3rd to 7th day and in 7th day respectively. Only one previous study has examined significant decreases in the number of lymphocytes between MOF and non-MOF groups appear after day 2 [5].

Despite improvements in prognosis and survival, there is a lack of validated diagnostic tools for post-traumatic complications. In a retrospective study involving 188 patients after severely injured trauma the immunoregulatory index, in the polytrauma patients with purulent-inflammatory complications, was lower than in the patients without purulent-inflammatory complications due to the reduced values of Th [7]. In our study the levels of Tregn in 1st and 5th day and Th17n, T127hin in 5th day after injury differed significantly in groups without and with IC: patients from the latter group had it considerably lower. Study on trauma patients admitted to the ICU ward have identified increased Th17 cells and serum IL-17 levels during the first week after admission, which are correlated with development of early poor outcomes [2].

A comparative analysis of the post-traumatic period data from SInjfav and SInjunfav groups has shown a significant increase Tregn in 1st day and T127hin in 7th day after injury in the SInjunfav group. At the same time, there were no differences between groups in terms of Th17n. It has been observed that the decrease and increase of Treg cells in the early and late phases of the disease, respectively, are associated with poor prognosis of trauma [2, 9, 12].

Conclusions

The results of the study indicate that in children the levels of Treg, T127hi and Th17 is significantly associated with the severity of injury and may be used to predict outcome of the traumatic disease and assess the risk of infectious complications and multiple organ dysfunction syndrome. The lower-level absolute cell count Treg in trauma patients admitted to the ICU is significantly associated with develop the infectious complications and outcome of the traumatic disease. The dynamics of absolute cell count Th17 in the post-injury period are important for development multiple organ dysfunction in children with severe trauma.

Compliance with ethical standards

The study was conducted in accordance with the Declaration of Helsinki and approved by the Committee on Biomedical Ethics of Institute of Urgent Children Surgery and Traumatology (Protocol No. 2 of 26.05.2020). All study participants signed an informed consent.

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ДИФФЕРЕНЦИРОВАННЫЕ НАРУШЕНИЯ ИММУННОЙ СИСТЕМЫ ПРИ ОСТРОМ ГЕМАТОГЕННОМ И ОСТРОМ ПОСТТРАВМАТИЧЕСКОМ ОСТЕОМИЕЛИТАХ У ДЕТЕЙ

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Резюме. Остеомиелит – воспаление кости и костного мозга, вызванное распространением *S. aureus* из локального очага гематогенным путем или из открытого травматического перелома, которое трудно поддается лечению и остается серьезной проблемой. Условиями распространения инфекционного процесса в кости является влияние *S. aureus*, нарушение его элиминации из-за дисфункции иммунной системы (ИС). Разноречивые данные об иммунопатогенетических механизмах развития острого остеомиелита требуют изучения, позволяющего разработать обоснованную иммунотерапию. Цель исследования – уточнить варианты нарушений противобактериальной иммунной защиты у детей с острым гематогенным и острым посттравматическим остеомиелитами. Исследованы дети 8-15 лет (n = 22): группа исследования 1 (ГИ1) – 12 пациентов с острым гематогенным остеомиелитом (ОГО); группа исследования 2 (ГИ2) – 10 детей с острым посттравматическим остеомиелитом (ОПО). Группу сравнения (ГС) – 13 здоровых детей соответствующего возраста. Тестировали Т-лимфоциты ($CD3^+CD19^-$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+/CD3^+CD8^+$), В-лимфоциты ($CD3^-CD19^+$), NK ($CD3^-CD16^+CD56^+$) и TNK ($CD3^+CD16^+CD56^+$) лимфоциты, CD16, CD32, CD64 рецепторы на нейтрофильных гранулоцитов (НГ) (FC-500 Beckman Coulter, США); уровень сывороточных IgA, IgM, IgG (ИФА). Оценивали фагоцитарную функцию НГ по отношению к *S. aureus*: количество активно фагоцитирующих НГ (%ФАН), процессы захвата (ФЧ, ФИ) и киллинговую активность (%П, ИП). В группах ОГО и ОПО выявлено снижение количества Т-лимфоцитов, Т-хелперов, TCTL и NK ($p_{1\text{-}4} < 0{,}05$). Также установлено, что при ОГО уровень IgA, IgM, IgG не отличался от показателей ГС, тогда как при ОПО отмечалось повышение уровня IgA и IgG ($p_{1,2} < 0{,}05$). Показано, в группах с ОГО и ОПО отмечается разная плотность экспрессии рецепторов CD64, CD16, CD32 на НГ, предопределяющая несостоятельность фагоцитарной функции. Дефекты фагоцитоза, при ОГО в первую очередь, связанны с нарушениями захвата и киллинга, а при ОПО только с процессами переваривания бактериального антигена. Выявленные комбинированные дефекты функционирования ИС диктуют

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необходимость разработки новых подходов в лечении ОГО и ОПО у детей, патогенетически обосновывающих использование иммунотерапии в комплексном этиопатогенетическом лечении, что будет способствовать восстановлению противоинфекционного иммунитета, улучшению клинического течения заболеваний, а также препятствовать хронизации воспалительного процесса и усугублению дисфункции ИС.

Ключевые слова: острый гематогенный остеомиелит, острый посттравматический остеомиелит, дети, иммунная система, дисфункции, противобактериальный иммунитет

DIFFERENTIATED DISORDERS OF THE IMMUNE SYSTEM IN ACUTE HEMATOGENIC AND ACUTE POSTTRAUMATIC OSTEOMYELITIS IN CHILDREN

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Abstract. Osteomyelitis is an inflammation of bone and bone marrow caused by the spread of *S. aureus* from a local focus by the hematogenous route or from an open traumatic fracture; it is difficult to treat and remains a serious problem. The condition for spreading of the infectious process into bone is the effect of *S. aureus* and its impaired elimination due to immune system (IS) dysfunction. Controversial information on the immunopathogenetic mechanisms of acute osteomyelitis needs study, which would allow the development of sound immunotherapy. Purpose of the study: to specify the variants of antibacterial immune protection disorders in children with acute hematogenous and acute posttraumatic osteomyelitis. Materials and methods. Children 8-15 years old (n = 22) were studied: Study Group 1 (SG1, n = 12) – with acute hematogenous osteomyelitis (AHO); Study Group 2 (SG2, n = 10) – with acute post-traumatic osteomyelitis (APTO). The comparison group (CG) – 13 healthy children. Tested: T lymphocytes ($CD3^+CD19^-$, $CD3^+CD4^+$, $CD3^+CD8^+$), B lymphocytes ($CD3^-CD19^+$), NK ($CD3^-CD16^+CD56^+$) and TNK ($CD3^+CD16^+CD56^+$) lymphocytes, neutrophil granulocytes (NG, CD16, CD32, CD64) (FC-500 Beckman Coulter, USA); the level of serum IgA, IgM, IgG (ELISA). Phagocytic function of NGs in relation to *S. aureus* was assessed: the number of actively phagocytizing NGs (%PhAN), capture processes (PhN, PhI) and killing activity (%D, DI). Results. In both groups was revealed a decrease of T lymphocytes, T helpers, T_{CTL} and NK quantity ($p_{1-4} < 0.05$). In AHO, the levels of IgA, IgM, IgG did not differ from that in GS, while in APTO the levels of IgA and IgG increased ($p_{1,2} < 0.05$). The density of CD64, CD16, CD32 receptor expression on NG in the studied groups has been a different equipping, predetermining an incompetence of the phagocytic function: in AHO associated with abnormalities in the function capture and killing, in APTO only with the *S. aureus* digestion. Conclusion. The revealed combined defects of IS functioning necessitate the development of new approaches in the treatment of AHO and APTO in children, pathogenetically substantiating the use of immunotherapy in the complex etiopathogenetic treatment. This approach will contribute to the restoration of mechanisms of anti-infective immunity, timely elimination of pathogens, improve the clinical course of the diseases, prevent the chronic inflammatory process.

Keywords: acute hematogenous osteomyelitis, acute post-traumatic osteomyelitis, children, immune system, dysfunctions, antibacterial immunity

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Introduction

Deep infections, such as osteomyelitis, which are difficult to treat, remain a serious health problem worldwide [2, 11]. Osteomyelitis is an infectious inflammation of the bone and bone marrow caused by the spreading of an agent from a local focus by hematogenous route and/or from an open fracture to all parts of the bone and its surrounding soft tissues, which ultimately leads to progressive destruction of the bone [7]. Acute osteomyelitis is predominantly a childhood disease with a peak incidence occurs at 10-14 years (60-80%) [3]. In difficult cases of acute

osteomyelitis, children continue to die, even in our era of significant advances in surgical procedures and the strongest antibiotics. The causative agents of osteomyelitis are commensal staphylococci, *S. epidermidis*, and the most common etiological agent is *S. aureus* [6]. *S. aureus* is highly virulent, expressing immunomodulatory proteins, adhesins, toxins, and superantigens, and is able to adapt to the immune response and evade it.

In addition, *S. aureus* has many mechanisms promoting tolerance to antibiotic treatment [9]. In particular, it is able to create three-dimensional conglomerates consisting of bacteria surrounded by neutrophilic granulocytes (NGs) and macrophages. *S. aureus* forms biofilms on necrotic bone; it secretes SpA proteins that bind to IgG Fc-fragments and block antibody-mediated phagocytosis, or to Fab-domains of the VH3 chain of IgM antibodies, which causes proliferative expansion of B cells ending in apoptosis [9]. Recently, the mechanism of *S. aureus* evasion from the response of the immune system (IS), which leads to its persistence, was discovered: the invasion into submicron channels deep in the bone cortex, where bacteria can survive for many years, dissolving the surrounding bone mineral matrix [4].

On the other hand, it has been established that the most important condition for the spread of the infectious process in the bone is both the negative impact of *S. aureus* itself and the violation of its elimination, and IS dysfunction [8, 12]. The imbalance of the immune system in AHO in young children has been confirmed in various studies [1, 3, 12]. There is the information of the presence of immune deficiency before the onset of the disease, which caused the onset and progression of a focus in the bone tissue in chronic osteomyelitis [1]. At the same time, there are many conflicting facts of the pathogenetic mechanisms in the development of acute osteomyelitis, which, from our point of view, remain insufficiently studied. It should be noted that an in-depth approach to the study of the immunopathogenesis of acute osteomyelitis is needed, which would allow to develop the pathogenetically substantiated immunotherapy.

Purpose of the study: to clarify the variants of violations of antibacterial immune defense in children with acute hematogenous and acute post-traumatic osteomyelitis.

Materials and methods

The study included children aged 8-15 years with acute osteomyelitis (n = 22) hospitalized at the Regional Children's Clinical Hospital of the Ministry of Health of the Krasnodar Krai.

Based on clinical and laboratory data, 2 study groups were formed. Study group 1 (SG 1) included 12 patients (1 girl, 11 boys) with acute hematogenous osteomyelitis (AHO). For patients from SG1, at the time of admission to the hospital the febrile temperature was for the 4 (2.5-6.5) days from the onset of the disease, a high level of CRP – 60 (13-158) mg/L. Before hospitalization, 1 person took antibiotics.

Study group 2 (SG2) consisted of 10 children (2 girls, 8 boys) with acute post-traumatic osteomyelitis (APTO). In SG2 patients, at 9 (7-14) from the onset of the disease, subfebrile temperature was noted, the level of CRP was 8 (5-30) mg/L. Prior to hospitalization, 5 people were taking antibiotics (a broad-spectrum synthetic penicillin antibiotic with a beta-lactamase inhibitor or a third-generation cephalosporin with a beta-lactamase inhibitor). The comparison group (CG) consisted of 13 conditionally healthy children of the corresponding age.

We have determined the content of T lymphocytes ($CD3^+CD19^-$, $CD3^+CD4^+$, $CD3^+CD8^+$, IRI_1 – $CD3^+CD4^+/CD3^+CD8^+$), and B lymphocytes ($CD3^-CD19^+$), as well as NK lymphocytes ($CD3^-CD16^+CD56^+$), TNK lymphocytes ($CD3^+CD16^+CD56^+$), CD16, CD32, CD64 NGs receptors on Cytomics FC-500 cytometer (Beckman Coulter, USA) using monoclonal antibodies (Beckman Coulter, USA). The level of serum IgA, IgM, IgG was determined (ELISA, test systems of CJSC Vector-Best, Russia). The phagocytic activity of NG was assessed with the determination of the number of actively phagocytic NG (%PhAN) uptake processes (PhN, PhI) and the degree of completion of the phagocytic act with an assessment of the digestive activity (% D, DI) in relation to *S. aureus* (strain 209)

The conducted study complies with the requirements of the WMA Declaration of Helsinki (DoH), approved by the Social Ethics Committee of the Kuban State Medical University of the Ministry of Health of Russia.

Statistical processing of the study results was carried out using computer programs Microsoft Excel 2016 and StatPlus 2020. Nonparametric statistics methods were used: Me ($Q_{0.25}$-$Q_{0.75}$), Mann–Whitney U test. Differences were determined to be statistically significant at $p < 0.05$

Results and discussion

An analysis of the total number of leukocytes and their morphology made it possible, already at the level of general clinical studies, to identify an inadequate response to the inflammatory process in AHO and APTO. Thus, in children with AHO in SG1, a slight increase in WBC was found to 9.6 (8.4-10.0) × 10^9/L *versus* 4.6 (4.1-6.2) × 10^9/L in CG ($p < 0.05$), by against the background of an unchanged absolute and percentage content of NG with an increase in the proportion of stab forms – 7.0 (5.0-10.3) % ($p_1 < 0.05$; $p_2 > 0.05$), a decrease in the number of lymphocytes (LY) – 29 (19.5-30.0) % *versus* 37,3 (33.4-38.5) %

($p < 0.05$) and 2.1 (1.7-2.9) × 10^9/L *versus* 2.5 (2.4-2.5) × 10^9/L in CG ($p > 0.05$) (Table 1).

Evaluation of leukocyte parameters in children with APTO in SG 2 revealed a blockade of the response to the infectious and inflammatory process. Tendencies were noted: an increase in the number of WBCs to the upper limit of the reference values of CG ($p > 0.05$), an increase in the absolute content of NG ($p > 0.05$), against the background of a decrease in the proportion of segmented NG due to an increase in banded NG ($p < 0.05$). At the same time, the indicators of the relative amount and absolute LY did not differ from the values of CG ($p_{1,2} > 0.05$) (Table 1).

In the study of cellular immunity in children with AHO and APTO, unidirectional, but with varying degrees of dysfunction, are revealed. Thus, in SG 1 with AHO, there was a 1.4-fold decrease in the level of T lymphocytes – $CD3^+CD19^-$ to 1.3 (1.1-1.6) × 10^9/L against 1.9 (1.7-2.0) × 10^9/L in CG ($p < 0.05$), due to a parallel decrease in T helpers – $CD3^+CD4^+$ by 1.6 times ($p < 0.05$) and 1.9 times the amount of T_{CTL}-$CD3^+CD8^+$ ($p < 0.05$), implementation index inversion (IRI_1) – 1.3 (0.9-1.6). Also, a pronounced trend of a 2.5-fold decrease in NK-$CD3^-CD16^+CD56^+$ to 0.2 (0.2-0.4) × 10^9/L *versus* 0.5 (0.3-0.4) × 10^9/L in CG ($p > 0.05$), while the level of TNK-$CD3^+CD16^+CD56^+$ and the content of B lymphocytes $CD3^-CD19^+$ did not differ from the values determined in the CG ($p > 0.05$) (Table 2).

The SG2 of APTO children also showed a 1.3-fold decrease in Tl-$CD3^+CD19^-$ ($p < 0.05$) and Th-$CD3^+CD4^+$ ($p < 0.05$) and 1.6-fold decrease in T_{CTL}-$CD3^+CD8^+$ ($p < 0.05$) and 2,5-fold NK-$CD3^-CD16^+CD56^+$ ($p < 0.05$). In contrast to SG1, there was a 2,3-fold increase in TNK-$CD3^+CD16^+CD56^+$ ($p < 0.05$) in SG2. The content of Bl-$CD3^-CD19^+$ did not differ from that of CG ($p > 0.05$) (Table 2).

It is interesting to note that only 1 child from SG1 with AHO and 1 child from SG 2 with APTO had leukocytosis, an adequate increase in the amount of NG necessary to eliminate the bacterial pathogen. Against the background of leukocytosis in patients with AHO and APTO, there were similar changes in the relative parameters of cellular immunity noted in the corresponding groups of SG1 and SG2, which were partially leveled due to an increase in the total number of leukocytes.

In the analysis of humoral immunity in children with AHO in SG1, the concentrations of the main classes of immunoglobulins IgA, IgM, IgG did not differ from the values of GS ($p_{1-3} > 0.05$), and for IgA and IgG, a downward trend was recorded (Table 2). In GI2 of children with APTO, there is an increase in the concentration of IgG to 18.1 (15.9-22.2) g/L ($p < 0.05$) and IgA to the upper limits of the CG

TABLE 1. LEUKOCYTES IN CHILDREN WITH ACUTE HEMATOGENOUS AND ACUTE POST-TRAUMATIC OSTEOMYELITIS, Me ($Q_{0.25}$-$Q_{0.75}$)

Indicators	SG1 acute hematogenous osteomyelitis n = 11	SGI 2 acute post-traumatic osteomyelitis n = 9	Comparison group healthy children aged 8-15 years. n = 13
WBC, 10^9/L	9.6* (8.40-9.96)	6.3^ (6.0-7.0)	4.6 (4.1-6.2)
LY, %	29.0* (19.5-30.0)	36.5 (25.5-40.8)	37.3 (33.4-38.5)
LY, × 10^9/L	2.1 (1.7-2.9)	2.3 (1.8-2.8)	2.5 (2.4-2.5)
NG, %	51.0 (36.8-68.3)	51.5 (46.8-54.8)	57.8 (54.3-59.8)
NG, × 10^9/L	3.9 (2.6-6.6)	3.7 (3.3-5.3)	2.7 (2.6-3.3)
segmented NG, %	44.5 (33.3-59.5)	47.0* (42.5-49.5)	55.5 (54.1-58.0)
banded NG, %	7.0* (5.0-10.3)	5.00* (3.0-7.0)	2.5 (1.0-3.5)
MON, %	9.0* (7.0-13.3)	5.00 (4.0-8.0)	4.0 (3.3-5.8)
EOS, %	1.0 (0.1-5.0)	6.0 (3.0-7.0)	3.5 (3.0-4.0)

Note. *, differences in the indicators of study groups with osteomyelitis from those of healthy children; ^, differences between study groups, statistically justified with an error of the 1st kind $p < 0.05$ (Mann–Whitney test).

TABLE 2. INDICATORS OF CELLULAR AND HUMORAL IMMUNITY IN CHILDREN WITH ACUTE HEMATOGENOUS AND ACUTE POST-TRAUMATIC OSTEOMYELITIS, Me ($Q_{0.25}$-$Q_{0.75}$)

Indicators	SG1 acute hematogenous osteomyelitis n = 11	SG 2 acute post-traumatic osteomyelitis n = 9	Comparison group healthy children aged 8-15 years n = 13
WBC, × 10^9/L	9.6* (8.40-9.96)	6.3^ (6.0-7.0)	4.6 (4.1-6.2)
LY, %	29.0* (19.5-30.0)	36.5 (25.5-40.8)	37.3 (33.4-38.5)
LY, × 10^9/L	2.1 (1.7-2.9)	2.3 (1.8-2.8)	2.5 (2.4-2.5)
T lymphocytes $CD3^+CD19^-$, %	60.8* (52.4-67.5)	58.7* (50.7-66.2)	75.8 (71.8-78.2)
T lymphocytes $CD3^+CD19^-$, × 10^9/L	1.3* (1.1-1.6)	1.5 (1.1-1.8)	1.9 (1.7-2.0)
T helpers $CD3^+CD4^+$, %	33.2* (29.5-34.5)	35.7* (29.0-36.9)	46.9 (41.3-58.6)
T helpers $CD3^+CD4^+$, × 10^9/L	0.6* (0.5-0.9)	0.8 (0.5-1.0)	1.1 (0.9-1.9)
CTL $CD3^+CD8^+$, %	27.7* (22.8-30.4)	22.5* (22.4-24.9)	34.7 (31.4-38.9)
CTL $CD3^+CD8^+$, 10^9/L	0.5 (0.4-0.8)	0.6 (0.5-0.7)	0.9 (0.7-1.0)
IRI CD4/CD8	1.3 (0.9-1.6)	1.4 (1.2-1.6)	1.8 (1.5-2.0)
NK, % $CD3^-CD16^+CD56^+$	11.9* (7.3-15.0)	7.7* (5.2-11.3)	19.8 (17.1-19.9)
NK, × 10^9/L $CD3^-CD16^+CD56^+$	0.2* (0.2-0.4)	0.2* (0.1-0.3)	0.5 (0.3-0.5)
TNK, % $CD3^+CD16^+CD56^+$	0.8 (0.6-2.2)	2.8* (1.4-3.7)	0.7 (0.5-0.9)
TNK, × 10^9/L $CD3^+CD16^+CD56^+$	0.03 (0.01-0.05)	0.06 (0.02-0.06)	0.03 (0.02-0.06)
B lymphocytes $CD3^-CD19^+$, %	17.2 (11.8-19.7)	10.3 (9.9-14.1)	11.4 (9.2-7.7)
B lymphocytes $CD3^-CD19^+$, × 10^9/L	0.3 (0.2-0.4)	0.2 (0.1-0.3)	0.3 (0.2-0.3)
IgA g/L	1.2 (1.1-1.8)	2.1^ (1.8-2.2)	1.5 (1.4-2.6)
IgM g/L	1.3 (1.1-1.4)	0.9 (0.8-1.3)	1.4 (1.1-1.6)
IgG g/L	12.5 (11.4-15.6)	18.1* ^ (15.9-22.2)	13.2 (12.8-13.6)

Note. As for Table 1.

quartile zone – 2.1 (1.8-2.2) g/L ($p > 0.05$) against the background of low IgM values of 0.9 (0.8-1.3) g/L ($p > 0.05$) (Table 2).

NG dysfunctions common to all children with AHO and APTO were also found. The functional activity of NG, in particular, the phagocytic function, depends on the number and density of expressed receptors [10]. It is known that CD64 (FcγRI), CD16 (FcγRIII), CD32 (FCγRII) receptors trigger immune phagocytosis and killing processes, antibody-dependent cellular cytotoxicity (ADCC).

It was shown that children with SG1 with AHO, there was a 1,2-fold decrease in the number of NG expressing CD16 ($p < 0.05$), and a 29-fold increase in the level of $CD64^+$NG ($p < 0.05$) against the background of an unchanging quantity of CD32

TABLE 3. INDICATORS OF RECEPTOR AND PHAGOCYTIC FUNCTIONS OF NEUTROPHILIC GRANULOCYTES IN CHILDREN WITH ACUTE HEMATOGENOUS AND ACUTE POST-TRAUMATIC OSTEOMYELITIS, Me ($Q_{0.25}$-$Q_{0.75}$)

Indicators	SG1 acute hematogenous osteomyelitis n = 11	SG 2 acute post-traumatic osteomyelitis n = 9	Comparison group healthy children aged 8-15 years n = 13
% PhAN	51.0* (42.8-58.3)	67.0* ^ (58.5-71.5)	54.7 (51.0-57.0)
PhN	1.9* (1.7-2.3)	3.2* ^ (2.4-3.7)	4.4 (3.8-4.7)
PhI	1.0* (0.9-1.5)	2.0 (1.5-2.3)	1.9 (1.7-2.2)
% D	41.9* (37.8-44.8)	46.0* ^ (40.3-47.0)	64.5 (62.6-66.9)
DI	0.5* (0.3-0.7)	1.0* ^ (0.6-1.2)	1.7 (1.5-2.0)
CD16, % NG	86.5* (80.5-96.4)	93.1^ (91.1-96.0)	98.3 (96.8-99.4)
CD32, % NG	93.8 (90.8-96.9)	93.7 (92.7-94.3)	93.1 (91.1-96.6)
CD64, % NG	14.5* (5.9-15.7)	3.4* ^ (2.1-4.7)	0.5 (0.4-0.7)

Note. As for Table 1.

($p > 0.05$) in relation to the indicators of CG (Table 3). At the same time, depression of phagocytic activity is observed in SG1 with AHO, which is associated both with a decrease in the number of actively phagocytic NG (% PhAN) ($p > 0.05$), impaired capture functions (PhN, PhI) ($p_{1, 2} < 0.05$) and processes of killing (%D, DI) ($p_{1, 2} < 0.05$) due to impaired NG receptor function.

Meanwhile, in children with AHO in SG1, the content of NGs expressing CD16 and CD32 receptors did not significantly differ from CG values ($p_{1, 2} > 0.05$), while there was a 6,8-fold increase in the level of $CD64^+NG$ ($p < 0.05$) (Table 3). When assessing phagocytic activity in children with SG2, a slight increase in % PhAN to 67.0 (58.5-71.5) % *versus* 54.7 (51.0-57.0) % ($p < 0.05$), however, there was a decrease in 1,4 times the index of PhN reflecting the ability of NG to capture ($p < 0.05$) and, as in SG1, the killing ability of cells was reduced (% D, DI, $p < 0.05$).

Thus, combined defects in the functioning of IS indicators of different severity were revealed in children with AHO s and APTOs aged 8-15 years old.

So, in both studied groups, general dysfunctions of the cellular link of IS were revealed: a decrease in the number of T lymphocytes with a parallel decrease in the proportion of T helpers and T_{CTL} lymphocytes, a decrease in NK cells against the background of an unchanged content of B lymphocytes. At the same time, it was found that in AHO, the level of immunoglobulins of the main classes did not change and did not differ from the CG indicators, while in APTO in SG 2, an increase in the level of IgA and IgG was noted. The received data obtained partly coincide with the trends noted by other authors in children of other age groups with AHO [3, 12].

When analyzing the receptor and phagocytic activity, it was found that the expression levels of the CD64, CD16, CD32, and NG receptors in the studied groups of children with AHO and APTO demonstrate different equipment, which predetermines the failure of the phagocytic function. Defects in phagocytosis, in AHO, are primarily associated with impaired functions of NG capture and killing of the bacterial antigen, and in APTO, only with the completion of the phagocytic act.

Conclusion

The identified combined defects in the functioning of the immune system necessitate the development of new approaches in the treatment of AHO and APTO in children, pathogenetically substantiating the use of immunotropic medicines in the complex etiopathogenetic treatment of this pathology, which will help restore disturbed mechanisms of anti-infective immunity and, as a result, timely elimination of pathogens, improve clinical the course of diseases, as well as to prevent the chronicity of the inflammatory process and the aggravation of the dysfunction of the immune system.

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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-

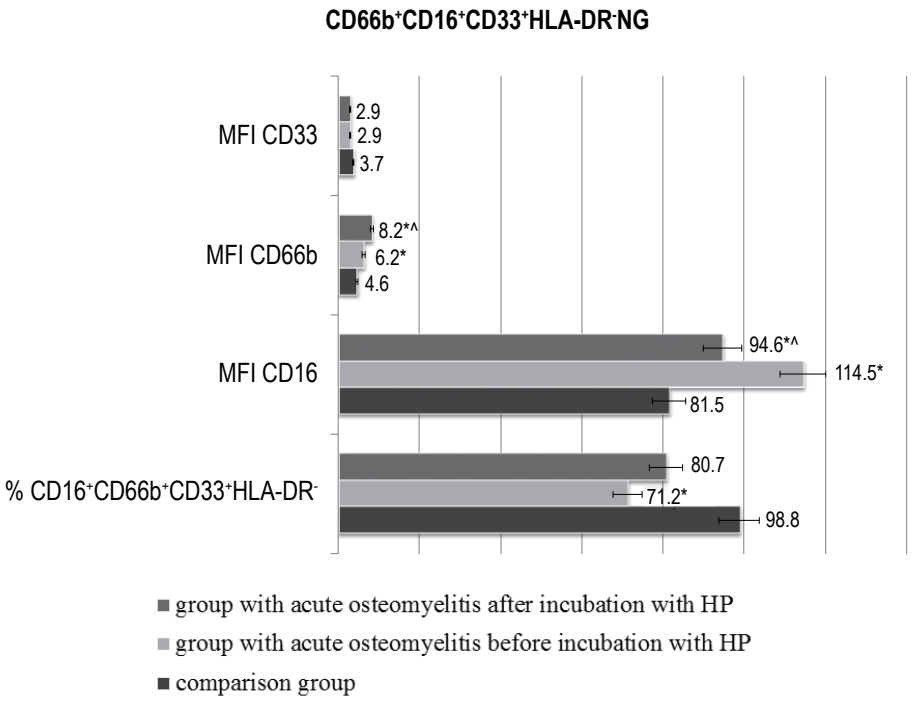

Figure 1. Number of CD66b$^+$CD16$^+$CD33$^+$HLA-DR$^-$NG subset and density of receptor expression (MFI) receptor expression density in acute osteomyelitis before and after *in vitro* incubation of the system with hexapeptide

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, $p < 0.05$.

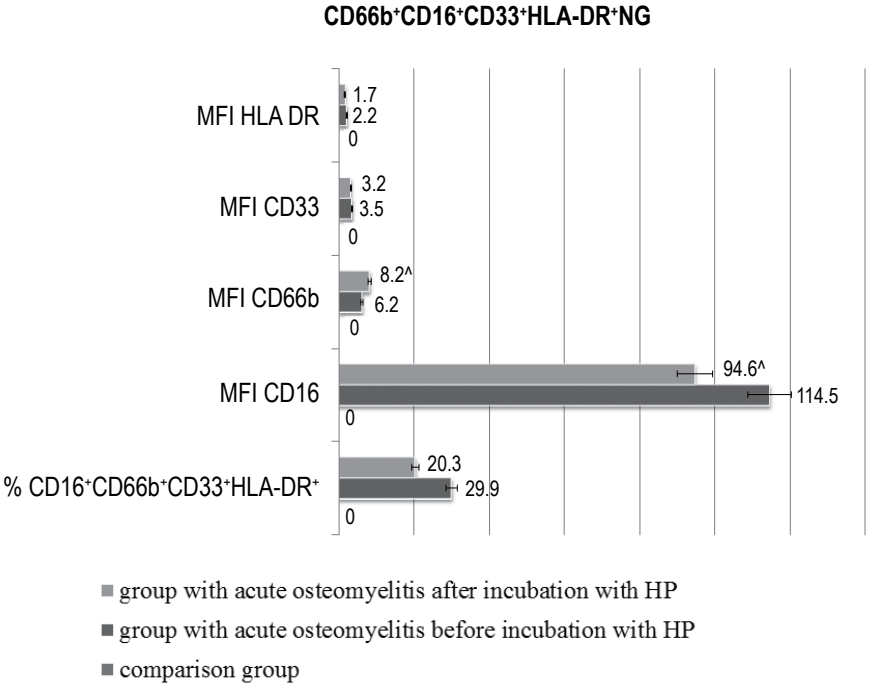

Figure 2. Number of CD66b$^+$CD16$^+$CD33$^+$HLA-DR$^+$ subset and density of receptor expression (MFI) in osseous osteomyelitis before and after incubation of the system *in vitro* with hexapeptide

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.

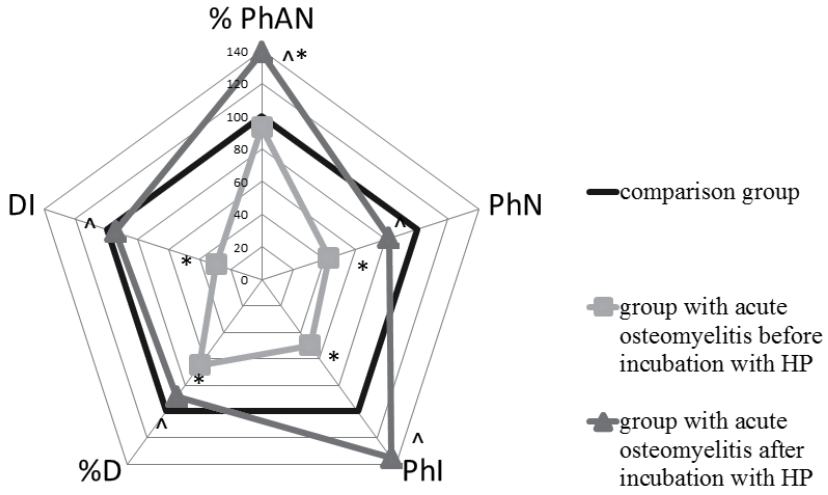

Figure 3. Phagocytic activity of neutrophil granulocytes in acute osteomyelitis before and after incubation of the system *in vitro* with hexapeptide

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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ИММУНОЛОГИЧЕСКИЕ ИНДИКАТОРЫ ОСЛОЖНЕНИЙ ХИРУРГИЧЕСКИХ ЗАБОЛЕВАНИЙ КИШЕЧНИКА У ДЕТЕЙ

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Резюме. Статья посвящена разработке иммунологических индикаторов воспаления кишечника у детей, что имеет большое значение для органов здравоохранения при организации специализированной педиатрической и хирургической службы. Предложенный метод способствует ранней диагностике и профилактике развития осложнений воспалительных хирургических заболеваний кишечника у детей, что имеет важное практическое значение. Авторами проведено иммунологическое исследование 91 пациента детского возраста. Цель исследования явилась разработка иммунологических показателей осложнений хирургических заболеваний кишечника у детей.

Проведен ретроспективный анализ 867 историй болезни детей, получавших стационарное лечение в отделении детской хирургии Бухарского филиала Республиканского научного центра неотложной медицинской помощи с 2019 по 2022 год по поводу хирургических заболеваний желудочно-кишечного тракта.

Всем детям было проведено иммунологическое исследование крови: изучены клеточный и гуморальный иммунитет, цитокины (TNFα, IFNα, IL-8, MCP-1 и сосудистый эндотелиальный фактор роста VEGF-A). Для профилактики после операционных осложнений ХЗК у детей рекомендуется определение IFNα в сыворотке крови в период до операции для решения показаний к иммунокоррекции. Авторами установлено, что для корректного определения показаний к антибактериальной терапии и оценки ее эффективности необходимо определение прокальцитонина в сыворотке крови в течении первых 2 суток после операции и в динамике. Была установлена заметная положительная взаимосвязь между IFNα и CD8 – r = 0,34, между IFNα и CD23 – r = 0,38, между IFNα и IgA – r = 0,39, между IFNα и PCT – r = 0,36. В то же время PCT имеет заметную негативную взаимосвязь с CD16 – r = -0,31 и с CD8 – r = -0,31 на фоне заметной положительной взаимосвязи с IgG – r = 0,32 и IFNα – r = 0,36. Установлено, что IFNα является более информативным показателем эффективности иммунного ответа, а PCT – показателем эффективности антибактериальной терапии при хирургических заболеваниях кишечника у детей.

Ключевые слова: клеточный иммунитет, цитокины, хирургические заболевания кишечника, хронический запор, дети, кишечная непроходимость

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IMMUNOLOGICAL INDICATORS OF COMPLICATIONS OF SURGICAL BOWEL DISEASE IN CHILDREN

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Abstract. The article is devoted to the development of immunological indicators of intestinal inflammation in children, which is of great importance for health authorities when organizing specialized pediatric and surgical services. The proposed method contributes to the early diagnosis and prevention of complications of inflammatory surgical bowel diseases in children, which is of great practical importance.

The purpose of the study: To develop immunological indicators of complications of surgical bowel diseases in children.

A retrospective analysis of 867 case histories of children who received inpatient treatment at the Department of Pediatric Surgery of the Bukhara branch of the Republican Scientific Center of Emergency Medical Care from 2019 to 2022 for surgical diseases of the gastrointestinal tract was carried out.

The authors conducted an immunological study of 91 pediatric patients. All children underwent immunological blood tests: cellular and humoral immunity, cytokines (TNFα, IFNα, IL-8, MCP-1 and vascular endothelial growth factor VEGF-A) were studied. For the prevention of postoperative complications of CKD in children, it is recommended to determine IFNα in the blood serum in the period before surgery to solve the indications for immunocorrection. A noticeable positive relationship was established between IFNα and CD8 – r = 0.34, between IFNα and CD23 – r = 0.38, between IFNα and IgA – r = 0.39, between IFNα and PCT – r = 0.36. At the same time, PCT has a noticeable negative relationship with CD16 – r = -0.31 and with CD8 – r = -0.31 against the background of a noticeable positive relationship with IgG – r = 0.32 and IFNα – r = 0.36. It was found that IFNα is a more informative indicator of the effectiveness of the immune response, and PCT is an indicator of the effectiveness of antibacterial therapy in surgical bowel diseases in children

Keywords: cellular immunity, cytokines, surgical bowel disease, chronic constipation, children, intestinal obstruction

Introduction

In the structure of mortality of patients with acute surgical pathology of the abdominal organs, this disease occupies one of the first places, amounting to 4.3–18.9%, and among people over 70 years of age – up to 36.0% [3]. In acute intestinal obstruction (AIO) complicated by peritonitis, the mortality rate approaches 100% [1]. Among acute surgical diseases of the abdominal cavity, acquired intestinal obstruction ranks second in frequency, second only to acute appendicitis; at the same time, the number of deaths in it is greater than in other acute surgical diseases of the abdominal cavity combined. The frequency of intestinal obstruction in relation to acute surgical diseases of the abdominal cavity can reach 9.4%. Intestinal intussusception and adhesive intestinal obstruction are most common in children, much less often - obstruction on the basis of Meckel's diverticulum, inversions and nodules of the small and large intestine, strangulated internal hernias [2].

Despite significant achievements in modern surgery, the development and introduction of new methods of early diagnosis and surgical treatment of patients with AIO, there is a large number of unsatisfactory results and deaths in this pathology. All this ultimately necessitates the search and clinical application of reliable methods for determining the type and level of intestinal obstruction, the choice of adequate surgical aids in each case, and the improvement of existing diagnostic and treatment algorithms that help save patients' lives [4].

Inflammatory bowel diseases (IBD) often develop in patients with pathology of adaptive immunity. Numerous genetic defects that can disrupt T and B differentiation and activation eventually lead to the development of complex dysfunctions of adaptive immunity, including immunodeficiency and autoimmune inflammation. Diseases manifested by IBD-like symptoms include B lymphocyte defects, such as general variable immunodeficiency, hyperimmunoglobulinemia M, agammaglobulinemia. A number of severe combined primary immunodeficiencies (Wiskott–Aldrich syndrome, Omenne syndrome) may also be accompanied by IBD-like intestinal inflammation.

Understanding the pathophysiology of a disease caused by a genetic defect can serve as a justification

for choosing an unconventional therapy due to specific pathogenetic effects. For example, patients with mevalonate kinase deficiency or chronic granulomatous disease accompanied by an increase in IL-1β levels may be indicated therapy with an IL-1β receptor antagonist. In cases of phagocytosis and/or neutropenia disorders, the introduction of a granulocyte colony-stimulating factor may be effective. This is not a standard therapy for IBD, but it is justified from the point of view of pathophysiology and, in the future, may be very effective in relation to this group of patients.

The purpose of the study: To develop immunological indicators of complications of surgical bowel diseases in children.

Materials and methods

A retrospective analysis of 867 case histories of children who received inpatient treatment at the Department of Pediatric Surgery of the Bukhara branch of the Republican Scientific Center of Emergency Medical Care from 2019 to 2022 for surgical diseases of the gastrointestinal tract was carried out. The total number of hospitalized sick children for 3 years was 5,650, in 2019 – 1,551 (27.5%), in 2020 – 1,149 (20.4%), in 2021 – 1,353 (23.9%), in 2022 – 1,597 (28.3%).

Of these, 2853 sick children were admitted for IBD, which is 50.5% of all hospitalized in the surgery department. To develop informative indicators of complications of surgical bowel diseases in children, patients were divided into 3 groups: group 1 (control) consisted of 30 healthy children; group 2 consisted of 31 sick children with intestinal obstruction (IO); and group 3 consisted of 30 sick children with chronic constipation (CC). All children underwent immunological blood tests: cellular and humoral immunity, cytokines (TNFα, IFNα, IL-8, MCP-1 and vascular endothelial growth factor VEGF-A) were studied. The study was conducted in accordance with the Helsinki Declaration.

Statistical processing of the results was carried out using Excel programs from the Microsoft Office XP application package (Microsoft, USA), correlation analysis was performed using the Pearson method and evaluated on the Cheddock scale.

Results and discussion

The relative number of CD3 lymphocytes was reduced in patients of both the main group and the comparison group to 46.3±0.31% and 34.2± 0.47%, respectively, against the control indicators – 51.7±0.62% ($p < 0.05$-0.001). The relative percentage of CD4 helper lymphocytes was significantly reduced in patients of the main group to 27.0±0.41% *versus* the control – 34.4±0.43% ($p < 0.01$).

When studying the level of CD8-immunoregulatory subpopulation of T suppressors/cytotoxic lymphocytes, a significantly reduced content was revealed at IO to – 19.5±0.59% ($p < 0.05$), and at CC in patients of the main group there was a statistically significant increase to 28.0±0.94% ($p < 0.05$).

Thus, at IO in children have a deficiency of cellular immunity. There is a decrease in the relative number of CD3, CD4 and CD8 lymphocytes.

The B system is represented by the quantitative content of B lymphocytes with CD20 and CD23 molecules and the level of immunoglobulins of IgG, IgA, IgM and IgE classes. $CD20^+$ lymphocytes are known to be directly involved in specific immune defense reactions of the body.

A comparative assessment of the content of circulating $CD20^+$ cells in the blood showed that with IO and CC, the level of these cells significantly increased to 31.7±0.52% and 28.9±0.5%, respectively ($p < 0.01$) compared with the control group – 23.2±0.63%. Our research data showed that in the main group of patients, the level of relative values of $CD23^+$ cells was significantly increased by 2.3 times ($p < 0.001$).

A study of the concentration of the main classes of IgG, IgA and IgM, as well as IgE, showed that with CC, there was a 3.2 – fold decrease in IgG against the background of an increase in IgM to 1.7±0.09 g/L *versus* control – 1.2±0.13 g/L. In the main group of patients with IO, IgG concentration was at the level of control values, and IgM was increased to 1.6±0.11 g/L *versus* control – 1.2±0.13 g/L.

As is known, this type of antibody is produced against infectious agents, activates complement and enhances phagocytosis. It is possible that the increased synthesis of IgM in the main and comparative group of patients with IO and with CC is associated with the addition of an infectious process. Very important properties of IgM are their attraction of phagocytic cells to the location of the antigen or to the focus of infection and activation of phagocytosis.

IgG is the primary antibody of the secondary immune response. The main biological function of immunoglobulins of this class is to protect the body from pathogens of infection and their waste products. Being thymus-dependent, IgG is produced only with the obligatory participation of T lymphocytes.

As can be seen from the above data, the most increased synthesis of IgA occurs in the group of patients with IO – 1.4±0.08 g/L ($p < 0.01$), and in the comparative group its concentration was increased to 0.9±0.04 g/L, against the control – 0.4±0.03 g/L, $p < 0.05$.

With an immediate type of hypersensitivity reaction, specific antibodies (reagins) with the ability to sensitize their own tissues are detected in the body.

Its concentration in the blood serum in the control group averaged 105.4±11.4 ng/mL. In all groups of examined patients, its concentration was at the level of control values.

The special attention of researchers is attracted by the class of immunocompetent cells, which performs a killer function. We are talking about natural killer cells, NK cells ($CD16^+$). The control group contains natural killer cells ($CD16^+$ cells) with an average of 16.4±1.0%. The absolute value of this indicator averaged 182±9.0 in 1 μl. The relative content of NK cells in the bloodstream of patients with CN and CD was increased to 23.2±0.9% and 20.6±0.23%, respectively, in relation to the data of the control group – 17.1±0.44% ($p < 0.05$).

Analysis of the results showed that with IO, there was a significant increase in the expression of activation markers of early activation – $CD25^+$ cells to 20.4±0.28% in patients of group 2 and to 25.2±0.62% in patients of group 3 of the examination, against control values – 18.0±0.41%, $p < 0.05$.

The level of lymphocytes with a receptor for apoptosis (CD95) in our studies in the main group of patients with IO was at the level of control values, and in patients with CC was significantly increased by 1.65 times. Thus, the maximum increase in their relative number is observed, where the level is increased by 1.65 times and averages 29.8±0.47% ($p < 0.001$).

During the immunological assessment of blood parameters in patients, a statistically significant decrease in the level of IFNα with CC was found to 10.7±0.23 pg/mL compared to the control – 11.6±0.22 pg/mL, which is explained by the chronization of the pathological process and the depletion of the body's defense mechanisms with the formation of a state of immunodeficiency. In patients of the main group, the IFNa was at the level of control values, which confirms the acute onset of IO.

To study the nature of inflammation in the intestine, IL-8 was studied in the blood serum of patients and healthy children. Its significant increase in patients of the main group was found to be 1.33 times, on average up to 48.7±3.39 pg/mL compared to the control values – 36.8±1.44 pg/mL. At the same time, in patients with IO, a tendency to decrease IL-8 to 32.6±1.93 pg/mL was revealed, which confirms the importance of dysbiosis in constipation in children.

As a result of apoptosis and cell death, the intestines disintegrate and destructurize, which is confirmed paraclinically by the level of TNFα in the blood serum. In our studies, the greatest increase in its level was found in patients of the 3rd group (comparison) to 149.7±1.29 pg/mL, and in the main group to 136.7±10.89 pg/mL against the control – 58.4±1.84 pg/mL ($p < 0.001$). The results obtained, obviously, prove the disintegration of tissue at the intestinal level in our studies by the formation of megacolons or other secondary changes in the intestinal tract in CC and IO in case of obstruction.

Taking into account the above facts, the assessment of the chemotaxis process in patients selected for the study showed an increase in the level of MCP-1 in patients of the main group by 1.3 times (366.7±20.69 pg/mL), compared to the control – 279.8±28.6 pg/mL, which confirms the presence of an acute inflammatory process and activation of macrophages. In patients of the comparative group, MSR-1 was reduced to 183.1±25.17 pg/mL, which is 1.5 times lower than the control values. The obtained result indicates the chronization of the pathological process and a decrease in macrophage activity.

In the study, we found a tendency to increase VEGF-A to 208.4±13.05 pg/mL in patients of the main group and a statistically significant decrease to 144.3±9.48 pg/mL in patients of the 3rd group in relation to the indicators of the control group – 191.3±14.76 pg/mL. Consequently, with IO, activation of chemotaxis with the participation of endothelial growth factor is noted.

One of the promising directions for improving the quality of diagnosis and stratification of patients according to the severity of the condition in scientific and practical research is the determination of the concentration of procalcitonin (PCT), a marker of systemic inflammatory reaction and bacterial infection [1].

The study of the nature of inflammation and the activity of inflammatory markers made it possible to determine bacterial infection. Thus, in patients of the main group, there was a 7.25-fold increase in PCT (up to 2.9±0.64 ng/mL) in relation to the control group – 0.4±0.44 ng/mL ($p < 0.05$) and 3.2-fold increase against the comparison group – 0.9±0.06 ng/mL ($p < 0.05$).

Thus, with IO, an increase of IL-8 by 1.33 times, TNFα by 2.4 times, MCP-1 by 1.3 times, PCT by 7.25 times was found against the background of activation of chemotaxis with the participation of endothelial growth factor VEGF-A. The obtained results of the immunological study indicate the activation of the body's defense system in children with IO and allow determining the prognosis of the outcome of surgical corrections and postoperative complications. Therefore, for the prevention of postoperative complications in IO, it is important to take into account the immune status.

To develop indicators of postoperative complications, a correlation analysis of immunological parameters was performed in patients of the main group (Figure 1, see 3rd page of cover). As a result, a noticeable positive relationship was established between IFNα and CD8 – $r = 0.34$, between IFNα

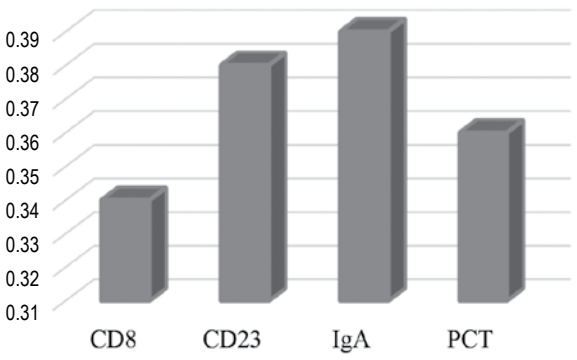

Figure 2. Relationship of INFα in intestinal obstruction in children

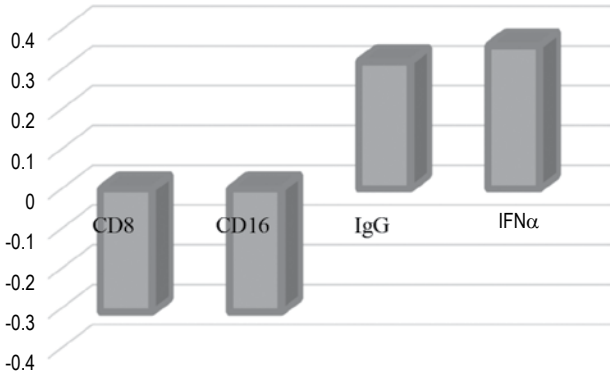

Figure 3. Correlation relationship of PCT in intestinal obstruction in children

and CD23 – r = 0.38, between IFNα and IgA – r = 0.39, between IFNα and PCT – r = 0.36 (Figure 2). At the same time, PCT has a noticeable negative relationship with CD16 – r = -0.31 and with CD8 – r = -0.31 against the background of a noticeable positive relationship with IgG – r = 0.32 and IFNα – r = 0.36 (Figure 3).

Consequently, among all the studied immunological indicators, IFNα and PCT with a noticeable correlation dependence were determined to be the most informative indicators. As an indicator of immunity, IFNα has the most noticeable positive association with CD23, with IgA and PCT. According to the level of IFNα, it is possible to predict the outcome of the postoperative period. And PCT, as an indicator of the activity of inflammation and bacterial infection, due to the noticeable links with the studied immunological parameters of the blood, it acts as an indicator for the indication of antibacterial therapy and its effectiveness.

The greater the activation of protective mechanisms of protection (for example, the level of IFNα), the better the prognosis of the outcome of surgical correction of the intestine in children. That is, the body's response with an increase in the level of the above-mentioned immunological (CD8, CD23, IgA and PCT) indicators confirms the compensatory phase of the immune response.

This leads to the conclusion that IFNα is a more informative indicator of the effectiveness of the immune response, and PCT is an indicator of the effectiveness of antibacterial therapy in surgical bowel diseases in children.

Conclusions

1. There was a deficiency of cellular immunity in intestinal obstruction in children, while there was a decrease in the relative number of CD3, CD4 and CD8 lymphocytes against the background of an increase in CD20 and CD23 lymphocytes (2.3 times).

2. Surgical bowel diseases in children aged 6 years and older are characterized by an increase in IgM and IgA against the background of normal IgG and IgE values and an increase in the expression of activation markers of early (CD25) and killer (CD16) activation.

3. With intestinal obstruction, an increase of IL-8 by 1.33 times, TNFα by 2.4 times, MCP-1 by 1.3 times, PCT by 7.25 times was found against the background of activation of chemotaxis with the participation of endothelial growth factor VEGF-A.

4. A noticeable positive relationship was established between IFNα and CD8 – r = 0.34, between IFNα and CD23 – r = 0.38, between IFNα and IgA – r = 0.39, between IFNα and PCT – r = 0.36. At the same time, PCT has a noticeable negative relationship with CD16 – r = -0.31 and with CD8 – r = -0.31 against the background of a noticeable positive relationship with IgG – r = 0.32 and IFNα – r = 0.36.

5. It was found that IFNα is a more informative indicator of the effectiveness of the immune response, and PCT is an indicator of the effectiveness of antibacterial therapy in surgical bowel diseases in children

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ОСОБЕННОСТИ ЦИТОКИНОВОГО ПРОФИЛЯ КРОВИ ПРИ ГАСТРОЭЗОФАГЕАЛЬНОЙ РЕФЛЮКСНОЙ БОЛЕЗНИ У ШКОЛЬНИКОВ С ГАСТРИТОМ И СЕМЕЙНЫМ ОТЯГОЩЕНИЕМ ПО ЯЗВЕННОЙ БОЛЕЗНИ

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Резюме. Гастроэзофагеальная рефлюксная болезнь (ГЭРБ) представляет собой распространенное кислотозависимое заболевание среди населения, в том числе детского с мультифакториальным генезом. Она, как и многие другие кислотозависимые заболевания (язвенная болезнь и др.), ассоциирована с семейной предрасположенностью к заболеванию. Интерес представляет изучение роли цитокинов в регуляции патологии в детском возрасте в зависимости от отягощенности семейного анамнеза по язвенной болезни. Цель – оценить показатели цитокинов в сыворотке крови при семейном отягощении по язвенной болезни у школьников с гастритом, ассоциированным с ГЭРБ. В ходе научного исследования обследовано 142 ребенка с гастроэнтерологическими жалобами в возрасте 7-17 лет. Диагноз «ГЭРБ» выставлялся при наличии еженедельной изжоги в соответствии с глобальным консенсусом по патологии у детей. Всем обследуемым была проведена гастроскопия с взятием биопсийного материала из слизистой желудка и морфологическим подтверждением у них диагноза гастрит в соответствии с Сиднейской классификацией. Методом иммуноферментного анализа получена концентрация цитокинов в сыворотке крови (IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-1β, IFNα, TNFα). При статистической обработке использовались критерии χ^2 и Манна–Уитни. Исследования одобрены этическим комитетом и до начала исследования получены информированные согласия пациентов и их родителей. Результаты исследования не показали значимых различий концентрации цитокинов у школьников в зависимости от наличия ГЭРБ. У детей с семейным отягощением по язвенной болезни ГЭРБ определялась чаще ($p = 0,054$), что, вероятно, является следствием наличия у них повышенного кислотообразования. Отмечены изменения в цитокиновом профиле крови. В течение ГЭРБ при отягощении по язвенной болезни было усиление репликации IL-4 ($p = 0,027$) и IFNα

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(p = 0,001). Увеличение IFNα в крови у детей с ГЭРБ при семейном отягощении очевидно направлено на усиление иммунных реакций с участием всего организма на повреждение. Это обусловлено его функциональной ролью – участие в иммунном ответе. Усиление репликации IL-4, очевидно, обеспечивает усиление метаболических, иммунных процессов в организме, направленных на обеспечение оптимизации течения пролиферативных процессов в слизистой пищевода в условиях повышенной секреции соляной кислоты в желудке. Таким образом, при отягощении семейного анамнеза по язвенной болезни у школьников с гастритом, ассоциированным с ГЭРБ наблюдается переход ряда звеньев цитокиновой сети (IL-4, IFNα) на системный уровень регуляции.

Ключевые слова: цитокины, дети, гастроэзофагеальная рефлюксная болезнь, гастрит, язвенная болезнь, семейная предрасположенность

FEATURES OF THE BLOOD CYTOKINE PROFILE IN GASTROESOPHAGEAL REFLUX DISEASE IN SCHOOLCHILDREN WITH GASTRITIS AND FAMILY HISTORY OF PEPTIC ULCER

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Abstract. Gastroesophageal reflux disease (GERD) is a common acid-dependent disease among the population, including children, with multifactorial genesis. It, like many other acid-dependent diseases (peptic ulcer, etc.) is associated with a family predisposition to the disease. Of interest is the study of the role of cytokines in the regulation of pathology in childhood, depending on the severity of a family history of peptic ulcer disease. Aim: to evaluate the levels of cytokines in the blood serum in case of family history of ulcerative diseases in schoolchildren with gastritis associated with GERD. In the course of a scientific study, 142 children with gastroenterological complaints aged 7-17 years were examined. The diagnosis of GERD was made in the presence of weekly heartburn in accordance with the global consensus on pathology in children. All subjects underwent gastroscopy with taking biopsy material from the gastric mucosa and morphological confirmation of their diagnosis of gastritis in accordance with the Sydney classification. The concentration of cytokines in blood serum (IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-1β, IFNα, TNFα) was obtained by enzyme immunoassay. During statistical processing, the χ^2 and Mann–Whitney tests were used. The studies were approved by the ethics committee and informed consents of patients and their parents were obtained prior to the start of the study. The results of the study did not show significant differences in the concentration of cytokines in schoolchildren depending on the presence of GERD. In children with a family burden of peptic ulcer, GERD was detected more often (p = 0.054), which is probably a consequence of their increased acid formation. Changes in the cytokine profile of the blood were noted. During GERD, with aggravation of peptic ulcer, there was an increase in the replication of IL-4 (p = 0.027) and IFNα (p = 0.001). The increase in blood IFNα in children with GERD with family burden is obviously aimed at enhancing immune responses involving the whole body to damage. This is due to its functional role – participation in the immune response. Increased replication of IL-4, obviously, provides an increase in metabolic, immune processes in the body aimed at optimizing the course of proliferative processes in the esophageal mucosa under conditions of increased secretion of hydrochloric acid in the stomach. Thus, when a family history of peptic ulcer is aggravated in schoolchildren with gastritis associated with GERD, a number of links in the cytokine network (IL-4, IFNα) move to the systemic level of regulation.

Keywords: cytokines, children, gastroesophageal reflux disease, gastritis, peptic ulcer, family predisposition

Introduction

Gastroesophageal reflux disease (GERD) is a common pathology in all age groups, which has a multifactorial origin and is related to acid-mediated diseases [3, 4]. The latter explains the increase in the prevalence and severity of esophageal lesions in GERD in adults compared with children, which is largely predetermined by the functional state of acid formation in the population in age populations [6, 9]. In a number of studies, scientists emphasize the close relationship between acid-dependent diseases of the stomach, in particular, with peptic ulcer. Such a close relationship between diseases is due, according to researchers, to the presence of common pathogenetic mechanisms in their development: the presence of an imbalance of protective and aggressive factors of the stomach and motor disorders of the organ [5, 13]. Similarly, like any other diseases with high acid production in genesis, they have a genetic nature associated with a family predisposition to the disease, which manifests itself already in childhood and adolescence [2, 10].

In this context, of undoubted interest is the question of the role of regulatory mechanisms (cytokine link) in the course of GERD in childhood and whether there is an influence of family predisposition to peptic ulcer on the formation of the disease. This issue has not been studied in child populations.

The aim of this work was to evaluate the levels of cytokines in the blood serum (IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-1β, IFNα, TNFα) in case of family history of ulcerative diseases in schoolchildren with gastritis associated with GERD.

Materials and methods

The concentration levels of cytokines in the blood of 142 children with gastroenterological complaints were studied. All existing complaints and their characteristics were recorded in the questionnaires. In parallel, the questionnaire method collected data on the presence of diseases of the gastrointestinal tract in relatives (1-2 degrees of kinship) of the examined children.

All schoolchildren underwent endoscopic examination of the digestive system (esophagogastroduodenoscopy) with the taking of biopsy material from two sections of the stomach (antral, body). The biopsies were placed in separate vials containing 10% buffered formalin. Staining with hematoxylin-eosin and Giemsa was performed, followed by a morphological assessment of the state of the mucous membrane in accordance with the Sydney classification [1, 12]. In all cases, gastritis was morphologically confirmed.

The study does not include schoolchildren: less than 7 years old and over 17 years old; in the presence of acute inflammatory diseases during the last month; with recrudescence of chronic diseases of other organs; in the presence of functional insufficiency of organs and systems of the body; in the presence of allergic diseases; in the absence of morphological signs of inflammation in the gastric mucosa; in the presence of organic pathology of the stomach (peptic ulcer, erosive gastritis).

The presence of GERD was determined based on the global pediatric consensus definition of GERD [11]. This nosology was registered in schoolchildren with complaints of heartburn at least once a week. Heartburn was considered as a feeling of discomfort and/or burning behind the sternum.

The study of the cytokine profile of blood serum (IL-2, IL-4, IL-6, IL-8, IL-18, IL-1β, IFNα, TNFα, IL-10) was carried out using a set of reagents from the company "Vector-Best" (Russia) for the ELISA method. Direct preparation of biological material for enzyme immunoassay included taking blood from children with a volume of 5 ml. Next, the serum was separated from the blood sample by centrifugation and stored until the time of the study itself at a temperature of -20 °C.

The study protocol complies with the ethical principles of the Helsinki Declaration of the World Medical Association (1964) and Article 24 of the Russian Constitution. The Ethics Committee of the Research Institute of Medical Problems of the North (Krasnoyarsk) reviewed and approved the plan and protocol of the scientific study (No. 9 dated September 12, 2016). Written informed consents were obtained from all examined patients prior to the start of the study.

SPSS software, version 23.0 (IBM) was used for statistical processing of the received scientific data. The significance of differences was calculated for qualitative features using the χ^2 criterion; and to compare the severity of quantitative traits, the nonparametric Mann–Whitney U test was used for unrelated samples. The data obtained were calculated for samples that do not correspond to the normal distribution of feature values and are described by the median (Me) and interquartile interval ($Q_{0.25}$-$Q_{0.75}$). The significance level of differences in variables was taken equal to 0.05.

Results and discussion

The results of the study did not show significant differences in the cytokine profile in schoolchildren with GERD in comparison with children with other gastroenterological complaints (Table 1). It is important that both groups of children were dominated by those examined with functional diseases related to other nosologies (dyspepsia syndrome, irritable bowel

TABLE 1. INDICATORS OF CYTOKINES IN BLOOD SERUM IN CHILDREN DEPENDING ON THE PRESENCE OF GASTROESOPHAGEAL REFLUX DISEASE

Cytokine	Gastroesophageal reflux disease + (n = 42)			Gastroesophageal reflux disease – (n = 100)			p
	Me	$Q_{0.25}$	$Q_{0.75}$	Me	$Q_{0.25}$	$Q_{0.75}$	
IL-2	0.1	0.1	0.2	0.1	0.1	0.4	0.791
IL-4	1.5	0.8	2.0	1.2	0.6	1.8	0.304
IL-6	0.1	0.1	0.1	0.1	0.1	0.1	0.363
IL-8	16.4	0.1	73.9	17.4	0.1	89.6	0.985
IL-18	117.9	64.5	215.9	129.0	74.2	182.0	0.968
IL-1β	0.1	0.1	0.1	0.1	0.1	0.1	0.762
IFNα	0.1	0.1	1.3	0.1	0.1	1.5	0.747
TNFα	0.1	0.1	0.1	0.1	0.1	0.1	0.621
IL-10	0.1	0.1	0.1	0.1	0.1	0.1	0.442

Note. n, number of children; p, level of significance.

TABLE 2. FREQUENCY OF GASTROESOPHAGEAL REFLUX DISEASE IN CHILDREN WITH A FAMILY HISTORY OF PEPTIC ULCER DISEASE

Family history of peptic ulcer disease	Gastroesophageal reflux disease	
	n	%
Yes (n = 57)	22	38.6
No (n = 85)	20	23.5
p	0.054	

Note. n, number of children; p, level of significance.

syndrome, abdominal migraine, etc.). The absence of differences obviously indicates a certain commonality of dysfunction of regulatory systems, regardless of the nosology of functional pathology.

In children with a family burden of peptic ulcer, GERD was determined much more often (Table 2), which is probably a consequence of their increased acidity of gastric contents. In the presence of motor disorders of the gastroesophageal region, this leads to its damaging effect on the mucous membrane of the esophagus, which leads to the occurrence of clinical manifestations of heartburn.

The presence of a family predisposition to peptic ulcer was associated not only with an increase in the clinical manifestations of GERD, but was also reflected in the cytokine regulation of the disease. In particular, the course of GERD in the presence of a family history of peptic ulcer disease was associated with increased replication of both IL-4 and IFNα (Table 3).

The increase in IFNα replication ($p = 0.001$) in the blood serum of children with GERD with a family burden of peptic ulcer is obviously aimed at enhancing immune responses involving the whole body to damage. This is due to its functional role in the body: participation in the immune response. At the same time, the considered cytokine has not only immunomodulatory and antiviral effects, but also antibacterial activity due to the ability to induce the activity of enzymes with antibacterial activity in the damaged cell [8].

IL-4 performs a diverse function in the body: it reduces macrophage activity; activates the replication of such cytokines as TNFα, IL-6; regulates proliferative processes [7]. Enhancement of IL-4 replication ($p = 0.027$), obviously, enhances various metabolic and immune processes in the body, one of the main purposes of which is to maintain homeostasis of proliferative processes in the esophageal mucosa under conditions of hyperproduction of hydrochloric acid in the

TABLE 3. INDICATORS OF CYTOKINES IN BLOOD SERUM IN CHILDREN WITH GASTROESOPHAGEAL REFLUX DISEASE, DEPENDING ON THE PRESENCE OF A FAMILY HISTORY OF PEPTIC ULCER DISEASE

Family history of peptic ulcer disease	Cytokine	GERD + (n* = 22; n** = 20)			GERD – (n* = 35; n** = 65)			p
		Me	$Q_{0.25}$	$Q_{0.75}$	Me	$Q_{0.25}$	$Q_{0.75}$	
Yes	1. IL-2	0.1	0.1	0.3	0.1	0.1	0.5	0.578
	2. IL-4	1.7	1.4	2.4	1.2	0.5	1.8	0.035
	3. IL-6	0.1	0.1	0.1	0.1	0.1	0.1	0.869
	4. IL-8	15.4	0.1	73.0	8.3	0.1	44.7	0.895
	5. IL-18	132.1	66.6	213.5	113.0	79.1	149.6	0.442
	6. IL-1β	0.1	0.1	0.1	0.1	0.1	0.1	0.914
	7. IFNα	1.1	0.1	1.9	0.2	0.1	1.5	0.200
	8.TNFα	0.1	0.1	0.1	0.1	0.1	0.1	0.604
	9. IL-10	0.1	0.1	0.1	0.1	0.1	0.1	0.672
No	10. IL-2	0.1	0.1	0.2	0.1	0.1	0.1	0.984
	11. IL-4	1.1	0.1	1.9	1.2	0.6	1.8	0.427
	12. IL-6	0.1	0.1	0.1	0.1	0.1	0.1	0.679
	13. IL-8	16.4	0.1	91.7	21.6	0.1	89.8	0.996
	14. IL-18	99.6	9.6	228.9	146.6	44.2	231.1	0.414
	15. IL-1β	0.1	0.1	0.1	0.1	0.1	0.1	0.441
	16. IFNα	0.1	0.1	0.1	0.1	0.1	1.3	0.142
	17. TNFα	0.1	0.1	0.1	0.1	0.1	0.1	0.573
	18. IL-10	0.1	0.1	3.7	0.1	0.1	0.1	0.143
p	1-10	0.832			0.293			
	2-11	0.027			0.915			
	3-12	0.865			0.770			
	4-13	0.712			0.638			
	5-14	0.562			0.306			
	6-15	0.142			0.184			
	7-16	0.001			0.097			
	8-17	0.172			0.269			
	9-18	0.500			0.483			

Note. GERD, Gastroesophageal reflux disease; n, number of children; n*, number of children with a family history of peptic ulcer disease; n**, number of children without a family history of peptic ulcer disease; p, level of significance.

stomach, which is characteristic of people with family burden for peptic ulcer disease. In addition, in children with a family history of peptic ulcer, there was an increase in serum IL-4 in children with GERD compared with children who did not have clinical signs of GERD ($p = 0.035$).

Conclusion

When a family history of peptic ulcer is aggravated in schoolchildren with gastritis associated with GERD, a number of links in the cytokine network (IL-4, IFNα) transition to the systemic level of regulation.

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СРАВНИТЕЛЬНАЯ ХАРАКТЕРИСТИКА ЦИТОКИНОВОЙ РЕГУЛЯЦИИ У ПАЦИЕНТОВ С ЗАБОЛЕВАНИЯМИ ЖЕЛУДКА, АССОЦИИРОВАННЫМИ С *HELICOBACTER PYLORI*-ИНФЕКЦИЕЙ

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Резюме. При инфицировании организма бактерией *Helicobacter pylori* запускается цитокиновый каскад, играющий ключевую роль в прогрессировании хронических воспалительных и деструктивных процессов в слизистой оболочке желудка. Таким образом, происходит стимуляция секреции целого ряда цитокинов, которые в свою очередь способствуют привлечению иммунокомпетентных клеток, развитию воспалительных изменений. Однако гиперпродукция цитокинов может привести к атрофическим изменениям СОЖ и как следствие перерождение в рак желудка. Таким образом, роль цитокинов в предраковых состояниях неоднозначна, с одной стороны, они активируют иммунный ответ, направленный на элиминацию патогена, с другой, сами способствуют прогрессированию заболевания.

В комплексное клинико-лабораторное исследование были включены больные: 60 – с хроническим гастритом (ХГ), 55 – с хроническим атрофическим гастритом (ХАГ), 50 – с раком желудка (РЖ, I-II стадии, морфологический вариант – аденокарцинома) и 60 – контрольная группа. Диагнозы верифицировались согласно международным и Российским рекомендациям и подтверждались лабораторно-инструментальными исследованиями. Все больные были сопоставимы по гендерно-возрастным характеристикам ($p > 0,05$). У всех больных выявлялись специфические IgG к *H. pylori*. Исследование было одобрено локальным этическим комитетом ФИЦ КНЦ СО РАН (протокол № 11 от 11.11.2013), соблюдались все этические требования, больные подписывали форму информированного согласия на участие. У больных и лиц контрольной группы проводился однократный забор крови из локтевой вены при поступлении в вакутейнеры с гепарином.

Уровни IL-2, IL-4, IL-8, TNF-α, интерферона-γ в сыворотке крови больных и здоровых лиц определяли с помощью метода иммуноферментного анализа с использованием наборов реагентов производства АО «Вектор-Бест». Статистическая обработка данных проводилась с помощью пакетов прикладных программ Statistica for Windows 8.0.

У всех больных с *H. pylori* – ассоциированными заболеваниями (ХГ, ХАГ, РЖ) выявляется увеличение провоспалительных (IL-2, IL-8, IFNγ) со значительным ростом IL-8 у всех больных и IFNγ при РЖ и противовоспалительного цитокина (IL-4) с максимальным значением при раке желудка. Обнаруживается сочетанный Th1- и Th2-опосредованный иммунный ответ с максимальным нарушением цитокиновой регуляции при РЖ.

Ключевые слова: интерлейкины, Helicobacter pylori, хронический гастрит, хронический атрофический гастрит, рак желудка, иммунитет

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COMPARATIVE CHARACTERISTICS OF CYTOKINE REGULATION IN PATIENTS WITH GASTRIC DISEASES ASSOCIATED WITH *HELICOBACTER PYLORI* INFECTION

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Abstract. When the body is infected with the bacterium *Helicobacter pylori*, a cytokine cascade is launched, which plays a key role in the progression of chronic inflammatory and destructive processes in the gastric mucosa. Thus, the secretion of a number of cytokines is stimulated, which in turn contribute to the attraction of immunocompetent cells and the development of inflammatory changes. However, hyperproduction of cytokines can lead to atrophic changes in the gastric mucosa and, as a result, degeneration into gastric cancer. Thus, the role of cytokines in precancerous conditions is ambiguous. On the one hand, they activate the immune response aimed at eliminating the pathogen. On the other hand, they themselves contribute to the progression of the disease.

The complex clinical and laboratory study included patients: 60 with chronic gastritis (CG), 55 with chronic atrophic gastritis (CAG), 50 with gastric cancer (GC, stage I-II, morphological variant – adenocarcinoma) and 60 – control group. The diagnoses were verified according to international and Russian recommendations and confirmed by laboratory and instrumental studies. All patients were comparable in terms of gender and age characteristics ($p > 0.05$). All patients had specific IgG to *H. pylori*. The study was approved by the Local Ethics Committee of the FRC KSC SB RAS (protocol No. 11 dated November 11, 2013). All ethical requirements were observed, and the patients signed the informed consent form for participation. Patients and persons of the control group underwent a single blood sampling from the cubital vein upon admission to vacutainers with heparin.

The levels of IL-2, IL-4, IL-8, TNFα, interferon-γ in the blood serum of patients and healthy individuals were determined using the enzyme immunoassay method using reagent kits manufactured by JSC "Vector-Best". Statistical data processing was carried out using the Statistica for Windows 8.0 application package.

All patients with *H. pylori*-associated diseases (CG, CAG, GC) showed an increase in pro-inflammatory (IL-2, IL-8, IFNγ) with a significant increase in IL-8 in all patients and IFNγ in gastric cancer and anti-inflammatory cytokine (IL-4) with a maximum value in gastric cancer. A combined Th1 and Th2 is found – a mediated immune response with a maximum violation of cytokine regulation in gastric cancer.

Keywords: interleukins, Helicobacter pylori, chronic gastritis, chronic atrophic gastritis, stomach cancer, immunity

Introduction

Currently, *H. pylori* infection is classified as a carcinogen, and long-term persistence of infection in the gastric mucosa initiates a cascade of pathogenetic disorders from inflammatory changes to atrophy and even metaplasia of gastric epithelial cells [2]. The number of patients with diseases of the stomach increases annually, while even in practically healthy volunteers, infection of the gastric mucosa with a pathogenic bacterium is found. All this determines the relevance of studying this topic [1, 3, 4, 6]. **The aim of our work** was to compare the content of cytokines and study the features of cytokine regulation in chronic gastritis, chronic atrophic gastritis and gastric cancer infected with *H. pylori*. We assume that a common pathogenetic mechanism caused by *H. pylori* infection will be revealed in different pathogenetic diseases.

Materials and methods

A comprehensive clinical and laboratory study included patients: 60 with chronic gastritis (CG), 55 with chronic atrophic gastritis (CAG), 50 with stomach cancer (SC, stages I-II, morphological variant – adenocarcinoma) and 60 – control group. The diagnoses were verified according to international and Russian recommendations and confirmed by laboratory and instrumental studies. All patients were comparable in terms of gender and age characteristics ($p > 0.05$). All patients had specific IgG to *H. pylori*. The study was approved by the Local Ethics Committee of the FRC KSC SB RAS (protocol No. 11 dated November 11, 2013). All ethical requirements were observed, and the patients signed the informed consent form for participation. Patients and persons of the control group underwent a single blood sampling from the cubital vein upon admission to vacutainers with heparin.

The content of cytokines was determined in blood plasma by enzyme immunoassay using a Multiskan FC (Thermoscientific) analyzer. Based on the analyses, a database was compiled in Excel, and statistical processing was carried out in the Statistica 8.0 program [5, 7, 8].

Results and discussion

In patients with CG, CAG and SC there is an increase in the content of IL-2 compared with the control group, with the maximum value of the indicator in CG (Table 1). In patients of all the studied groups, there is a significant increase in the median

concentration of IL-8 compared with practically healthy volunteers, with the maximum value of the median parameter in SC. The median of the indicator increases by about 20 times in all the studied patients. In all patients, there is a significant increase in the median content of IFNγ relative to the control, 5 times in patients with CG and CAG and 8 times in patients with SC.

Thus, in all patients with inflammatory changes in the gastric mucosa (chronic gastritis), degenerative-inflammatory processes (chronic atrophic gastritis) and with metaplasia of epithelial cells of the gastric mucosa (cancer of the stomach), an increase in the content of pro-inflammatory cytokines (IL-2, IL-8, IFNγ) with a significant increase in IL-8 in all patients and IFNγ in stomach cancer.

The content of anti-inflammatory cytokine (IL-4) was assessed in CG, CAG and SC. There is a large increase in the median concentration of IL-4 in all patients; the median content of IL-4 is approximately increased by 13 times with a maximum level of IL-4 in stomach cancer.

Thus, in all patients there is an increase in pro-inflammatory and anti-inflammatory cytokines, which indicates the activation of immune cells and an imbalance in the cytokine regulation system. An increase in the production of Th1 and Th2 helper cytokines indicates a combined Th1- and Th2-mediated immune response in *H. pylori*-associated diseases (CG, CAG, and SC).

At the next stage, the immune responses in CAG were characterized depending on the severity of atrophy relative to the control group, patients with CG and SC. In general, regardless of the severity of degenerative-atrophic changes in the gastric mucosa, there was an increase in pro-inflammatory cytokines (IL-2, IL-8, IFNγ) and anti-inflammatory cytokine (IL-4) relative to the control group, while there was a tendency to increase medians of all indicators in severe atrophy of the gastric mucosa.

When comparing the parameters in CAG depending on atrophy relative to those in CG and SC, no statistically significant changes in the parameters of pro- and anti-inflammatory cytokines were obtained, while the trend towards an increase in the median of all cytokines in severe CAG versus CG and in SC versus severe CAG remains. Taking into account that all patients with CG, CAG and SC had specific antibodies to *H. pylori*, in this case we regard all conditions as *H. pylori*-associated diseases. The inflammatory process in the cancer of the stomach is

TABLE 1. CONTENT OF CYTOKINES IN PATIENTS WITH CHRONIC GASTRITIS (CG), CHRONIC ATROPHIC GASTRITIS (CAG), GASTRIC CANCER (GC) COMPARED WITH THE CONTROL GROUP (Me, $Q_{0.25}$-$Q_{0.75}$, p_{M-U})

Indicator	Control group, n = 60 (1)		Patients with CG, n = 60 (2)		Patients with CAG, n = 55 (3)		Patients with CAG PI 25 -50 μg/L, n = 24 (4)		Patients with CAG PI < 25 μg/l, n = 31 (5)		Patients with GC, n = 50 (6)	
	Me	$Q_{0.25}$-$Q_{0.75}$	Me	$Q_{0.25}$-$Q_{0.75}$	Me	$Q_{0.25}$-$Q_{0.75}$	Me	$Q_{0.25}$-$Q_{0.75}$	Me	$Q_{0.25}$-$Q_{0.75}$	Me	$Q_{0.25}$-$Q_{0.75}$
TNFα (pg/mL)	0.54	0.38-0.87	0.67	0.44-0.93	0.78	0.56-1.30	0.7	0.54-1.20	0.78	0.56-1.30	0.76	0.45-0.95
IL-2 (pg/mL)	1.1	0.50-3.05	5.7	3.6-10.3	4.9	3.8-9.5	4.4	3.8-10.1	4.9	3.8-9.5	5.2	3.0-8.7
			$p_{1-2} < 0.001$		$p_{1-3} < 0.001$		$p_{1-2} < 0.001$		$p_{1-3} < 0.001$		$p_{1-4} < 0.001$	
IL-8 (pg/mL)	2.1	0.5-4.0	40.5	7.5-97.2	38.1	5.5-87.3	37.3	4.2-85.6	38.1	5.5-87.3	41.1	12.2-99.5
			$p_{1-2} < 0.001$		$p_{1-3} < 0.001$		$p_{1-2} < 0.001$		$p_{1-3} < 0.001$		$p_{1-4} < 0.001$	
IFNγ (pg/mL)	0.6	0.22-4.00	2.9	2.2-4.0	3.2	2.3-4.8	3.1	1.9-5.1	3.2	2.3-4.8	4.4	3.3-6.9
			$p_{1-2} < 0.001$		$p_{1-3} < 0.001$		$p_{1-2} < 0.001$		$p_{1-3} < 0.001$		$p_{1-4} < 0.001$; $p_{2-4} = 0.02$	
IL-4 (pg/mL)	7.0	5.6-7.8	86.8	76.8-103.5	91.4	73.2-112.3	88.7	68.2-105.4	91.4	73.2-112.3	93.3	68.6-122.1
			$p_{1-2} < 0.001$		$p_{1-3} < 0.001$		$p_{1-2} < 0.001$		$p_{1-3} < 0.001$		$p_{1-4} < 0.001$	

Note. p_{1-2}, statistically significant differences between the group of CG patients and the control group; p_{1-3}, statistically significant differences between the group of patients with CAG and the control group; p_{1-4}, statistically significant differences between the control group and the group of patients with CAG with a PI level of 25-50 μg/L; p_{1-5}, statistically significant differences between the control group and the group of CAG patients with PI < 25 μg/L; p_{1-6}, statistically significant differences between the group of patients with gastric cancer and the control group; p_{2-3}, statistically significant differences between the group of patients with CG and the group of patients with CAG; p_{2-4}, statistically significant differences between the group of patients with CG and the group of patients with CAG with a PI level of 25-50 μg/L; p_{2-5}, statistically significant differences between the group of patients with CG and the group of patients with CAG with PI < 25 μg/L; p_{4-5}, statistically significant differences between the group of patients with CAG with a PI level of 25-50 μg/L and the group of patients with CAG with a level of PI < 25 μg/L; p_{2-6}, statistically significant differences between the group of patients with chronic hepatitis and the group of patients with GC; p_{3-6}, statistically significant differences between the group of patients with CAH and the group of patients with GC; p_{4-6}, statistically significant differences between the group of patients with CAH with a PI level of 25-50 μg/L and the group of patients with GC; p_{5-6}, statistically significant differences between the group of patients with CAH with PI < 25 μg/L and the group of patients with gastric cancer;

initiated by *H. pylori*, followed by other pathogenetic changes, such as atrophy and metaplasia.

The criterion for inflammation according to a clinical blood test is leukocytosis. We studied the correlation relationships between leukocytosis and indicators of pro-inflammatory and anti-inflammatory cytokines in control, in chronic hepatitis, CAG and SC. The following relationships were obtained: in the control group, five to four straight lines between the number of leukocytes and the content of TNFα ($r = 0.7$; $p = 0.05$), IFNγ ($r = 0.76$; $p = 0.01$), IL-2 ($r = 0.84$; $p = 0.015$), IL-8 ($r = 0.88$; $p = 0.02$) and inverse with IL-4 ($r = -0.64$; $p = 0.03$), which is natural, since pro-inflammatory cytokines increase leukocytosis, anti-inflammatory cytokine reduces the number of leukocytes. In patients with CG and CAG, 4 direct correlations are found: IFNγ (CG – $r = 0.81$; $p = 0.013$, CAG – $r = 0.69$; $p = 0.04$), IL-2 (CG $r = 0$ .8; $p = 0.015$, CAG – $r = 0.71$; $p = 0.02$), IL-8 (CG – $r = 0.82$; $p = 0.04$, CAG – $r = 0.9$; $p = 0.01$) and inverse with IL-4 (CG – $r = -0.6$; $p = 0.05$, CAG – $r = -0.7$; $p = 0.02$), there is no connection with TNFα, and with stomach cancer there are only 3: direct – IFNγ ($r = 0.72$; $p = 0.03$), IL-8 ($r = 0.89$; $p = 0.02$) and inverse with IL-4 ($r = - 0.78$; $p = 0.01$), TNFα and IL-2 exist autonomously.

All patients with *H. pylori*-associated diseases (CG, CAG, SC) showed an increase in pro-inflammatory (IL-2, IL-8, IFNγ) with a significant increase in IL-8 in all patients and IFNγ in stomach cancer and anti-inflammatory cytokine (IL-4) with a maximum value in cancer of the stomach. A combined Th1 and Th2 is found – a mediated immune response with a maximum violation of cytokine regulation in stomach cancer.

Conclusion

Correlation analysis confirmed the pathogenetic significance of the parameters IL-8, IL-2, IFNγ, IL-4 for CG and CAG, and IL-8, IFNγ, IL-4 for SC. Despite the different diseases, a single common pathogenetic mechanism caused by *H. pylori* infection was identified, which proves the need for mandatory eradication of the pathogen when it is detected, even in the absence of clinical symptoms in practically healthy people.

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ФУНКЦИОНАЛЬНАЯ И МЕТАБОЛИЧЕСКАЯ АКТИВНОСТЬ НЕЙТРОФИЛЬНЫХ ГРАНУЛОЦИТОВ КРОВИ У ДЕТЕЙ С ЭРОЗИВНО-ЯЗВЕННЫМ ПОРАЖЕНИЕМ ЖЕЛУДКА И 12-ПЕРСТНОЙ КИШКИ С ВЫЯВЛЕННОЙ ИНФЕКЦИЕЙ *HELICOBACTER PYLORI*

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Резюме. Одним из ведущих этиопатогенетических факторов формирования язвенной болезни желудка и двенадцатиперстной кишки у детей и взрослых в настоящее время рассматривается бактерия *Helicobacter pylori*. Несмотря на множество исследований в данной области, механизмы фагоцитарной активности в ответ на воздействие *Helicobacter pylori* до конца не ясны. Целью работы являлось получение результатов по функциональной и метаболической активности нейтрофильных гранулоцитов крови у детей с *Helicobacter pylori*-ассоциированным эрозивно-язвенным поражением желудка и двенадцатиперстной кишки. Объектом исследования являются нейтрофильные гранулоциты, выделенные из крови больных и контрольной группы. Образцы были взяты у 46 лиц с *Helicobacter pylori*-ассоциированным эрозивно-язвенным поражением желудка и 12-перстной кишки в возрасте от 11 до 18 лет и контрольную группу, которую составляли 55 практически здоровых лиц, у которых было исключено данное заболевание в аналогичном возрастном диапазоне. Проведен сравнительный анализ функциональной активности клеток с помощью хемилюминесцентного анализа и метаболической активности биолюминесцентным методом. В качестве активатора хемилюминесценции использовался люминол. Измерение функциональной активности фагоцитов основывалось на определении базовой активности (спонтанная реакция) и резервных возможностей клеток при воздействии на них неспецифическим индуктором зимозаном. Наблюдается пониженный индекс активации нейтрофилов больных относительно контрольной группы, что может характеризовать пониженные метаболические резервы клеток. В нейтрофильных гранулоцитах происходит снижение Г6ФДГ-фермента, который запускает гликолиз по пентозофосфатному пути и способствует восстановлению никотинамидаденин-динуклеотидфосфата (NADP) до NADPH, который необходим для образования восстановленного глутатиона, связывающего окислители. При его недостаточности происходит снижение энергетических запасов клеток. В нейтрофильных гранулоцитах крови у детей с эрозивно-язвенным поражением желудка и 12-перстной кишки с выявленной инфекцией *H. pylori* наблюдается понижение метаболических резервов, что связано с ингибированием метаболических процессов в клетках.

Ключевые слова: Helicobacter pylori, гастрит, язва, нейтрофильные гранулоциты, функциональная активность, метаболизм, ферменты

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FUNCTIONAL AND METABOLIC ACTIVITY OF BLOOD NEUTROPHILIC GRANULOCYTES IN CHILDREN WITH EROSIVE AND ULCERATIVE LESIONS OF THE STOMACH AND DUODENUM WITH DETECTED *HELICOBACTER PYLORI* INFECTION

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Abstract. The *Helicobacter pylori* bacterium is currently considered one of the leading etiopathogenetic factors in the formation of gastric and duodenal ulcer in children and adults. Despite many studies in this area, the mechanisms of phagocytic activity in response to exposure to *Helicobacter pylori* are not completely clear. The aim of the work was to obtain results on the functional and metabolic activity of blood neutrophilic granulocytes in children with *Helicobacter pylori*-associated erosive and ulcerative lesions of the stomach and duodenum. The object of the study are neutrophilic granulocytes isolated from the blood of patients and the control group. Samples were taken from 46 persons with *Helicobacter pylori*-associated erosive – ulcerative lesions of the stomach and duodenum aged 11 to 18 years and the control group, which consisted of 55 practically healthy persons who had this disease excluded in the same age range. A comparative analysis of the functional activity of cells using chemiluminescent analysis and metabolic activity by the bioluminescent method was carried out. Luminol was used as a chemiluminescence activator. The measurement of the functional activity of phagocytes was based on the determination of the base activity (spontaneous reaction) and the reserve capacity of the cells when they were exposed to the nonspecific inducer zymosan. There is a reduced activation index of neutrophils in patients relative to the control group, which may characterize reduced metabolic reserves of cells. In neutrophilic granulocytes, there is a decrease in G6PDG, an enzyme that triggers glycolysis along the pentose phosphate pathway and contributes to the reduction of nicotinamide adenine dinucleotide phosphate (NADP) to NADPH, which is necessary for the formation of reduced glutathione that binds oxidants. With its insufficiency, a decrease in the energy reserves of cells occurs. In neutrophilic blood granulocytes in children with erosive and ulcerative lesions of the stomach and duodenum with *H. pylori* infection, a decrease in metabolic reserves is observed, which is associated with inhibition of metabolic processes in cells

Keywords: Helicobacter pylori, gastritis, ulcer, neutrophilic granulocytes, functional activity, metabolism, enzymes

Introduction

One of the leading etiopathogenetic factors in the formation of peptic ulcer of the stomach and duodenum in children and adults is currently considered the bacterium *Helicobacter pylori*. The severity of the clinical course of helicobacteriosis largely depends on the degree of pathogenicity of the pathogen strains. Despite many studies in this area, the mechanisms of phagocytic activity in response to exposure to *H. pylori* are not completely clear [1, 2, 5].

Materials and methods

The objects of the study were neutrophilic granulocytes isolated from venous blood in 101 people: 46 people with *H. pylori*-associated erosive and ulcerative lesions of the stomach and duodenum aged 11 to 18 years and the control group, which consisted of 55 practically healthy individuals of the same age range, and who hadn't had the disease.

The indicators of the following oxidoreductases were determined: glucose-6-phosphate dehydrogenase (G6PDH), glycerol-3-phosphate dehydrogenase (G3PDH), malic enzyme (NADPMDH), NAD^+ and NADH-dependent reaction of lactate dehydrogenase (NALDDH and NADHLDH), NAD^+ and NADH-dependent reaction of malate dehydrogenase (NADMDH and NADHMDG), $NADP^+$ and NADPH-dependent reaction of glutamate dehydrogenase (NADPGDH and NADPHNGDG), NAD^+ and NADH-dependent reaction of glutamate dehydrogenase (NADHDH and NADHMDG), NAD^+ and NADP-dependent isocitrate dehydrogenases (NADICDH) and NADFITSDG). Enzyme activity is expressed in enzymatic units (1 E = 1 μmol/min) per mg of protein. The study was carried out on the NAD(P)H:FMN oxidoreductase-luciferase enzymatic system.

One of the most sensitive methods for assessing the formation of ROS (reactive oxygen species) is

chemiluminescence analysis. Luminol is used as a chemiluminescence activator. The determination of the functional activity of phagocytes was based on the determination of the base activity (spontaneous reaction) and the reserve capacity of the cells when exposed to the nonspecific inductor zymosan. Bioluminescent and chemiluminescent analyzes were carried out on a BLM-3607 chemiluminescent analyzer (Russia).

Results and discussion

In the study of chemiluminescence in patients' neutrophil fraction with the inducer luminol, a decrease in the time to reach the peak is observed, but an increase in intensity and an area under the curve relative to the control. The activation index is more than 2 times lower in the patients' group (Figure 1).

Metabolic enzymes of neutrophilic granulocytes, which are indicators of intracellular metabolism, were also studied. They take an active part in bioenergetic processes. Also, they participate in the directed coordination of conjugated metabolic flows, largely determine the adaptive changes in cellular metabolism. Thus, there was a decrease in G6PDH, NADHLDH, NADFICDH, NADICDH, NADLDH, NADMDH, NADFMDH, NADDMDH, NADPHNG, NADNGDH with an increase in the activity of G3PDH and NADHDG (Figure 2).

It was found that in the luminol-dependent process, an increase in the area under the curve and the maximum intensity of the luminescence of neutrophils in the blood of patients relative to control neutrophils determines an increased level of production of reactive oxygen species, which in general may reflect the cytotoxic activity of neutrophilic granulocytes in the focus of inflammation. However, there is a reduced activation index of neutrophils in patients relative to the control group, which may characterize reduced metabolic reserves of cells [4].

Thus, in neutrophilic granulocytes, there is a decrease in G6PDG, an enzyme that triggers glycolysis along the pentose phosphate pathway and contributes to the reduction of nicotinamide adenine dinucleotide phosphate (NADP) to NADPH, which is necessary for the formation of reduced glutathione that binds oxidants. With its insufficiency, there is a decrease in the energy reserves of cells [1]. Also, in the group of patients, the LDH enzyme, one of the key enzymes of glycolysis, is reduced. At the same time, the enzymatic reaction, which can compensate for the outflow of substrates from glycolysis, is catalyzed by G3PD, the activity of which is increased in neutrophils in the group of patients [3]. There is also an increase in NADHDH activity in patients, which indicates substrate stimulation of the Krebs cycle by products of amino acid metabolism reactions. One of the metabolic systems maintaining the hydrogen gradient is the malate-aspartate shunt, the key reaction of which is carried out by NADHMDH. In blood neutrophils in the group of patients, its activity is reduced relative to the parameters of the control group.

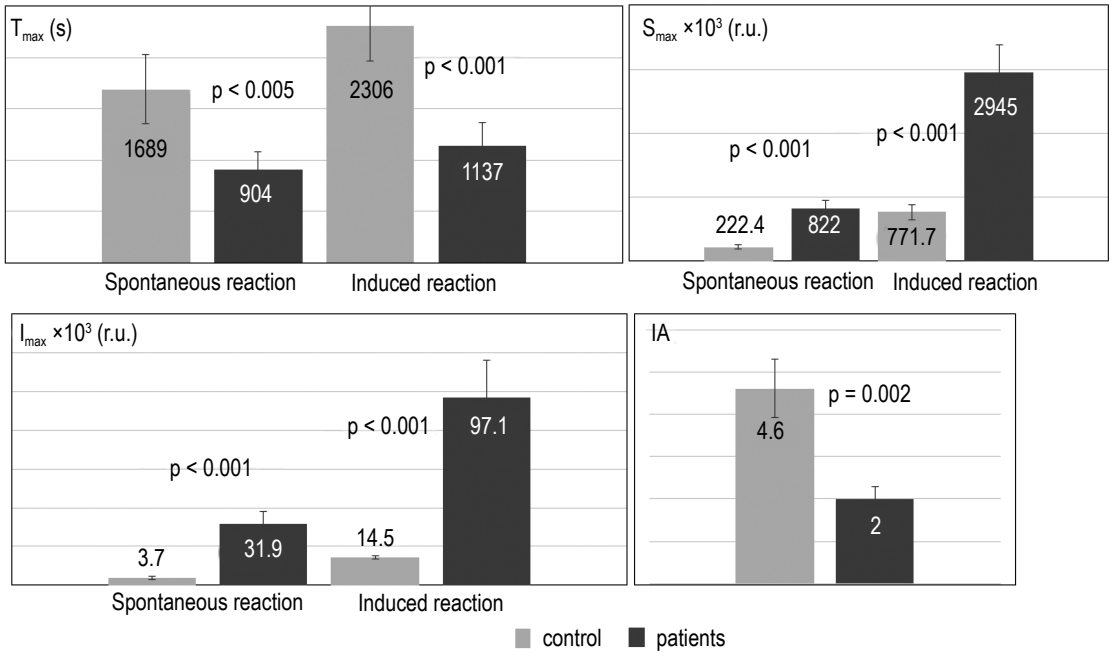

Figure 1. Luminol-dependent chemiluminescence of the patients' neutrophilic granulocytes and the control group

Note. T_{max}, time to reach the reaction peak; I_{max}, maximum luminescence intensity; S_{max}, area under the curve; IA, activation index.

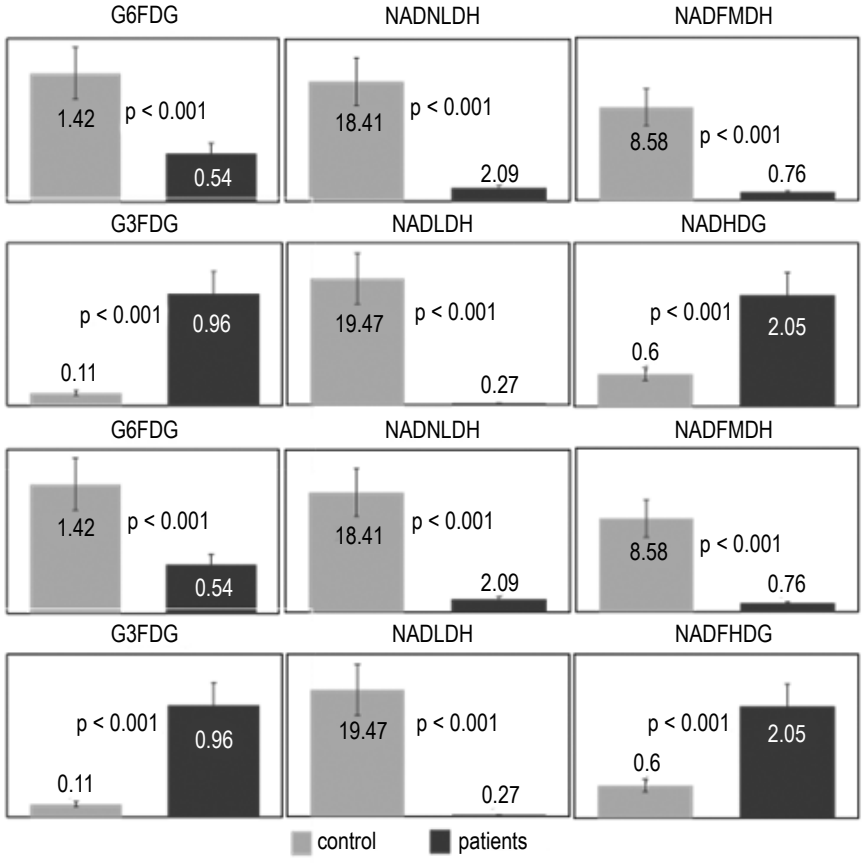

Figure 2. Bioluminescent activity of NAD(P)H-dependent enzymes in the patients' neutrophilic granulocytes of patients and the control group

Conclusion

In neutrophilic blood granulocytes in children with erosive and ulcerative lesions of the stomach and duodenum with *H. pylori* infection, a decrease in metabolic reserves is observed, which is associated with inhibition of metabolic processes in cells.

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ОСОБЕННОСТИ ИММУНОЛОГИЧЕСКИХ ПРОЯВЛЕНИЙ У ПАЦИЕНТОВ С РЕВМАТОИДНЫМ АРТРИТОМ ПРИ НАЛИЧИИ ХРОНИЧЕСКОГО ИНФИЦИРОВАНИЯ ШТАММОМ *HELICOBACTER PYLORI*, КОДИРУЮЩИМ АССОЦИИРОВАННЫЙ С ЦИТОТОКСИНОМ ГЕН *A*

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Резюме. Цель исследования – оценить взаимосвязь между серопозитивностью по антителам к циклическому цитруллинированному пептиду и хронической инфекцией *Helicobacter pylori* (*H. pylori*) у пациентов с ревматоидным артритом (РА). В исследование были включены 92 женщины с умеренной активностью РА. В иммуноферментном анализе определяли сывороточные антитела к циклическому цитруллинированному пептиду (anti-CCP), антитела к *H. pylori* (anti-*H. pylori*-IgG), суммарные антитела к антигену CagA *H. pylori* (anti-CagA); наличие позитивного результата anti-CagA-IgG подтверждали методом иммуноблота. 68,5% больных РА показали положительный результат anti-*H. pylori*-IgG, причем среди пациентов данной группы 40% дали положительный результат на anti-CagA-IgG. Все участники исследования были распределены на группы: I – *H. pylori* серонегативные пациенты (*H. pylori*$^-$); II – *H. pylori* положительные, но CagA отрицательные (*H. pylori*$^+$/CagA$^-$); III – пациенты сероположительные и на *H. pylori*, и на CagA (CagA$^+$). Показатели anti-CCP у больных РА с CagA$^+$ (группа III) были достоверно выше не только по сравнению с группой больных, серонегативных по *H. pylori* ($p < 0,001$), но и по сравнению с пациентами из группы II (*H. pylori*$^+$/CagA$^-$) ($p < 0,041$). Изучение влияния активности РА, наличия РФ и *H. pylori* данных факторов на содержание anti-CCP продемонстрировало незначительную долю изменчивости anti-CCP при высоком удельном вкладе

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H. pylori (beta = 0,25). Добавление в представленную модель показателя CagA(+) (beta = 0,503) позволило описать изменчивость anti-CCP практически в 30% случаев (R^2 = 0,29). В группе больных РА с показателями anti-CCP, превышающими установленное пороговое значение в 20 МЕ/мл (показатель нормы), наблюдалось повышение доли больных, инфицированных *H. pylori* (p < 0,001), но не доля CagA-положительных пациентов (p = 0,06). При увеличении порогового уровня до 60 МЕ/мл (трехкратное превышение верхней границы нормы) у пациентов с достоверно высоким anti-CCP связь с позитивностью на CagA стала значимой (p = 0,005). CagA, обладая высокой иммуногенностью, способен вызывать воспалительную реакцию в организме хозяина, которая выходит за рамки действия самой *H. pylori*. Необходимы дополнительные экспериментальные исследования для изучения возможных клинических и лабораторных ассоциаций, которые могут оказать влияние на тактику лечения $CagA^+$ пациентов с РА, серопозитивных по антицитруллинированным антителам, а также оценить эффективность эрадикации *H. pylori* в данной группе.

Ключевые слова: ревматоидный артрит, антитела к циклическому цитруллинированному пептиду, инфекция, диагностика, хеликобактер пилори, белок CagA

PECULIARITIES OF IMMUNOLOGICAL MANIFESTATIONS IN PATIENTS WITH RHEUMATOID ARTHRITIS IN THE PRESENCE OF CHRONIC INFECTION WITH *HELICOBACTER PYLORI* VARIANT ENCODING CYTOTOXIN-ASSOCIATED GENE *A*

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Abstract. The study aimed to evaluate the association between cyclic citrullinated peptide antibody seropositivity and chronic *Helicobacter pylori* (*H. pylori*) infection in patients with rheumatoid arthritis (RA). We examined 92 women with moderate RA activity. Serum antibodies to cyclic citrullinated peptide (anti-CCP), antibodies to *H. pylori* (anti-*H. pylori*-IgG), and total antibodies to *H. pylori* CagA antigen (anti-CagA) were determined by enzyme immunoassay; the presence of anti-CagA-IgG positivity was confirmed by immunoblot. 68.5% of RA patients were positive for anti-*H. pylori*-IgG, and 44.4% of patients in this group were positive for anti-CagA-IgG. All the study participants were divided into three groups: I – *H. pylori* seronegative (*H. pylori*$^-$); II – *H. pylori* positive, CagA negative (*H. pylori*$^+$/CagA$^-$); III – *H. pylori* positive and CagA positive ($CagA^+$). The anti-CCP values in RA patients with $CagA^+$ (group III) were significantly higher not only in comparison with patients seronegative for *H. pylori* (p < 0.001), but also in comparison with patients from group II (*H. pylori*$^+$/CagA$^-$) (p = 0.041). A study of the influence of the RA activity, the presence of RF and *H. pylori* on anti-CCP content demonstrated a small proportion of anti-CCP variability (R2 = 0.09), with a high contribution of *H. pylori* (beta = 0.25). The addition of the CagA(+) index (beta = 0.503) to the presented model allowed us to describe the variability of anti-CCP in almost 30% of cases (R2 = 0.29). In the group of RA patients with anti-CCP values exceeding the established threshold value of 20 U/mL (normal index), there was an increase in the proportion of patients infected with *H. pylori* (p < 0.001), but not the proportion of CagA-positive patients (p = 0.06). When the threshold level was increased to 60 U/mL (three times the upper limit of normal) in patients with significantly high anti-CCP, the association with positivity for CagA became significant (p = 0.005). CagA is highly immunogenic and is capable of inducing an inflammatory response in the host that goes beyond the effect of *H. pylori* itself. Additional experimental studies are needed to investigate possible clinical and laboratory associations that may influence the treatment tactics of $CagA^+$ patients with RA who are seropositive for anti-citrullinated antibodies, as well as to evaluate the possible effects of therapeutic intervention aimed at the eradication of *H. pylori* in this group.

Keywords: rheumatoid arthritis, antibodies to cyclic citrullinated peptide, infection, diagnostics, Helicobacter pylori, CagA protein

Introduction

In recent years, the association of *Helicobacter pylori* (*H. pylori*) infection with the spread of autoimmune pathology has attracted much attention because *H. pylori* can participate in the etiopathogenesis of a number of autoimmune diseases and persist chronic inflammation by stimulating the immune overall response [13]. Various mechanisms associated with *H. pylori* can cause loss of cellular tolerance and the production of various autoantibodies (IgM-rheumatoid factor, antinuclear and antiphospholipid antibodies, etc.) [4]. According to statistical analysis, infection with a more virulent variant of *H. pylori* encoding cytotoxin-associated gene *A* (CagA) can significantly increase the risk of autoimmune diseases [14]. This strain of *H. pylori* has an increased ability to stimulate the secretion of pro-inflammatory cytokines, which ultimately leads to modulation of the patient's immune responses [2].

The results of the many studies suggest a possible pathogenetic role of *H. pylori* infection in the progression of non-digestive diseases [3], such as chronic autoimmune rheumatic diseases, including rheumatoid arthritis (RA). Although a number of studies do not support a significant association between *H. pylori* infection and RA [1, 14], there is evidence of significant improvement in both clinical and laboratory parameters after successful eradication of *H. pylori* infection in patients with RA [2].

It should be stated that the relationship between *H. pylori* infection and RA is understudied nowadays [4].

The study aimed to evaluate the association between cyclic citrullinated peptide antibody seropositivity and chronic *H. pylori* infection in patients with RA.

Materials and methods

We examined 92 women (mean age, 55.5±9.6 years old; mean disease duration, 8.5 (7.0-9.5) years; body mass index, 29.1 (24.8-32.9) points) with moderate ($3.2 < DAS28 \leq 5.1$) RA activity. The mean value of the DAS28-ESR index was 3.83 (3.47-4.38) points. Rheumatoid factor (RF) seropositivity was found in 56 (61%) people. Rheumatoid arthritis diagnosis was based on the diagnostic criteria defined by the "American College of Rheumatology/European League Against Rheumatism Collaborative initiative" (2010).

Serum samples were obtained after centrifugation (5 mL of whole blood at 3000 rpm for 5 minutes), then samples were kept at -20°C until testing. Serum antibodies to cyclic citrullinated peptide (anti-CCP) were determined by enzyme immunoassay (Anti-CCP hs kit, Cat. No. 416-6010, Orgentec Diagnostika, Germany). The presence of helicobacteriosis was established using the Anti-*Helicobacter pylori* ELISA (IgG) ELISA test (EI2080-9601G, Euroimmun, Germany) designed for semi-quantitative/quantitative determination of human IgG antibodies to *Helicobacter pylori* (anti-*H. pylori*-IgG) in blood serum and plasma. All serum samples from RA patients were also analyzed for the presence of total antibodies to *Helicobacter pylori* CagA antigen (anti-CagA) (Vector-Best, Russia). The threshold values were determined at the levels recommended in the manufacturer's instructions. The immunoblot method and the recomLine Helicobacter IgG 2.0 kit (Cat. No. 4774, Mikrogen, Germany) were additionally used to confirm *Helicobacter pylori* CagA-IgG associated infection.

Statistical analysis

The results were statistically analyzed using Microsoft Excel 2011 and Statistica 10.0 (Stat Soft Inc., USA).

Data were displayed as median and lower/upper quartile Me ($Q_{0.25}$-$Q_{0.75}$) for variables with asymmetric distribution, and as mean ± standard deviation (M±SD) for variables with normal distribution. The test of variance analysis (ANOVA) and Kruskal–Wallis H test (as appropriate) were used in intergroup comparisons. Categorical variables were presented as percentages (%). Correlation analysis was performed using Spearman's coefficient (r_s). We used the χ^2 criterion to analyze differences in the frequencies of the variables between the groups. Differences were considered statistically significant at $p < 0.05$.

Results and discussion

In determining anti-CCP, the mean interquartile range was 63.4 (9.49-312) U/mL. About half of the patients (47.8%) had a significantly high level of anti-CCP (> 60 U/mL; three times the upper limit of normal). In 18.5% of the patients the index was in the range of 20 U/mL to 60 IU/mL (moderate increase).

68.5% of RA patients (n = 63) were positive for anti-*H. pylori*-IgG, and 44.4% (n = 28) of patients in this group were positive for anti-CagA-IgG.

All the study participants were divided into three groups: group I (n = 29; *H. pylori*$^-$) – *H. pylori* seronegative (no anti-*H. pylori*-IgG); group II (n = 35; *H. pylori*$^+$/CagA$^-$) – *H. pylori* positive, CagA negative (positive anti-*H. pylori*-IgG, but no anti-CagA-IgG); group III (n = 28; CagA$^+$) – *H. pylori* positive and CagA positive (positive anti-*H. pylori*-IgG and anti-CagA-IgG).

There were no intergroup differences in determining C-reactive protein (CRP) and rheumatoid factor class IgM (IgM-RF) ($p > 0.05$), except for group 3 patients, whose IgM-RF levels were significantly

TABLE 1. LEVELS OF LABORATORY MARKERS OF RHEUMATOID ARTHRITIS IN GROUPS OF PATIENTS WITH CHRONIC *H. PYLORI* INFECTION

	Group I	Group II	Group III
CRP, mg/L	17.0 (9.4-19)	12.5 (4.8-17.7)	13.3 (9.4-19.7)
IgM-RF, IU/mL	$13.5\pm10.9^{I-III}$	20.6±17.7	23.2±19.9
anti-CCP, U/mL	$9.07\ (3.77\text{-}32.4)^{I-II,\ I-III}$	$63.7\ (14.6\text{-}149.0)^{II-III}$	788 (74.3-1392.0)

Note. CRP, C-reactive protein; IgM-RF, rheumatoid factor class IgM; anti-CCP, antibodies against cyclic citrullinated peptide; the upper register indicates intergroup differences at $p < 0.05$.

higher ($p = 0.026$) than those of *H. pylori* seronegative patients (group I) (Table 1).

The anti-CCP values in RA patients with CagA(+) (group III) were significantly higher, not only in comparison with patients seronegative for *H. pylori* (group I), but also in comparison with patients from group II (*H. pylori*$^+$/CagA$^-$) ($p < 0.001$ and $p = 0.041$, respectively) (Table 1).

Previous studies have noted the correlation between *H. pylori* infestation, confirmed by histological method and the immunological manifestations of RA [5]. The research on laboratory parameters in RA patients with the presence of *H. pylori* demonstrates an increase in inflammatory markers and various autoantibodies [13].

Mechanisms of *H. pylori* evasion of the humoral immune response remain unclear. The persistence of *H. pylori* in the human body is accompanied, as a rule, by a successive change in the pronounced immune response at the introduction of the bacterium to the further development of immune tolerance [10].

The immune response to the soluble protein CagA present in highly virulent strains of *H. pylori* is often accompanied by increased levels of RF, ESR, CRP, and anti-mutant citrullinated vimentin (anti-MCV) [2]. CagA is highly immunogenic, has a direct effect on cancer-promoting signaling pathways, and causes an inflammatory response in the host body that goes beyond the action of *H. pylori* itself [9]. And if in our study the absence of correlation between CagA(+) and the studied markers of inflammatory reaction (CRP, CRP) can be explained by the homogeneity of RA patients by sex, age, activity and duration of the disease (the association with Helicobacter infection was previously established for all parameters), the presence of close association of anticitrullinated proteins and CagA(+) is most indicative.

Anti-CCP levels correlated with the RA activity index DAS28 ($r_s = 0.19$), the presence of RF ($r_s = 0.29$), and *H. pylori* ($r_s = 0.45$). The study of the influence of these factors on anti-CCP content demonstrated a small proportion of anti-CCP variability ($R^2 = 0.09$), with a high contribution of *H. pylori* (beta = 0.25). The addition of the CagA(+) index (beta = 0.503) to the presented model allowed us to describe the variability of anti-CCP in almost 30% of cases ($R^2 = 0.29$).

In the group of RA patients with anti-CCP values exceeding the established threshold value of 20 IU/mL (normal index), there was an increase in the proportion of patients infected with *H. pylori* (Chi-square = 13.5; $p < 0.001$), but not the proportion of CagA-positive patients (Yates Chi-square = 3.55; $p = 0.06$). When the threshold level was increased to 60 IU/mL (three times the upper limit of normal) in patients with significantly high anti-CCP (n = 44), the association with positivity for CagA became significant (Yates Chi-square = 7.67; $p = 0.005$).

The high level of *H. pylori* infection is apparently associated with impaired immune system formation in RA patients due to pronounced immune disorders and the influence of immunosuppressive therapy. Autoreactive B cells actively producing antibodies to citrullinated proteins in RA are exposed to *H. pylori*. Using modeling techniques to detect specific physiological free amino acids as biomarkers of *H. pylori*-related peptic ulcer disease, citrulline has been identified as one of the key traits [6]. It has been argued that citrullination processes could potentially play a role in bacterial and viral sepsis because high levels of circulating citrullinated histone H3 have been found in patients with sepsis and coronavirus [11, 12]. According to Kastbom et al., decreased mucosal immunity to citrullinated proteins/peptides and recruitment of new B-cells are important characteristics of the response to antirheumatic therapy in patients with early RA [8].

Based on the results of our study, we can conclude that chronic infection with *Helicobacter pylori* strain encoding cytotoxin-associated gene *A* (CagA) and immunological processes occurring in anti-CCP-

positive patients, regardless of the activity and duration of rheumatoid arthritis, are closely associated.

Conclusion

The role of *H. pylori* infection in the induction and maintenance of autoimmunity remains unclear. Clarification of the mechanisms of the influence of chronic H. pylori infection on the course of immunological processes in RA, especially in $CagA^+$ patients, is of clinical significance due to the fact that an infection with CagA-positive strains leads to increased resistance to antibiotics [7] used in eradication therapy.

Additional experimental studies are needed to investigate possible clinical and laboratory associations that may influence the treatment tactics of $CagA^+$ patients with RA who are seropositive for anti-citrullinated antibodies, as well as evaluate the possible effects of therapeutic intervention aimed at the eradication of *H. pylori* in this group.

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ИММУНОВОСПАЛИТЕЛЬНЫЕ МАРКЕРЫ СИНТРОПНЫХ СЕРДЕЧНО-СОСУДИСТЫХ ЗАБОЛЕВАНИЙ

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Резюме. Целью исследования явилось изучить иммуновоспалительные маркеры ишемической болезни сердца, артериальной гипертензии и их сочетанного течения.

В исследовании приняли участие 116 пациентов с сердечно-сосудистыми заболеваниями среднего и пожилого возраста. Средний возраст пациентов составляет 62,4±1,27 года. Все пациенты были обследованы в Бухарском филиале Республиканского научно-практического центра неотложной медицинской помощи. В ходе анализа было установлено, что уровень систолического артериального давления напрямую зависит от концентрации фибриногена – r = 0,3 и противоположно зависит от концентрации прокальцитонина (ПКТ) – r = -0,3 и IL-6 – r = -0,26. В то же время также была выявлена заметная положительная связь частоты сердечных сокращений с уровнем креатинина в крови – r = 0,35. Установленные связи показывают вклад синдрома воспаления (иммунного) в прогрессирование артериальной гипертензии, точнее, показателями прогрессирования артериальной гипертензии при ишемической болезни сердца являются креатинин, фибриноген, ПКТ и IL-6. Благодаря высокой и заметной корреляции с изучаемыми иммунобиохимическими показателями крови и функциональными показателями сердца, IL-6 является более информативным показателем прогрессирования ишемической болезни сердца с риском осложнений и полиорганной недостаточности. Таким образом, установленные связи в наших исследованиях позволяют включить вывод о том, что, наряду с вышеизложенным, факторами риска развития разрыва и/или аневризмы аорты при ИБС являются повышение уровня комплемента C3, IL-17 и ПКТ в крови. Следовательно, с повышением уровня VEGF в крови при ИБС AS возрастает риск увеличения толщины ЛЖ в диастолу, а повышение IL-6, IL-17A и мочевины в крови свидетельствует об уменьшении толщины ЛЖ в диастолу. диастола, которая позволяет дифференцировать рестриктивный вариант от гипертрофической формы при атипичной стенокардии. Благодаря высокой и заметной корреляции с изучаемыми иммунобиохимическими показателями крови и функциональными показателями сердца, IL-6 является более информативным показателем прогрессирования ишемической болезни сердца с риском осложнений и полиорганной недостаточности.

Ключевые слова: сердечно-сосудистые заболевания, ишемическая болезнь сердца, артериальная гипертензия, иммунитет

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IMMUNO-INFLAMMATORY MARKERS OF SYNTROPIC CARDIOVASCULAR DISEASES

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Abstract. Objective: To study the immuno-inflammatory markers of coronary heart disease, arterial hypertension and their combined course.

The research work included 116 patients with cardiovascular disease of middle and old age. The average age of patients is 62.4±1.27. All patients were examined at the Bukhara branch of the Republican scientific and practical Center for emergency medical care. During the analysis, it was found that the level of systolic arterial blood pressure directly depends on the concentration of fibrinogen – r = 0.30 and oppositely depends on the concentration of procalcitonin (PCT) – r = -0.3 and IL-6 – r = -0.26. At the same time, a noticeable positive association of heart rate with creatinine in the blood – r = 0.35 was also revealed. The established connections show the contribution of inflammation syndrome (immune) in the progression of hypertension, or rather, indicators of the progression of hypertension in coronary heart disease are creatinine, fibrinogen, PCT and IL-6. Due to the high and noticeable correlation with the studied immuno-biochemical parameters of blood and functional parameters of the heart, IL-6 is a more informative indicator of the progression of coronary heart disease with the risk of complications and multiple organ failure. Thus, the established connections in our studies allow us to include the conclusion that, along with the above, risk factors for the development of rupture and/ or aneurysm of the aorta in CHD are an increase in the blood level of complement C3, IL-17 and PCT. Consequently, with an increase in the level of VEGF in the blood in CHD AS, the risk of an increase in the thickness of the LV in the diastole increases, and an increase in IL-6, IL-17A and urea in the blood shows a decrease in the thickness of the LV in the diastole, which makes it possible to differentiate the restrictive variant from the hypertrophic form in atypical angina. Due to the high and noticeable correlation with the studied immuno-biochemical parameters of blood and functional parameters of the heart, IL-6 is a more informative indicator of the progression of coronary artery disease with the risk of complications and multiple organ failure.

Keywords: cardiovascular diseases, ischemic heart disease, arterial hypertension, immunity

Introduction

In modern literature, the term “remodeling of the heart” has appeared, which includes the whole complex of changes in the mass, volume and shape of the left ventricle due to cardiomyocyte hypertrophy, as well as hypertrophy and hyperplasia of interstitial cells and endothelium, leading to a violation of the biochemical and functional properties of the myocardium under the influence of various factors, including hypertension [1].

Age is a recognized risk factor for cardiovascular diseases and mortality, including in patients with hypertension. In many ways, this influence is realized through age-related changes in the structure and function of blood vessels [6]. The results of research in recent decades confirm the crucial role of vascular endothelium in the regulation of vascular homeostasis, while a significant contribution of endothelial dysfunction (ED) to the development of cardiovascular diseases (CVD) has been established, in particular, participation in the pathogenesis of hypertension. It is generally recognized that the endothelium maintains a balance between the processes of vasoconstriction and vasodilation, produces inflammatory factors and vascular proliferation, participates in vascular remodeling and in thrombosis [2, 3, 4].

Transforming growth Factor-β (TGF-β) is a cytokine, a protein growth factor that plays an important role in the regulation of cell growth, differentiation and regeneration of various tissues. In the heart, TGF-β1 is induced by MI, pressure overload, with the introduction of angiotensin II, norepinephrine and is inhibited by nitric oxide [5]. In the myocardium, TGF-β1 is synthesized by fibroblasts and cardiomyocytes and plays a key role in the development of tissue fibrosis. Thus, along with the already “gold standard” biomarker of heart failure pro-BNP, new biomarkers are being intensively studied, such as markers of apoptosis, remodeling of connective tissue extracellular matrix and inflammation, which allow not only to more accurately diagnose, but also to determine the risk of developing or progressing heart failure and death [7, 8].

Objective: to study the immuno-inflammatory markers of coronary heart disease, arterial hypertension and their combined course.

Materials and methods

The study included 116 middle-aged and elderly patients with an average age of 62.4±1.27 years.

All patients were examined for cytokine status: IL-17A, TNFα, C3 complement component, VEGF

and a marker of the acute phase of inflammation – procalcitonin (PCT), the lipid spectrum of blood was studied, an echocardiogram (ECHOCG) and a biochemical blood test were performed.

The inclusion criteria were patients aged 45 to 74 years with a diagnosis of arterial hypertension (AH), coronary heart disease (CHD), confirmed by clinical and laboratory-instrumental methods, hospitalized in a hospital.

The patients of the study groups were comparable in age, gender, and the presence of CVD risk factors. AH verification was carried out according to the requirements of the World Health Organization (WHO), classified according to the International Classification of Diseases (ICD-10).

At the same time, the ACC/AHA Hypertension Guidelines (2017) classification was adhered to.

The exclusion criteria from the study were patients with acute myocardial infarction, acute coronary syndrome, acute infectious diseases, myocarditis and cardiomyopathies, chronic renal and hepatic insufficiency, pulmonary hypertension, congenital and acquired heart defects, systemic diseases, oncological and hematological diseases.

The research was carried out in accordance with the Helsinki Declaration.

Statistical processing of the results was carried out using Excel programs from the Microsoft Office XP application package (Microsoft, USA), correlation analysis was carried out using the Pearson method and evaluated on the Cheddock scale.

Results and discussion

To develop specific indicators of the progression of AH in CHD, a correlation analysis of the relationship of the studied blood parameters with the parameters of ECHOCG in patients with CHD selected for examination was carried out.

The dependence of the aortic diameter on the studied immunological parameters of the blood was established: a weak positive relationship with C3 – $r = 0.20$, with IL-17A – $r = 0.21$, with PCT – $r = 0.25$, a negative relationship between the aortic diameter and TNFα – $r = -0.21$, VEGF – $r = -0.2$.

The established relationships show a directly proportional positive dependence of the aortic diameter on the level of inflammatory markers: complement C3, IL-17A and PCT ($r = 0.2$-0.25).

In patients of this group, the average concentration of PCT was 0.2±0.01 ng/mL, which indicates the absence or low risk of infectious complications. The established weak positive association of PCT with the diameter of the aorta shows the risk of developing infectious complications in coronary heart disease and the importance of dynamic determination of PCT in this case.

Thus, the change in the diameter of the aorta depends on the degree of the infectious process in the body. Therefore, in case of CHD, it is important to take into account the state of syntropy, that is, the presence of concomitant diseases, for the prognosis of complications. At the same time, the longer the duration of chronization of the infectious process and the dynamic increase in the level of complement C3, IL-17 and PCT, the greater the risk of an increase in the diameter of the aorta in CHD.

It is known that the aorta regulates blood pressure and heart rate. Age-related enlargement of the aortic root leads to thinning of the aortic wall, increases the risk of aortic rupture and/or aortic aneurysm. Hypertension, hyperglycemia and hypercholesterolemia are risk factors for changes in the state of the vascular wall.

At the same time, negative weak connections of the aortic diameter with TNFα ($r = -0.2$) and VEGF ($r = -0.2$) were also revealed (Figure 1).

Consequently, the established negative associations of the aortic diameter with TNFα in CHD confirm the data of literature sources and allow the inclusion of TNFα in the list of indicators of the progression of hypertension in CHD as a marker of inflammation in CHD.

Taking into account the data of the above-mentioned modern literature sources, the negative connections between the diameter of the aorta and TNFα, VEGF in CHD indicate a compensatory phase of the body's defense system.

Thus, the established connections in our studies allow us to include the conclusion that, along with the above, risk factors for the development of rupture and/or aneurysm of the aorta in CHD are an increase in the blood level of complement C3, IL-17 and PCT. At the same time, the greater the degree of increase in the dynamics of the level of C3, IL-17 and PCT, the greater the risk of rupture and/or aneurysm of the aorta in CHD. Consequently, the more the level of TNFα, VEGF increases in coronary heart disease, the more the diameter of the aorta decreases. At the same time, it is necessary to take into account the state of syntropy.

In CHD revealed a noticeable positive association of final diastolic volume of the left atrium (FDV LA) with IGF-1 ($r = 0.30$), PCT ($r = 0.30$) and total blood protein ($r = 0.39$) against the background of a weak correlation with TGF-β1 – $r = 0.21$ (Figure 2).

The established features of the relationship show the effect of syntropy and chronic inflammation in CHD. At the same time, with coronary heart disease, an increase in the level of TGF-β1 and PCT in the blood against the background of an increase in total blood protein leads to an increase in FDV LA.

In CHD, the total blood protein has a noticeable negative relationship with final diastolic volume of the left ventricle (FDV LV) – $r = -0.31$, which makes it possible to determine it as an indicator of the shift of FDV LA and FDV LV. Consequently, in CHD, an increase in the level of total protein is accompanied

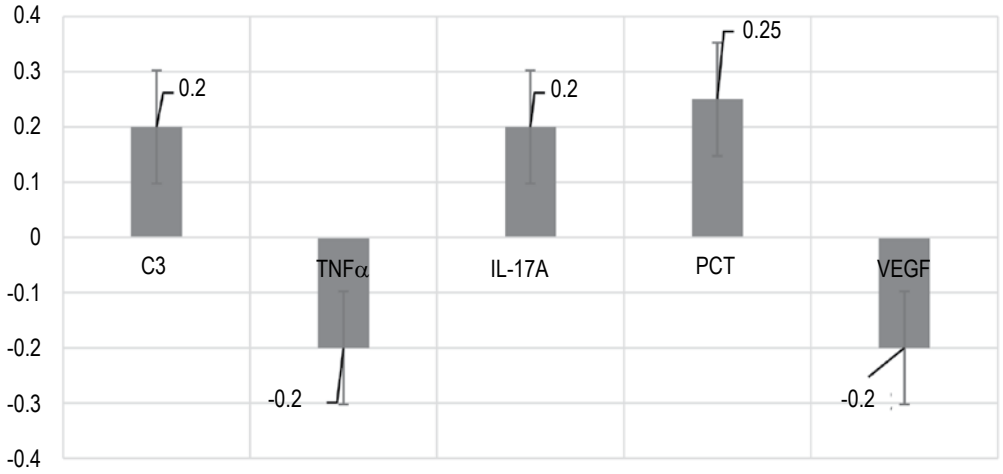

Figure 1. Relationship of aortic diameter with immuno-inflammatory markers in CHD

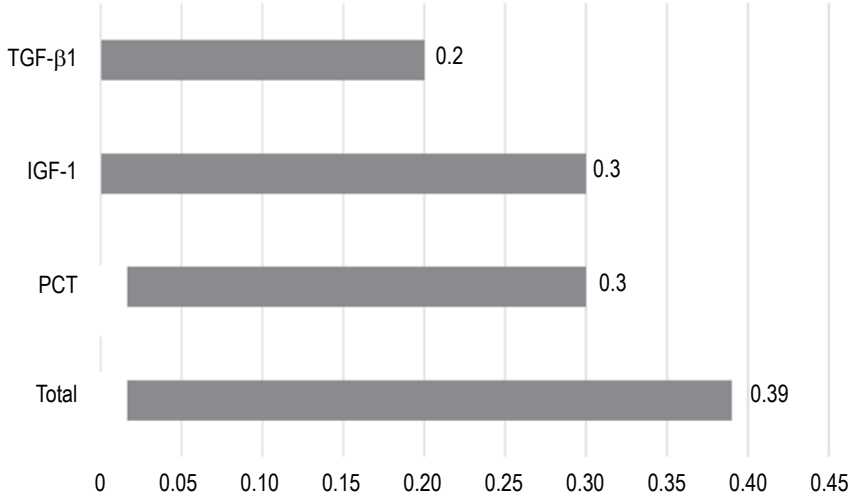

Figure 2. Relationship of the size of the left atrium with immuno-inflammatory markers in CHD

by an increase in FDV LA and a decrease in FDV LV. Thus, by determining the total protein in the blood, it is possible to predict the risk of developing LV dysfunction of the heart in CHD.

ECHOCG LV indices showed noticeable negative associations between muscle mass of the left ventricle (MMLV) and IL-6 – r = -0.31, a high negative association between LVEF and IL-6 – r = -0.41. At the same time, final systolic volume of the left ventricle (FSV LV) has a high positive relationship with IL-1 – r = 0.40, and specific volume of the left ventricle (SVLV) has a high positive dependence on the concentration of C3 in blood serum – r = 0.41 (Figure 3).

Consequently, SVLV decreases with an increase in IL-6, and MMLV increases with an increase in IL-1 in the blood, both cytokines are proinflammatory and show the role of immune inflammation at the level of the heart and blood vessels in coronary artery disease.

During the analysis, it was found that the level of SAP directly depends on the concentration of fibrinogen – r = 0.30 and oppositely depends on the concentration of PCT – r = -0.3 and IL-6 – r = -0.26.

At the same time, a noticeable positive association of heart rate with creatinine in the blood – r = 0.35 was also revealed.

The established connections show the contribution of inflammation syndrome (immune) in the progression of hypertension, or rather, indicators of the progression of hypertension in coronary heart disease are creatinine, fibrinogen, PCT and IL-6 (Figure 3).

Thus, revealed on the basis of correlation analysis of the relationship between the studied blood parameters and functional studies of the heart, the role of inflammation syndrome in the progression of hypertension in coronary heart disease has been established. At the same time, the development of cardiac restructuring dictates the need to develop

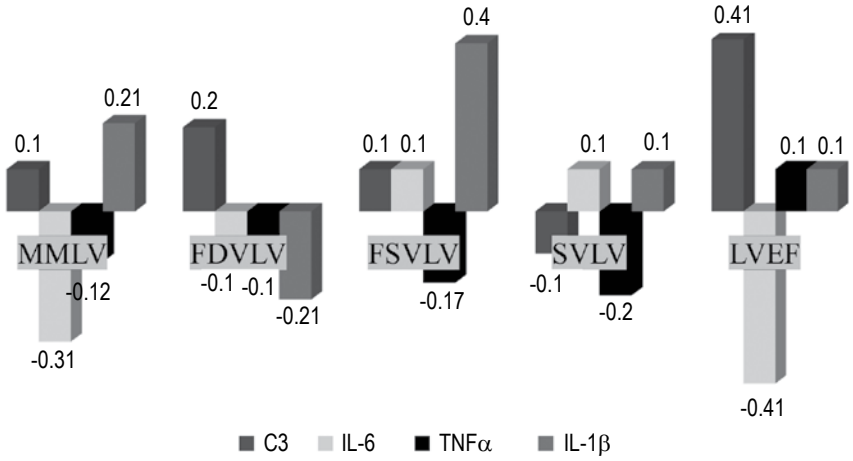

Figure 3. Relationship of ECHOCG indicators and cytokines in CHD

immunological and biochemical indicators for predicting the progression of hypertension in coronary heart disease in middle-aged and elderly people.

The dynamic study of immunological and biochemical blood parameters and the use of these recommendations allows the control and monitoring of hypertension, improving the effectiveness of diagnosis and the correct choice of treatment for patients with CHD, contributes to reducing mortality and disability at the same time.

In order to compare the relationship of immunological and biochemical parameters of blood with functional parameters of ECHOCG, a correlation analysis of the results obtained in patients with atypical angina was performed.

As a result, aortic diameter connections were established: weak positive with TNFα (r = 0.20), weak negative connections with IL-17A(r = -0.20), total blood protein (r = -0.20), PCT (r = -0.20) and VEGF (r = -0.26).

The obtained results of correlation analysis of the relationship allow a comparative assessment with the results of patients with CHD. Distinctive features of the relationship of the studied blood parameters with the diameter of the aorta in coronary artery disease were revealed: there is a weak positive relationship with TNFα (r = 0.20) against the background of a weak negative relationship between the diameter of the aorta and IL-17 (r = -0.20), total blood protein(r = -0.20), PCT(r = -0.20) and VEGF(r = -0.26). Consequently, in the atypical form of coronary angina, there is no risk of rupture and/or aneurysm of the aorta.

The study of the relationship between the FDV LA showed a noticeable positive relationship with the blood creatinine level in coronary artery disease (r = 0.32).

At the same time, weak negative associations of FDV LA with IL-17A (r = -0.21), fibrinogen (r = -0.20) and VEGF (r = -0.20). Consequently, in CHD, an increase in fibrinogen, IL-17A and VEGF in the blood is accompanied by a decrease in FDV LA. The degree of increase in creatinine shows the maximum size of the FDV LA. Therefore, with coronary artery disease, creatinine is an indicator of the prognosis of an increase in FDV LA.

In contrast to the correlation between FDV LA in CHD, in atypical angina, a noticeable positive relationship was established between FDV LV and PCT (r = 0.30), complement C3 (r = 0.40), weak noticeable connections with urea (r = 0.20), fibrinogen (r = 0.22), total blood protein (r = 0.22) against the background of a weak negative association with creatinine (r = -0.21).

The results obtained made it possible to determine the C3 complement of indicators of LV dysfunction in coronary artery disease in middle-aged and elderly people.

FDV LA in CHD has a high negative association with creatinine (r = -0.47) and TGF-β1 (r = -0.40), a noticeable negative association with IL-6 (r = -0.35), IL-1β (r = -0.30) and total blood protein (r = -0.30), a noticeable positive association with IGF-1 (r = 0.30). At the same time, the more creatinine and TGF-β1 increase in the dynamics in the blood, the more the FDV LA decreases. Consequently, creatinine and TGF-β1 are indicators of pancreatic hypertrophy in coronary artery disease atypical angina.

Consequently, in atypical angina, a decrease in TNFα in the blood in dynamics against the background of an increase in fibrinogen, VEGF and IL-1β in the blood indicates a threat of hypertrophy of the LV.

The state of the thickness of the posterior LV wall in the diastole (PWLV) is important. There was a noticeable positive association of PWLV with VEGF (r = 0.35) against the background of a noticeable negative association with IL-6 (r = -0.37), IL-17A (r = -0.30).

At the same time, weak associations of CSL were also revealed: negative with blood urea (r = -0.26) and positive with IGF-1 (r = 0.21), which shows a low risk of developing fibrosis in CHD.

The data obtained show that with coronary artery disease, an increase in IL-6, C3, and IGF-1 in the blood is accompanied by an increase in the size of the LV and vice versa, a decrease in these indicators in dynamics indicates an increase in LV with atypical angina in middle-aged and elderly people.

In general, a correlation analysis of blood parameters in CHD revealed a strong relationship between IL-6 and left ventricle ejection fraction (LVEF) (r = 0.44). At the same time, C3 complement and IL-6 were effective informative indicators of the severity of atypical angina.

Thus, the obtained high correlations made it possible to determine informative indicators of the risk of cardiac remodeling in CHD: an increase in IL-6 in the blood in dynamics predicts the risk of developing uremia (an increase in creatinine in the blood), tachycardia, a decrease in the thickness of the LV in the diastole, a decrease in LVEF, an increase in MMLV.

Conclusion

Thus, the established connections in our studies allow us to include the conclusion that, along with the above, risk factors for the development of rupture and/or aneurysm of the aorta in CHD are an increase in the blood level of complement C3, IL-17 and PCT.

Consequently, with an increase in the level of VEGF in the blood in CHD AS, the risk of an increase in the thickness of the LV in the diastole increases, and an increase in IL-6, IL-17A and urea in the blood shows a decrease in the thickness of the LV in the diastole, which makes it possible to differentiate the restrictive variant from the hypertrophic form in atypical angina.

Due to the high and noticeable correlation with the studied immuno-biochemical parameters of blood and functional parameters of the heart, IL-6 is a more informative indicator of the progression of coronary artery disease with the risk of complications and multiple organ failure.

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ВЫЯВЛЕНИЕ ВЗАИМОСВЯЗИ МЕЖДУ БИОМАРКЕРАМИ АУТОФАГИИ, АПОПТОЗА И ВОСПАЛЕНИЯ В ОСТРОМ ПЕРИОДЕ АТЕРОТРОМБОТИЧЕСКОГО ИШЕМИЧЕСКОГО ИНСУЛЬТА

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Резюме. Постишемический воспалительный ответ играет существенную роль в патогенезе острого ишемического инсульта (ИИ). Установлено, что острый ИИ сопровождается асептическим воспалением, индуцирующим активацию костимулирующих молекул в процессе ответной реакции врожденного иммунитета на повреждение ткани головного мозга. Постоянно прогрессирующая деструкция нейрональных антигенов способствует увеличению объема очага ишемического поражения. Продолжают накапливаться доказательства, свидетельствующие о важной роли NLRP3-опосредованного воспаления в патогенезе ишемического инсульта, реализуемого инфламмасомами – мультипротеиновыми олигомерными комплексами. В последнее время большой интерес вызывает перспектива модулирования аутофагии при остром ИИ, являющейся мощным регулятором воспалительных реакций. Показано, что при остром ИИ аутофагия задействована в важнейших этапах воспалительного каскада, выступая либо в качестве индуктора, либо в роли ингибитора постишемического нейровоспаления. Во многих противовоспалительных механизмах, реализуемых аутофагией при остром ИИ, участвуют ключевые белки аутофагического процесса Beclin-1, LC3 и p62. Экспериментальные исследования показали, что аутофагия подавляет активацию NLRP3-опосредованного воспаления. По-прежнему противоречивыми остаются данные о перекрестных взаимодействиях между апоптозом и

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аутофагией и их отдельными медиаторами в патогенезе острого ИИ. Исследования в области нейроиммунологии позволили установить сигнальные белки, инициирующие как апоптотическую, так и аутофагическую гибель клеток головного мозга при остром ИИ. Целью исследования явилась оценка взаимосвязи между биомаркерами аутофагии, воспаления, и апоптоза в динамике острого периода атеротромботического ИИ. В работе представлены результаты динамического исследования сывороточной концентрации ключевых биомаркеров аутофагии Beclin-1, LC3 и p62, показателей апоптоза Bcl-2 и p53, провоспалительных цитокинов IL-1β, TNFα, IL-8, IL-18, участвующих в постишемическом нейровоспалении. Установлено статистически достоверное повышение исследуемых показателей по сравнению с группой контроля. Максимальное повышение исследуемых биомаркеров отмечается в 1-е сутки после развития ишемии у пациентов с тяжелым течением заболевания. Выявлена взаимосвязь между активностью аутофагии, биомаркерами апоптоза и некоторыми показателями системной воспалительной реакции у пациентов с атеротромботическим инсультом среднетяжелого и тяжелого течения. Полученные результаты подтверждают данные литературы об участии аутофагии в регуляции постишемического воспалительного ответа.

Ключевые слова: аутофагия, апоптоз, острый ишемический инсульт, биомаркеры аутофагии, биомаркеры апоптоза, Beclin-1, LC3, p62, IL-1β, IL-18, NLRP3-инфламмасома, постишемическое нейровоспаление

IDENTIFICATION OF THE RELATIONSHIP BETWEEN BIOMARKERS OF AUTOPHAGY, APOPTOSIS AND INFLAMMATION IN THE ACUTE PERIOD OF ATHEROTHROMBOTIC ISCHEMIC STROKE

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Abstract. The postischemic inflammatory response plays a significant role in the pathogenesis of acute ischemic stroke (IS). It has been established that acute IS is accompanied by aseptic inflammation, which induces the activation of costimulatory molecules in the process of innate immunity response to brain tissue damage. The constantly progressive destruction of neuronal antigens contributes to an increase in the volume of the ischemic lesion. Evidence continues to accumulate indicating an important role of NLRP3-mediated inflammation in the pathogenesis of IS. It has been shown that autophagy is involved in the inflammatory cascade in acute IS. Many of the anti-inflammatory mechanisms mediated by autophagy in acute IS involve the key autophagic proteins Beclin-1, LC3, and p62. Experimental studies have shown that autophagy suppresses the activation of NLRP3 inflammation. Data on cross interactions between apoptosis and autophagy in the pathogenesis of IS are still controversial. The aim of the study was to evaluate the relationship between biomarkers of autophagy, inflammation, and apoptosis in the dynamics of the acute period of atherothrombotic IS. The article presents the results of a dynamic study of the serum concentration of the key autophagy biomarkers Beclin-1, LC3 and p62, apoptosis indicators Bcl-2 and p53, pro-inflammatory cytokines IL-1β, TNFα, IL-8, IL-18 which are involved in postischemic neuroinflammation. A statistically significant increase in the studied parameters was established in comparison with the control group. The maximum increase in the studied biomarkers is noted on the 1st day after the development of ischemia in patients with a severe course of the disease. The relationship between autophagy activity, apoptosis biomarkers, and some indicators of the systemic inflammatory response in patients with moderate and severe atherothrombotic stroke was revealed. The results obtained confirm the literature data on the involvement of autophagy in the regulation of the postischemic inflammatory response.

Keywords: autophagy, apoptosis, acute ischemic stroke, autophagy biomarkers, apoptosis biomarkers, Beclin-1, LC3, p62, IL-1β, IL-18, NLRP3-inflammasome, postischemic neuroinflammation

Introduction

Postischemic neuroinflammation is a critical pathophysiological process within the framework of the entire scheme of cerebral ischemia, covering early damage and the period of tissue repair. It is characterized by microglial and astroglial activation with increased expression of inflammatory mediators and development of innate and adaptive immune responses. After the onset of acute ischemic stroke (IS), damage to the blood-brain barrier (BBB) occurs in 2 stages. The first begins within the first hours with the participation of innate immunity, and the second – 24-48 hours after an ischemic attack. During extravasation, leukocytes attracted by chemokines release matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9), which cleave tight junctions between endothelial cells of cerebral vessels [12]. This leads to damage to the BBB, resulting in increased vascular permeability, which can contribute to the development of vasogenic edema, one of the most severe complications of cerebral infarction [9]. Violation of the integrity of the BBB contributes to the disintegration of brain tissue and the "leakage" of brain autoantigens (myelin basic protein, myelin oligodendrocyte glycoprotein (MOG), glial fibrillary acid protein (GFAP) into the peripheral blood. The release of brain autoantigens into circulation is accompanied by the activation of the immune system and the influx of peripheral immunocompetent cells into the central nervous system [12]. Thus, acute cerebral ischemia triggers a local and systemic immune response [5].

According to the literature, after acute IS, microglia are polarized into the pro-inflammatory phenotype M1, characterized by the secretion of pro-inflammatory cytokines, in particular TNFα (tumor necrosis factor α), IL-1β, IL-6, IL-18, IL-21 (interleukins-1β, interleukins-6, interleukins-18, interleukins-21) [3]. In the acute period of IS, patients have a significant increase in the concentration of pro-inflammatory cytokines in serum. It has been shown that a high level of serum IL-1β correlates with the severity of acute IS and is a predictor of a poor prognosis of the disease. These results are consistent with literature data on the key role of this interleukin in ischemic injury [15]. According to the latest experimental data, autophagy is associated with postischemic neuroinflammation and is involved in its regulation [7]. In recent reports, autophagy is considered as a negative regulator of NLRP3 inflammation (nucleotide-binding oligomerization domain-like receptor [NLR] family pyrin domain-containing 3), which plays a significant role in the pathogenesis of ischemic stroke [2]. This is supported by experimental data demonstrating that the use of autophagy inhibitors promotes the activation of the NLRP3 inflammasome. It has been shown that macrophages with knockout of the Atg7 autophagy gene are characterized by a significantly increased production of IL-1β in response to inflammation inducers compared to normal macrophages [10].

Numerous evidences have been provided for the involvement of autophagy in the regulation of the production of pro-inflammatory cytokines and chemokines [7, 8]. The interactions between autophagy and inflammation have been shown to work in a feedback manner. Autophagy is involved in the induction and suppression of inflammation and, in turn, can be both induced and suppressed by inflammatory mediators [4]. It has been established that basic autophagy under normal physiological conditions contributes to the maintenance of immunological tolerance, while excessive activation of autophagy in immunocompetent cells or the presence of defects in autophagy-inhibiting genes in them leads to the development of autoimmune processes [14].

A similar trend was revealed in the study of the mutual regulation of apoptosis and autophagy [6]. A number of recent studies show that basic autophagy, by stimulating the overexpression of anti-apoptotic proteins of the Bcl-2 family, inhibits apoptosis, protecting brain cells from delayed death. In contrast, activation autophagy acts in conjunction with apoptosis to exacerbate cell death. As a result of experimental studies, proteins were identified that are effectors of brain cell death both by apoptosis and by activation autophagy in acute IS. These proteins are the Bcl-2 and p53 antagonist proteins, as well as the autophagy initiator Beclin-1 (the protein encoded by the Atg6 gene initiates the initial stage of autophagy, the formation of a phagophore) [1,6]. Recent reports indicate that selective mitochondrial autophagy (mitophagy) with the participation of Beclin-1 and p62/SQSTM1 (LC3-binding adaptor p62/sequestosome 1) proteins can suppress the proapoptotic activity of the p53 protein and inhibit apoptosis [1].

Considering the latest literature data on the significant role of autophagy in the regulation of postischemic neuroinflammation and apoptosis processes in acute IS, it seems relevant to evaluate the relationship between biomarkers of autophagy, inflammation, and apoptosis in the dynamics of the acute period of IS and compare it with the severity of the clinical and neurological status of patients.

Objective: to quantify pro-inflammatory cytokines, C-reactive protein, autophagy biomarkers, apoptosis biomarkers in the serum of patients in the acute period of moderate and severe atherothrombotic IS. To reveal the relationship between biomarkers of autophagy, inflammation and apoptosis in the dynamics of the acute period of atherothrombotic IS.

Materials and methods

All studies were approved by the ethical committee of the Pavlov First St. Petersburg State Medical University of Russian Federation. We examined 92 patients (63 men and 29 women) in the acute

period of newly developed atherothrombotic IS and 56 healthy donors comparable in sex and age to patients with acute IS. Inclusion criteria for the study were: informed consent to participate in the study; age from 45 to 60 years; verified by magnetic resonance imaging (MRI) for the first time identified acute IS in the system of the internal carotid artery (atherothrombotic pathogenetic variant); gender of patients: male, female; no more than 24 hours from the onset of the disease; neurological symptoms no more than 14 points on the NIHSS scale (National Institutes of Health Stroke Scale).

According to the results of clinical neurological and laboratory examinations, as well as the results of neuroimaging diagnostic methods, all patients were divided into 3 groups: with mild (n = 6), moderate (n = 59) and severe disease (n = 27). Due to the small number of observations, patients with mild acute IS were not included in further analysis. Group I (moderate course) consisted of patients with a severity of neurological symptoms of no more than 10 points on the NIHSS scale, no more than 3 points on the Rankin scale (used to assess the degree of disability after a stroke), with a brain parenchymal lesion volume of less than 50 cm^3. Group II (severe course) consisted of patients with a severity of neurological symptoms of more than 10 points on the NIHSS scale, from 3 to 5 points on the Modified Rankin Scale (mRs), with a brain parenchymal lesion volume of more than 50 cm^3. Group III (control) consisted of donors (n = 56).

Patients underwent a dynamic clinical and neurological examination with an assessment of the severity of neurological deficit according to the NIHSS scale, a study of the volume of the lesion by brain MRI, testing by mRs on the 1st, 7th and 14th days from the onset of the disease. At the same time intervals, blood was taken for the study.

Serum concentrations of pro-inflammatory cytokines IL-1β, TNFα, IL-8, IL-18, apoptosis biomarkers p53, Bcl-2, and autophagy biomarkers Beclin-1, LC3, and p62 were determined by enzyme-linked immunosorbent assay using appropriate test systems (ELISA Kits; Abcam, UK) and (ELISA Kits; Enzo, UK).

A highly sensitive immunoturbidimetric method (Cobas 6000, Roche Diagnostics, Switzerland) was used to quantify C-reactive protein in peripheral blood.

For statistical processing of the obtained data, the nonparametric Wilcoxon–Mann–Whitney test was used to compare the means, Spearman's correlation analysis. The material was processed using the Statistica 10.0 software package (StatSoft, USA, Windows 10). The critical confidence level of the null hypothesis (the absence of significant differences) was taken equal to 0.05.

Results and discussion

The results of the assessment of the studied indicators in the dynamics of the acute period of IS are presented in Tables 1 and 2.

TABLE 1. COMPARATIVE CHARACTERISTICS OF THE CONTENT OF BIOMARKERS OF AUTOPHAGY, APOPTOSIS, AND PRO-INFLAMMATORY CYTOKINES IN THE PATIENT'S BLOOD SERUM IN THE DYNAMICS OF THE ACUTE PERIOD OF IS

Index	Groups of examined persons								
	I group	II group	III group	I group	II group	III group	I group	II group	III group
	1st day			7th day			14th day		
LC3, ng/L	180.9*	250.3**	89.3	215.6**	309.8**	–	198.5*	274.6**	–
Beclin-1, ng/L	150.6*	190.8*	75.6	199.8*	232.5**	–	170.6*	201.4**	–
p62, pg/mL	36.8*	21.9*	11.4	33.5*	40.9**	–	25.4*	37.8**	–
P53, U/mL	31.2***	19.9**	1.5	16.5**	23.5**	–	9.8*	15.9**	–
Bcl-2, ng/mL	6.6*	10.1**	1.8	11.3**	18.9**	–	19.4**	26.5***	–
TNFα, pg/mL	28.9**	65.3***	6.4	19.3*	28.8*	–	15.4*	21.5**	–
IL-1β, pg/mL	31.8**	54.6***	3.9	20.4**	30.6***	–	10.3*	19.8**	–
IL-8, pg/mL	40.5**	80.9***	12.8	31.1*	60.3**	–	25.4*	42.6***	–
IL-18, pg/mL	406.7*	894.9***	158.9	301.3*	503.9**	–	209.3*	418.4**	–

Note. I group, moderate course of the disease (n = 59); II group, severe course of the disease (n = 27); III group, control (n = 56). *, differences in the studied indicator with the control group are statistically significant ($p < 0.05$); **, differences in the studied indicator with the control group are statistically significant ($p < 0.01$); ***, differences in the studied indicator with the control group are statistically significant ($p < 0.001$).

The data obtained show that in the acute period of IS, there is a sharp increase in the concentration of the studied pro-inflammatory cytokines, which is most pronounced in patients with a severe course of the disease (group II), which, combined with a sharp increase in CRP, can be a criterion for a general inflammatory response. It is known that the pro-inflammatory cytokines IL-1β, IL-8 and TNFα, a sharp increase in which was revealed as a result of the study, play a key role in the development of the acute phase response to inflammation [5, 15]. Considering that the acute phase protein CRP is increased by 44.2 times in the group with severe IS and by 18.98 times in patients with a moderate course of the disease, it is probably possible to assume a systemic nature of inflammation in the acute period of IS, which is consistent with literature data [14].

As can be seen from the data presented in Tables 1 and 2, in both groups of patients there is a dynamic decrease in the concentration of inflammatory biomarkers. However, even by the 14th day, the median values of inflammation indicators are statically significantly increased compared to the control group, which indicates an ongoing inflammatory response. According to the literature, the postischemic inflammatory response, on the one hand, is aimed at removing necrotic tissue from the ischemic zone, pursuing protective goals. On the other hand, it leads to an increase in the volume of the lesion and aggravates the disease. The initial damage to neurons occurs within a few minutes after an ischemic attack, while the inflammatory response that contributes to the progression of the pathology can last from several days to several months [12].

The study found a statistically significant increase in biomarkers of apoptosis and autophagy compared to the control group, most pronounced in severe stroke (Table 1). According to the literature, this indicates that ischemic attack-induced activation autophagy acts in conjunction with apoptosis in the early stages of the acute period of IS, contributing to the progression of the disease. Of interest is the multidirectional dynamic change in the biomarkers of autophagy and the apoptosis inducer p53 protein. As can be seen from Table 1, the maximum values of the p53 protein concentration are observed on the 1st day after a stroke, with a further clearly defined downward trend.

These results are consistent with the literature data that the apoptotic cascade is triggered in the penumbra 1-2 hours after the development of ischemia and reaches its maximum activity on the 3rd day. In the future, with properly prescribed therapy, the process of apoptosis declines [8]. Against this background, as the results show, all studied biomarkers of autophagy reach maximum values on the 7th day with a slight downward trend on the 14th day of the study (Table 1). In addition, there is a dynamic increase in the concentration of the apoptosis inhibitor Bcl-2, reaching maximum values on the 14th day. Probably, a gradual increase in the level of serum Bcl-2 is associated with the time required for the activation of compensatory anti-apoptotic processes.

To identify the relationship between autophagy activity in the dynamics of the acute period of IS, indicators of apoptosis and inflammation, the microtubule-associated protein light chain 3 (LC3) protein, which is involved in the formation of autophagosome, was chosen as the studied parameter of autophagy. It is a reliable marker of autophagy, since its content in the studied biological material positively correlates with the number of active autophagosomes, the most important components of the autophagy process [1]. The results of the study of the relationship between the LC3 protein and the studied parameters are presented in Table 3.

A strong direct correlation was found, more pronounced in the group with severe stroke, between the level of autophagy and the concentration of serum protein p53 ($r = 0.74$; $p < 0.05$), CRP ($r = 0.81$;

TABLE 2. CONCENTRATION OF C-REACTIVE PROTEIN (CRP) IN THE PATIENT'S PERIPHERAL BLOOD IN THE DYNAMICS OF THE ACUTE PERIOD OF IS (HIGHLY SENSITIVE METHOD)

Groups of examined persons	Time from onset of stroke		
	1st day	7th day	14th day
	CRP concentration (mg/L)		
I (n = 59)	12.34**	7.65*	5.11*
II (n = 27)	28.75***	15.11*	10.89**
III (n = 56)	0.65	–	–
RI	0.00-1.00		

Note. As for Table 1. RI, abbreviation for the reference interval.

TABLE 3. CORRELATIONS BETWEEN THE CONCENTRATION OF THE KEY AUTOPHAGY BIOMARKER LC3 (ng/L) AND THE LEVEL OF INDICATORS OF APOPTOSIS AND INFLAMMATION IN THE DYNAMICS OF THE ACUTE PERIOD OF IS IN PATIENTS WITH DIFFERENT SEVERITY OF THE DISEASE

Index	Spearman's coefficient (r), p < 0.05					
	Groups of examined patients					
	I	II	I	II	I	II
	1st day		7th day		14th day	
p53, U/mL	0.67	0.74	0.51	0.63	0.39	0.31
Bcl-2, ng/mL	-0.59	-0.68	0.31	0.45	0.58	0.69
TNFα, pg/mL	0.69	0.73	0.42	0.34	0.44	0.36
IL-1β, pg/mL	0.79	0.83	0.31	0.23	-0.39	-0.45
IL-8, pg/mL	0.44	0.56	0.35	0.29	0.36	0.26
IL-18, pg/mL	0.71	0.79	0.33	0.21	-0.33	-0.53
CRP, mg/L	0.65	0.81	0.61	0.74	0.59	0.76

Note. I group, moderate course of the disease (n = 59); II group, severe course of the disease (n = 27). The revealed correlations between the concentration of the LC3 biomarker and the studied parameters are indicated in the table with a lowercase letter "r" (Spearman's coefficient).

$p < 0.05$), and IL-1β ($r = 0.83$; $p < 0.05$), respectively. At the same time, there is a pronounced inverse correlation between the key autophagy biomarker and the apoptosis inhibitor Bcl-2 ($r = -0.68$; $p < 0.05$). These results confirm the literature data on the synergistic destructive effect of autophagy and activation apoptosis at the beginning of the acute period of IS. Of particular interest is the dynamic change in the relationship between autophagy and the level of Bcl-2, which by the end of the observation acquired a pronounced positive character ($r = 0.69$; $p < 0.05$). These results may indicate that timely prescribed therapy inhibits activation and triggers basic autophagy, which performs a neuroprotective function and has a beneficial effect on the outcome of the disease.

The revealed relationship between the autophagy biomarker LC3 and the concentration of cytokines IL-1β and IL-18 draws attention (Table 3). As is known, IL-1β is a universal mediator of post-stroke inflammation [15]. In acute IS, this proinflammatory cytokine activates almost all inflammatory processes: it polarizes immune cells according to the pro-inflammatory phenotype, causes recruitment of leukocytes from the bloodstream, stimulates excessive activation of the NLRP3-inflammasome, leading to the death of neurons and microglia by the pyroptosis mechanism [7, 13]. Experimental animal studies have shown that the use of recombinant human IL-1RA (an antagonist of the IL-1 receptor) reduces the area of brain damage in ischemic stroke and its accompanying symptoms, and also helps to restore lost motor functions [14, 15]. Blocking of IL-1β and NLRP3 receptors is currently considered as a promising approach to limiting inflammation in ischemic stroke [8, 13]. The negative correlations between LC3, IL-1β and IL-18 ($r = -0.45$; $p < 0.05$ and $r = -0.53$; $p < 0.05$, respectively) revealed in our study indirectly confirm the literature data. that autophagy in acute IS plays the role of a negative regulator of NLRP3 inflammation [7].

Conclusion

Thus, the obtained results indicate a clear relationship between autophagy, apoptosis, and neuroinflammation in the pathogenesis of acute IS. Considering the numerous mechanisms by which autophagy affects individual stages of postischemic neuroinflammation, it is reasonable to consider possible ways of its modulation in order to influence certain targets of the inflammatory process. Such targets can be autoreactive T lymphocytes, NLRP3-inflammasome, proteins of signaling complexes that activate NLRP3-inflammation, receptors of some pro-inflammatory cytokines.

The authors declare that there is no conflict of interest and express their gratitude to the staff of the Department of Clinical Biochemistry and Laboratory Diagnostics of the S.M. Kirov, St. Petersburg for cooperation and providing a base for examining patients.

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ГЕНДЕРНЫЕ РАЗЛИЧИЯ СОДЕРЖАНИЯ В СЫВОРОТКЕ КРОВИ МАРКЕРОВ ВОСПАЛЕНИЯ И АКТИВАЦИИ ТРОМБОЦИТОВ У ПАЦИЕНТОВ С ФИБРИЛЛЯЦИЕЙ ПРЕДСЕРДИЙ НЕКЛАПАННОГО ГЕНЕЗА

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Резюме. Распространенность фибрилляции предсердий высока и сопоставима у обоих полов. Такие факторы, как различно экспрессируемые биомаркеры крови у женщин и мужчин могут играть определенную роль в возникновении фибрилляции предсердий и развитии тромботических осложнений.

Цель – исследование маркеров воспаления и активации тромбоцитов у больных с фибрилляцией предсердий неклапанного генеза, получающих антикоагулянтную терапию и имеющих в анамнезе тромботические осложнения и пациентов с фибрилляцией предсердий без тромботических осложнений в зависимости от гендерной принадлежности больных.

В исследование было включено 22 здоровых добровольца и 60 пациентов с диагнозом «фибрилляция предсердий», получающих антикоагулянтную терапию, из них у 21 пациента произошло развитие тромботических осложнений. Исследование содержания в сыворотке крови α2-macroglobulin, hsC-reactive protein, fetuin A, α-1-acid glycoprotein, L-selectin, serum amyloid P, adipsin, platelet factor 4 проводили на FLEXMAP 3D, с использованием диагностических тест-систем Acute Phase Panel 3.

Сравнительное исследование содержания биомаркеров продемонстрировало повышенное содержание С-реактивного белка у мужчин и женщин в обеих группах пациентов с фибрилляцией предсердий; снижение фетуина А и L-селектина в группе женщин с тромбозами по сравнению с женщинами без тромботических осложнений и по сравнению со здоровыми женщинами. Половых различий в содержании фетуина А и L-селектина в группе больных с фибрилляцией предсердий без тромботических осложнений и у здоровых добровольцев не обнаружено. Уровень адипсина не имел половых различий в группе пациентов с фибрилляцией предсердий с тромбозами и у здоровых добровольцев, однако он был значительно повышен у женщин без тромбозов. Содержание тромбоцитарного фактора 4 у женщин в обеих группах пациентов превышает значение данного показателя у здоровых женщин, половых различий в группах у пациентов с фибрилляцией предсердий не обнаружено.

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Низкие уровни фетуина А и L-селектина при одновременном повышении содержания С-реактивного белка и тромбоцитарного фактора 4 приводят к увеличению протромбогенного потенциала и изменению баланса про- и противоспалительных медиаторов в сторону усиления воспаления у пациентов с фибрилляцией предсердий женского пола.

Ключевые слова: фибрилляция предсердий, тромботические осложнения, воспаление, биомаркеры, белки острой фазы, гендерные различия

GENDER DIFFERENCES IN SERUM MARKERS OF INFLAMMATION AND PLATELET ACTIVATION IN PATIENTS WITH NON-VALVULAR ATRIAL FIBRILLATION

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Abstract. The prevalence of atrial fibrillation is high and comparable in both sexes. Such factors as differently expressed blood biomarkers in women and men may play a role in the occurrence of atrial fibrillation and the development of thrombotic complications.

To study markers of inflammation and platelet activation in patients with atrial fibrillation of non-valvular origin, receiving anticoagulant therapy and having a history of thrombotic complications and patients with atrial fibrillation without thrombotic complications, depending on the gender of the patients.

The study included 22 healthy volunteers and 60 patients diagnosed with atrial fibrillation receiving anticoagulant therapy, of which 21 patients developed thrombotic complications. Serum levels of α2-macroglobulin, hsC-reactive protein, fetuin A, α-1-acid glycoprotein, L-selectin, serum amyloid P, adipsin, and platelet factor 4 were studied on FLEXMAP 3D using Acute Phase diagnostic test systems Panel 3.

A comparative study of the content of biomarkers demonstrated an increased concentration of C-reactive protein in men and women in both groups of patients with atrial fibrillation; decrease in fetuin A and L-selectin in the group of women with thrombosis compared with women without thrombotic complications and compared with healthy women. There were no gender differences in the concentration of fetuin A and L-selectin in the group of patients with atrial fibrillation without thrombotic complications and in healthy volunteers. The level of adipsin had no gender differences in the group of patients with atrial fibrillation with thrombosis and in healthy volunteers, however, it was significantly increased in women without thrombosis. The content of platelet factor 4 in women in both groups of patients exceeded the value of this indicator in healthy women; no gender differences were found in the groups of patients with atrial fibrillation.

Low levels of fetuin A and L-selectin, with a simultaneous increase in C-reactive protein and platelet factor 4, lead to an increase of prothrombogenic potential and to a change in the balance of pro- and anti-inflammatory mediators towards increased inflammation in female patients with atrial fibrillation.

Keywords: atrial fibrillation, thrombotic complications, inflammation, biomarkers, acute phase proteins, gender differences

Introduction

Atrial fibrillation (AF) is one of the most common persistent arrhythmias and is known as a risk factor for the development of heart failure, cerebrovascular events, and sudden cardiac death. An increase in mortality associated with the presence of AF is observed in almost all groups of patients: with coronary heart disease, arterial hypertension, and also in individuals with an isolated form of AF [1]. Every year there is more and more data on the gender characteristics of cardiovascular diseases and factors predisposing to their development. The prevalence of atrial fibrillation is high and comparable in both sexes. Atrial fibrillation is associated with a 6-fold increase in the risk of thrombotic complications and stroke and a 2-fold increase in mortality compared with patients with sinus rhythm [1, 11]. It is now well known that men and women with atrial fibrillation differ in most clinical and demographic characteristics. Factors such as sex hormones or differently expressed blood biomarkers in women and men may play a role in the onset of AF.

Structural and electrophysiological changes in AF include many factors, among which inflammation plays an important role, its participation in the initiation, maintenance and progression of AF has been noted. Activation of the blood coagulation system and platelet aggregation is observed during atrial

fibrillation. In recent years, the relationship between thrombosis and inflammation has been actively studied, since it is known that not only inflammation is accompanied by an increase in the activity of the blood coagulation system, but also thrombosis, in turn, leads to the development of inflammation [2].

To date, the assessment of biomarkers is widely used in the diagnosis of myocardial infarction, heart failure, however, studies on gender differences in the composition of biomarkers in atrial fibrillation are not sufficient. Some biomarkers reflect the pathophysiological process of AF development, while others can be used as markers to predict the risk of thromboembolic complications.

Thus, it seems relevant to us to study gender differences in blood biomarkers in patients with atrial fibrillation in order to gain an understanding of the gender specificity of pathophysiological mechanisms in AF and their possible involvement in the development of thrombotic complications (TC).

Purpose of the study: to study markers of inflammation and platelet activation in patients with non-valvular atrial fibrillation, receiving anticoagulant therapy and having a history of thrombotic complications and patients with atrial fibrillation without thrombotic complications, depending on the gender of the patients.

Materials and methods

The study included 22 healthy volunteers and 60 patients over 18 years old with a diagnosis of atrial fibrillation, verified on the basis of clinical guidelines (ESC 2020 recommendations for the diagnosis and treatment of atrial fibrillation), confirmed by ECG, daily ECG monitoring, receiving anticoagulant therapy. Of these, 21 patients (35%) developed thrombotic complications at the background of adequate anticoagulant therapy. In the presence of indications and in the absence of contraindications, endocardial EPS and RFA were performed using the non-fluoroscopic navigation system CARTO 3 EP (Biosense Webster, USA). Exclusion criteria were as follows: contraindications to anticoagulants, chronic heart failure with a pronounced decrease in left ventricular ejection fraction of less than 40% and significant dilatation of the heart cavities, valvular heart disease, pregnant women or women of childbearing age planning a pregnancy at the time of the study, mentally incompetent patients – neurological conditions. All patients gave their written informed consent for inclusion in the study.

Serum lipid testing (total cholesterol, low density lipoproteins (LDL) cholesterol, high density lipoproteins (HDL) and triacylglycerol) was performed by standard methods on an automatic biochemical analyzer Cobas C311 (Roche Diagnostics, USA). Serum α2-macroglobulin (mg/mL), hsC-reactive protein (hsCRP) (mg/L), fetuin A (mcg/mL), α-1-acid glycoprotein (mcg/mL), L-selectin (mcg/mL), serum amyloid P (SAP) (mcg/mL), adipsin (mcg/mL), platelet factor 4 (PF4) (mcg/mL) was carried out on the equipment of the Center for Collective Use "Medical Genomics" of the Tomsk Research Center FLEXMAP 3D, using diagnostic test systems Acute Phase Panel 3 and MILLIPLEX Analyst 5.1 software (Merck KGaA, Milliplex; Darmshdadt) in accordance to the supplier's instructions. The multiplex analysis technology on the Luminex platform (xMAP® technology) is an important tool for the simultaneous quantification of a complex of different biomarkers in a single sample. The possibilities of xMAP® technology allow performing simultaneous quantitative analysis of a complex of 8 biochemical analytes contained in one blood serum sample of patients with AF and healthy volunteers with high sensitivity. Statistical processing of the obtained results was carried out using the Statistica 10.0 software (StatSoft Inc., USA). Quantitative data are presented medians and 25^{th}; 75^{th} percentiles ($Q_{0.25}$-$Q_{0.75}$), categorical data – in the form of n, % (the number of patients with this characteristic, the proportion of their frequency in the group). The statistical significance of differences between two independent quantitative variables was assessed using the Mann–Whitney U test. Differences were considered statistically significant at $p < 0.05$. Spearman's rank correlation coefficient (Spearman R) was used for correlation analysis.

Results and discussion

All patients were divided into groups: group 1 – patients with atrial fibrillation without thrombotic complications, group 2 – patients with atrial fibrillation with thrombotic complications. Within the group patients were divided by gender – group A – women and group B – men. The patients of the studied groups were comparable by age, gender, functional class of CHF, presence of coronary artery disease, arterial hypertension. The main clinical parameters and medical history of the patients of the study groups are presented in Table 1. Thrombotic complications (TC) in AF without damage to the valvular apparatus of the heart among all patients included in the study were as follows: thrombosis of the left atrial appendage was noted in 10 patients (17%), spontaneous echocontrast II degree in 5 patients (8.3%), cardioembolic stroke – in 3 patients (5%), thrombosis of peripheral arteries – in 1 (2%), thrombosis on the pacemaker electrodes – 2 (4%). The patients of the study groups were also comparable in terms of the frequency of use of the main groups of medications. Prescribed therapy, at the time of inclusion in the study, complied with contemporary recommendations and included standard conventional antiarrhythmic and anticoagulant therapy, as well as therapy for the underlying cardiovascular disease (beta-blockers, statins, angiotensin-converting enzyme inhibitors, diuretics), detailed characteristics are presented in Table 2.

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS WITH ATRIAL FIBRILLATION

Parameter	Women without thrombotic complications (n = 18)	Men without thrombotic complications (n = 21)	Women with thrombotic complications (n = 10)	Men with thrombotic complications (n = 11)
Age, years, Me ($Q_{0.25}$-$Q_{0.75}$)	66.5 (61.0-71.0)	56.5 (48.0-64.0)	66.0 (63.0-74.0)	68.0 (60.0-73.0)
Left atrium size, mm, Me ($Q_{0.25}$-$Q_{0.75}$)	39.0* (37.0-45.0)	41.0* (40.0-44.5)	45.5 (40.0-48.0)	47.0 (45.0-50.0)
Form of atrial fibrillation, n (%):				
paroxysmal form	8 (44.4)	10 (47.6)	4 (40)	3 (27.3)
persistent form	10 (55.6)	11 (52.4)	6 (60)	8 (72.7)
Arterial hypertension, n (%)	13 (72.2)	14 (66.7)	2 (20)	1 (9.1)
Coronary heart disease, n (%)	11 (61.1)	10 (47.6)	5 (50)	9 (81.8)
Functional class of heart failure (NYHA), n (%)				
I	5 (27.8)	4 (19.0)	1 (10)	1 (9.0)
II	9 (50)	8 (38.1)	–	4 (36.4)
III	–	2 (9.5)	–	–
Thrombotic complications				
Left atrial auricular thrombosis, n (%)	–	–	2 (20)	8 (72.7)
Spontaneous echocontrasting, n (%)	–	–	5 (50)	–
Right atrial thrombosis, n (%)	–	–	1 (10)	–
Cardioembolic stroke, n (%)	–	–	–	3 (27.3)
Right ventricular electrode thrombus, n (%)	–	–	–	2 (18.2)
Peripheral artery thrombosis, n (%)	–	–	–	2 (18.2)
Total cholesterol, mmol/L Me ($Q_{0.25}$-$Q_{0.75}$)	5.28* (3.91-6.19)	4.10 (3.43-4.72)	3.99 (3.32-4.77)	3.30 (2.99-4.45)
Triacylglycerol, Me ($Q_{0.25}$-$Q_{0.75}$)	1.29 (1.04-1.69)	1.09 (0.87-1.31)	1.12 (0.90-1.34)	1.12 (0.86-1.31)
HDL cholesterol, mmol/L, Me ($Q_{0.25}$-$Q_{0.75}$)	1.39 (1.12-1.56)	1.07 (1.01-1.22)	2.25 (1.50-2.95)	1.51 (1.23-3.00)
LDL cholesterol, mmol/L, Me ($Q_{0.25}$-$Q_{0.75}$)	3.46* (2.14-4.06)	2.43* (1.95-2.92)	1.24 (1.06-1.51)	1.11 (0.88-1.42)
LDL / HDL, Me ($Q_{0.25}$-$Q_{0.75}$)	2.43 (1.92-2.98)	2.06* (1.79-2.73)	3.06 (3.06-3.06)	1.34 (0.80-2.47)

Note. Data are presented as the % and median Me ($Q_{0.25}$-$Q_{0.75}$) for continuous non-normally distributed variables. *, statistical significance of differences between groups of patients with atrial fibrillation without vs with thrombotic complications ($p < 0.05$).

TABLE 2. MEDICATION THERAPY AT THE TIME OF THE STUDY

Parameter	Patients without thrombotic complications Group1 (n = 39)	Patients with thrombotic complications Group2 (n = 21)
Anticoagulant therapy:		
Warfarin, n (%)	8 (20)	8 (38)
Xarelto, n (%)	19 (48)	5 (24)
Pradaxa, n (%)	1 (2)	1 (5)
Eliquis, n (%)	11 (28)	8 (38)
Antiaggregant therapy:		
Aspirin, n (%)	–	2 (9)
Clopidogrel, n (%)	–	2 (9)
None, n (%)	39 (100)	15 (71)
Statins, n (%)	33 (85)	13 (85)
Beta-blockers, n (%)	12 (31)	13 (62)
ACE inhibitors	25 (64)	17 (81)
Diuretics	5 (13)	3 (14)
Antiarrhythmic therapy:		
Propanorm, n (%)	3 (8)	1 (5)
Cordarone, n (%)	6 (15)	7 (33)
Sotalex, n (%)	5 (13)	4 (19)

Note. Data are presented as the absolute value; (%).

A comparative study of the concentration of biomarkers in men and women in groups of patients and in healthy volunteers showed that both men and women in both groups of patients with AF had an increased concentration of C-reactive protein, which is a sensitive and specific laboratory marker of inflammation and tissue damage, the data are presented in Table 3. However, there was no difference in CRP concentration between men and women in groups of patients with AF and in healthy volunteers. When determining the concentration of α2-macroglobulin and α-1-acid glycoprotein in blood no gender differences were found, and their concentration in patients with AF did not differ from the level in the group of healthy volunteers. A decrease in fetuin A and L-selectin concentration was noted in blood serum in the group of women with thrombosis compared to women from the group without thrombotic complications and compared to healthy women. At the same time, in the group with thrombotic complications, the levels of fetuin A and L-selectin in women were reduced compared to men. The concentration of L-selectin in women without thrombotic complications exceeded the value of this indicator compared to healthy women. There were no gender differences in the content of fetuin A and L-selectin in the group of patients with AF without TC and in healthy volunteers.

In women, both from group without TC, and from the group with thrombosis, a reduced concentration of serum amyloid P was observed compared to men. In healthy volunteers there were no gender differences, the data were not statistically significant. One of the key adipokines that have a multidirectional effect on metabolic processes is adipsin secreted by fat cells. Adipsin acts as a regulator of carbohydrate and lipid metabolism possesses the same activity as complement factor D, which is necessary for the normal f activation of the alternative pathway of complement system. Due to this fact, adipsin acts as a link between the energy block of the endocrine system and the humoral block of the immune system [6, 9].

The data of our study demonstrated that the level of adipsin had no gender differences in the group of patients with atrial fibrillation with thrombosis and in healthy volunteers, however, it was significantly increased in women in the group without thrombosis compared to men from the same group, and also compared to women both from the group with thrombosis and the group of healthy volunteers. The proportion of patients included in the study and taking statins to correct lipid metabolism disorders was 87%. Statins are known to have pleiotropic effects: anti-inflammatory, antioxidant, cardioprotective, and antiarrhythmic. They participate in the normalization of body weight and hormonal levels, and lead to an improvement of endothelial function. The antithrombotic effect of statins is realized through the increase of thrombomodulin expression [8, 12]. Of all the examined patients, pronounced disorders of lipid metabolism, in the form of an increase in the content of total cholesterol and LDL cholesterol, were noted in women in the group without thrombosis.

The molecules associated with the cellular link of hemostasis include platelet factor 4, which is released into the plasma from platelet α- granules

TABLE 3. COMPARATIVE ANALYSIS OF ACUTE PHASE PROTEINS IN WOMEN AND MEN

	Patients without thrombotic complications Group 1 (n = 39)		Patients with thrombotic complications Group 2 (n = 21)		Healthy volunteers (n = 22)	
Parameters	**Group A (n = 18)**	**Group B (n = 21)**	**Group A (n = 10)**	**Group B (n = 11)**	**Group A (n = 11)**	**Group B (n = 11)**
hsC-reactive protein (mg/L)	26.33* (14.62-54.5)	27.96* (13.91-41.93)	23.961* (0.00-58.64)	29.01* (14.40-71.76)	16.716 (1.19-21.42)	11.61 (8.162-15.470)
α2-Macroglobulin (mg/mL)	3.81 (2.78-4.94)	3.51 (2.99-4.56)	2.86 (2.58-3.43)	3.55 (2.80-4.98)	2.71 (2.67-4.88)	3.16 (2.25-5.25)
α-1-Acid glycoprotein (mcg/mL)	4.28 (3.20-7.93)	4.19 (3.10-5.27)	3.89 (2.68-4.73)	3.48 (2.62-4.31)	4.16 (2.17-5.13)	3.86 (2.91-4.67)
Fetuin A (mcg/mL)	514.58 (396.12-689.14) ***	444.75 (337.28-623.06)	281.27 (169.720-311.245) * ** ***	375.229 (14.40-71.76)	412.33 (241.8028-478.2000)	448.95 (308.25-597.43)
L-selectin (mcg/mL)	3.075 (2.59-4.08) * ***	2.70 (1.82-3.47)	1.65 (1.09-1.95) * ** ***	2.04 (1.83-2.62)	2.53 (1.781-4.750)	2.54 (1.83-3.32)
Serum amyloid P (mcg/mL)	19.69 (15.93-35.52) ***	24.325 (14.24-31.97)	12.62 (10.63-17.05)	20.21 (11.67-25.14)	23.58 (9.30-27.93)	24.45 (13.83-28.12)
Adipsin (mcg/mL)	40.59 (31.20-47.87) * ** ***	27.83 (20.44-36.18)	32.745 (23.95-40.24)	33.43 (26.76-43.92)	27.04 (19.260-40.005)	27.9 (21.33-37.35)
Platelet factor 4 (mcg/mL)	27.70* (21.4-35.5)	23.10 (17.04-36.03)	21.29* (13.11-24.47)	29.81 (12.81-57.30)	13.90** (8.97-21.76)	21.77 (14.67-47.52)

Note. Data are presented as the % and median Me ($Q_{0.25}$-$Q_{0.75}$) for continuous non-normally distributed variables; *, statically significant groups of patients with atrial fibrillation with a group of healthy volunteers; **, statically significant difference between women and men in the group; ***, $p < 0,05$, statically significant within the group 1 vs group 2.

upon their activation. The content of platelet factor 4 in women in both groups of patients exceeded the value of this indicator in healthy women; no gender differences were found in the groups of patients with atrial fibrillation. However, in the group of healthy volunteers, the level of platelet factor 4 in women was lower than in healthy men. PF4 has high antiheparin activity, as well as the ability to potentiate the aggregation of platelets and erythrocytes. Correlation analysis in patients with thrombosis of the left atrial appendage, revealed a positive relationship between hsC-reactive protein with the size of the left atrium ($r = 0.77$, $p \leq 0.05$). Also, in the group of women with atrial fibrillation and with developed TC, there were statistically significant positive correlations of platelet factor 4 with the parameters of ADP and adrenaline-induced platelet aggregation ($r = 0.94$ and $r = 0.88$, respectively) $p < 0.05$ and inverse relationship of L-selectin concentration with ADP and adrenaline-induced aggregation ($r = -0.82$ and $r = -0.82$, respectively) $p < 0.05$. The level of fetuin A negatively correlated with adrenaline-induced platelet aggregation ($r = -0.90$, $p < 0.05$). Correlation analysis also demonstrated the existence of a relationship between the studied biomarkers and indicators of lipid metabolism. Thus, in the group of men with thrombosis, the level of fetuin A was negatively correlated with the concentration of total cholesterol in blood serum ($r = -0.64$, $p < 0.05$). The concentration of platelet factor 4 had a negative relationship with the level of HDL cholesterol ($r = -0.90$), and there was also a positive relationship with a high correlation

coefficient between the atherogenic index and the size of the left atrium ($r = 0.94$; $p < 0.05$).

The study revealed an imbalance of pro- and anti-inflammatory mediators, as well as gender differences in the content of inflammatory biomarkers and thrombosis markers in groups of patients with atrial fibrillation, as well as their differences compared to healthy volunteers. The most pronounced changes were found in women with thrombotic complications. It is known that in response to the electrical instability of the myocardium, the nervous and endocrine systems change their properties. Heart rhythm disturbances occur under the influence of various pathological conditions, as a result of which the neurohumoral mechanisms of homeostasis are activated. Since the nervous and endocrine systems complement each other, their restructuring triggers the immune system, which in turn can affect the neurohumoral system [1, 2]. Among the biological and immunological markers used to assess active inflammation, a special role is given to C-reactive protein, the protein of the acute phase of inflammation. In our study, the level of hsCRP was significantly increased in all the patients compared to healthy volunteers. In our opinion, these data coincide with other authors' observation that inflammatory processes play a significant role in the onset, maintenance, and preservation of AF [2].

Most cardiovascular diseases are accompanied by an imbalance between the synthesis of pro- and anti-inflammatory mediators. Fetuin A is a negative acute phase protein, and can be considered as a link between chronic inflammation and cardiovascular diseases. Fetuin A is considered to be an anti-inflammatory mediator involved in macrophage deactivation, antifibrotic activity and inhibition of apoptosis of vascular smooth muscle cells, has a proatherogenic effect, increasing insulin resistance, inhibits the production of pro-inflammatory cytokines TNFα and TGF-b, in the vascular system is an inhibitor of the formation of hydroxyapatites in the vascular system. A decrease in its content in the blood is a risk of cardiovascular calcification [3]. Studies investigating the role of fetuin A in cardiovascular disease provide conflicting results. Thus, it was shown that in patients with metabolic syndrome, fetuin A levels positively correlate with CRP, and higher biomarker levels are associated with an increased risk of myocardial infarction and ischemic stroke [4]. Other studies have shown that low concentrations of fetuin A can increase inflammation and overproduction of cardiotoxic cytokines such as tumor necrosis factor [10]. Studies conducted exclusively on patients with coronary artery disease indicate an association between low levels of fetuin A and mitral aortic calcification and stenosis [3]. In our study, a low level of fetuin A in men with thrombosis was negatively associated with lipid metabolism, and a decrease in the level of fetuin A in women with thrombotic complications negatively correlated with an increase in platelet aggregation activity. The effect of fetuin A appears to differ depending on the patient cohort studied, suggesting the need for further study of its role in various cardiovascular diseases.

L-selectin plays a key role in the adhesion of leukocytes to activated endothelium and their migration across the vascular barrier to the lymphoid tissue or area of inflammation. Participating in the regulation of selectin-dependent activation and adhesion of leukocytes, cell adhesion molecules function in various physiological and pathological processes, including the development of cardiovascular diseases. It is known that the level of soluble L-selectin is affected by inflammatory processes in the vascular wall. Circulating L-selectin maintains functional activity by preventing the interaction of leukocytes with L-selectin ligands on endothelial cells, which confirms the hypothesis that circulating L-selectin plays a protective role in the inflammatory process [5].

This hypothesis is consistent with the decrease in the amount of soluble L-selectin in the blood serum obtained in our study in women with atrial fibrillation and thrombotic complications and a negative relationship between its content and platelet aggregation activity. As is known, platelets take the most important and direct part in the reactions of hemostasis and thrombosis. Platelet activation leads to aggregation and exocytosis of the contents of the granules, the production of immunomodulatory molecules. One of the secreted factors is platelet factor 4, a positively charged glycoprotein of the alpha granule of platelets. An increase of the platelet factor 4 content is one of the markers of intravascular activation of platelet hemostasis [7]. Thus, inflammatory factors can contribute to the onset and maintenance of AF, causing structural and electrical atrial remodeling. An increase or decrease in the activity of the interactions between the inflammatory processes and the coagulation system can lead to the fact that coagulation and thrombosis become pathological factors, contributing to the development and progression of diseases.

Conclusion

Our data suggest that low levels of fetuin A and L-selectin, with a simultaneous increase in the content of CRP and platelet factor 4, lead to an increase in prothrombogenic potential and a change in the balance of pro- and anti-inflammatory mediators towards increased inflammation, which plays a role in the pathophysiology of thrombotic events complications in female patients with atrial fibrillation. Thus, our data suggest that the pathophysiological factors for the occurrence of thrombotic complications in atrial fibrillation may differ in women and men. This argues for further detailed and in-depth studies of sex differences in atrial fibrillation to support a gender-based personalized medicine approach.

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Т-РЕГУЛЯТОРНЫЕ ЛИМФОЦИТЫ И ЯДЕРНАЯ ТРАНСЛОКАЦИЯ FoxP3 В РАЗЛИЧНЫХ ДЕПО ЖИРОВОЙ ТКАНИ У ПАЦИЕНТОВ С ИШЕМИЧЕСКОЙ БОЛЕЗНЬЮ СЕРДЦА

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Резюме. Регуляторные Т-лимфоциты (Treg) присутствуют в жировой ткани. Их относительное содержание, а также уровень ядерной транслокации FoxP3 в эпикардиальной и тимус-замещающей жировой ткани остаются неизученными. В то же время свойства резидентных Treg в жировой ткани могут иметь большое значение у пациентов с ишемической болезнью сердца как потенциальный патофизиологический фактор развития атеросклероза. Целью исследования являлось сравнение содержания FoxP3$^+$Treg-лимфоцитов и ядерной транслокации FoxP3 в эпикардиальной, тимусной, подкожной жировой ткани и периферической крови у пациентов с ишемической болезнью сердца. Пилотное исследование включало 11 пациентов с ишемической болезнью сердца, у которых в плановом порядке было проведено аортокоронарное шунтирование после предшествующей селективной коронарографии. Частоту CD4$^+$CD25^{hi}FoxP3$^+$ и CD4$^+$CD25^{lo}FoxP3$^+$ лимфоцитов и уровень ядерной транслокации FoxP3 оценивали методом проточной цитометрии с визуализацией в периферической крови и стромально-сосудистой фракции эпикардиальной, подкожной и тимусной жировой ткани. Доля CD4$^+$CD25^{hi}FoxP3$^+$ и CD4$^+$CD25^{lo}FoxP3$^+$ лимфоцитов была выше в эпикардиальной жировой ткани по сравнению с кровью (в 3 и 5 раз, $p = 0,020$); доля CD4$^+$CD25^{lo}FoxP3$^+$ клеток в подкожной жировой ткани была в 4 раза выше, чем в крови ($p = 0,028$). Уровень ядерной транслокации FoxP3 был максимальным в крови и снижался в эпикардиальной, подкожной и тимусной жировой ткани ($p = 0,020$ как для CD4$^+$CD25^{hi}FoxP3$^+$, так и для CD4$^+$CD25^{lo}FoxP3$^+$ лимфоцитов). Доля CD4$^+$CD25^{lo}FoxP3$^+$ клеток была прямо связана с возрастом в тимусной ($r_s = 0,818$; $p = 0,002$) и обратно пропорционально – в

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эпикардиальной жировой ткани (r_s = -0,618; p = 0,043). Доли $CD4^+CD25^{hi}FoxP3^+$ и $CD4^+CD25^{lo}FoxP3^+$ клеток с ядерной транслокацией FoxP3 в подкожной жировой ткани отрицательно коррелировали с возрастом (r_s = -0,827; p = 0,002 и r_s = -0,648; p = 0,031 соответственно). Доля $CD4^+CD25^{lo}FoxP3^+$ клеток с ядерной транслокацией FoxP3 в тимусной жировой ткани отрицательно коррелировала с соотношением окружности талии и бедер (r_s = -0,700; p = 0,016). Тяжесть атеросклероза была связана только с долей $CD4^+CD25^{lo}FoxP3^+$ клеток в подкожной жировой ткани (r_s = -0,655; p = 0,029). Таким образом, эпикардиальная и подкожная жировая ткань обогащены Treg, но факторы, влияющие на накопление Treg и ядерную транслокацию FoxP3 в этих жировых депо, могут различаться. Полученные результаты в дальнейшем могут быть использованы для персонификации иммуномодулирующей терапии у больных атеросклерозом.

Ключевые слова: эпикардиальная жировая ткань, тимус, Т-регуляторные лимфоциты, FoxP3, субклеточная локализация, атеросклероз, ишемическая болезнь сердца

T REGULATORY LYMPHOCYTES AND FoxP3 NUCLEAR TRANSLOCATION IN VARIOUS ADIPOSE TISSUE DEPOTS IN PATIENTS WITH CORONARY ARTERY DISEASE

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Abstract. T regulatory lymphocytes (Treg) are present is adipose tissue. Their frequency, as well as the level of FoxP3 nuclear translocation, in epicardial and thymus adipose tissue remains unexplored. Properties of adipose-resident Tregs may be of high significance in patients with coronary artery disease as potential pathophysiological factor in the development of atherosclerosis. The aim of the study was to compare frequency of $FoxP3^+$Tregs and FoxP3 nuclear translocation in epicardial, thymus, subcutaneous adipose tissue and peripheral blood in patients with coronary artery disease. A pilot study was conducted in 11 patients with coronary artery disease scheduled for the coronary artery bypass graft surgery after prior selective coronary angiography. Frequency of $CD4^+CD25^{hi}FoxP3^+$ and $CD4^+CD25^{lo}FoxP3^+$ lymphocytes and FoxP3 nuclear translocation were evaluated by imaging flow cytometry in peripheral blood and in stromal vascular fraction of epicardial, subcutaneous and thymus adipose tissue. Frequencies of $CD4^+CD25^{hi}FoxP3^+$ and $CD4^+CD25^{lo}FoxP3^+$ lymphocytes were higher in epicardial adipose tissue compared to blood (3 and 5 times higher, p = 0.020); $CD4^+CD25^{lo}FoxP3^+$ cells frequency in subcutaneous adipose tissue was 4 times higher than in blood (p = 0.028). The level of FoxP3 nuclear translocation was the highest in blood and decreased in epicardial, subcutaneous and thymus adipose tissue (p = 0.020 both for $CD4^+CD25^{hi}FoxP3^+$ and $CD4^+CD25^{lo}FoxP3^+$ lymphocytes). Frequency of $CD4^+CD25^{lo}FoxP3^+$ cells was directly related to age in thymus (r_s = 0.818; p = 0.002), and inversely in epicardial adipose tissue (r_s = -0.618; p = 0.043). Frequencies of $CD4^+CD25^{hi}FoxP3^+$ and $CD4^+CD25^{lo}FoxP3^+$ with FoxP3 nuclear translocation in subcutaneous adipose tissue negatively correlated with age (r_s = -0.827; p = 0.002 and r_s = -0.648; p = 0.031, respectively). Frequency of $CD4^+CD25^{lo}FoxP3^+$ cells with FoxP3 nuclear translocation in thymus adipose tissue negatively correlated with waist-to-hip ratio (r_s = -0.700; p = 0.016). The severity of atherosclerosis was related only to the frequency of $CD4^+CD25^{lo}FoxP3^+$ cells in subcutaneous adipose tissue (r_s = -0.655; p = 0.029). Thus, epicardial and subcutaneous adipose tissue are enriched with Tregs, but factors that influence Treg accumulation and FoxP3 nuclear translocation in these fat depots may be different. The obtained results may further be used for personalized immunomodulatory therapy in patients with atherosclerosis.

Keywords: epicardial adipose tissue, thymus, T regulatory lymphocytes, FoxP3, subcellular localization, atherosclerosis, coronary artery disease

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Introduction

Adipose tissue plays one of the key roles in the regulation of metabolism, inflammation and endocrine functions through a finely tuned system of adipokines, chemokines, intercellular interactions both between adipocytes and between cells of the stromal vascular fraction of adipose tissue, composed of endothelial cells, pericytes, lymphocytes, monocytes, dendritic cells, and adipose-derived stromal stem cells [15]. Various fat depots have been demonstrated to possess unique properties and some of them may be directly involved in the pathogenesis of atherosclerosis. Epicardial adipose tissue (EAT) is a unique fat depot situated between the myocardium and the epicardium, and its dysfunction is associated with the development of coronary artery disease (CAD) [8]. Replacement of thymus with adipose tissue (thymus adipose tissue, TAT) takes place since young adulthood and continues throughout the lifetime. The development of atherosclerosis may be interconnected with the decline of thymus function, but the pathophysiology of this process remains unexplored [3].

T regulatory lymphocytes (Tregs) represent an important cell population in adipose tissue, regulating development of the local and systemic inflammation and maintaining insulin sensitivity in adipose tissue [15]. Decrease of adipose-resident Tregs was associated with increase of inflammatory cytokine production and adipose tissue dysregulation. Tregs in adipose tissue turned out to be primarily of thymus origin and express molecules typical to canonical Tregs, such as CD25, FoxP3, glucocorticoid-induced tumor necrosis factor receptor (GITR), cytotoxic T lymphocyte antigen-4 (CTLA-4), and OX40. The specificity of TCR-receptor was different in adipose-resident Tregs, and they appeared to be highly dependent on the activity of peroxisome proliferator-activated receptor-γ (PPAR-γ) and ST2, a receptor to IL-33 [12]. The majority of data on Tregs in adipose tissue was obtained in animal-studies and requires translation into clinics. Information on Tregs in EAT and TAT is absent.

Translocation of FoxP3 to the nucleus is an obligatory process for the stable suppressive activity of Tregs. Its perinuclear accumulation in cytoplasm was associated with the disruption of Treg regulatory functions [10]. The level of FoxP3 nuclear translocation in adipose tissue remains unexplored. The development of many autoinflammatory disorders was associated with the increase of $CD4^+CD25^{lo}FoxP3^+$ lymphocytes that presumably represent terminal differentiation stage of regulatory T cells [4]. Since atherosclerosis also represents a chronic inflammatory disorder with autoimmune component [11], one may expect increase of $CD4^+CD25^{lo}FoxP3^+$ lymphocytes during CAD as well, but their numbers have never been previously evaluated in adipose tissue of patients with CAD.

The aim of the study was to compare frequency of $FoxP3^+$Tregs in epicardial, thymus, subcutaneous adipose tissue and peripheral blood and explore FoxP3 nuclear translocation in patients with coronary artery disease.

Materials and methods

We have performed a pilot study which included 11 patients with CAD scheduled for the coronary artery bypass graft surgery (CABG) who underwent selective coronary angiography. All the procedures and tests were conducted in accordance with the guidelines of the Declaration of Helsinki and "Rules of Clinical Practice in the Russian Federation", approved by the Order of the Ministry of Health of the Russian Federation. The study's protocol was approved by the Biomedical Ethics Committee of Cardiology Research Institute, Tomsk NRMC (protocol № 210 from February 18, 2021). All patients recruited into the study signed an informed consent.

Exclusion criteria included: acute cardiovascular complications at least 6 months prior to the study (stroke, myocardial infarction, transient ischemic attack); active inflammatory disease other than atherosclerosis; chronic kidney disease class above C3b; decompensated diabetes mellitus; cancer; hematological and autoimmune disorders; change in body weight of more than 3% in the previous 3 months; refusal to participate in the study.

Anthropometric measurements were performed to assess total obesity according to the level of body mass index (BMI) and abdominal obesity according to the size of the waist circumference, hip circumference, and waist-to-hip ratio. The selective coronary coronary angiography was performed on angiographic complex Cardioscop-V and computer system Digitron-3NAC, Siemens (Germany). The severity of atherosclerosis was estimated via calculation of Gensini Score [7].

The basic characteristics of patients are presented in Table 1.

The samples of EAT, TAT and SAT were obtained in the amount 0.2-1.0 g during the CABG surgery. The samples were placed in M1999 medium preheated to 37 °C straight after obtainment and processed not later than 15 minutes after collection. To isolate the stromal-vascular fraction of adipose tissue, samples were minced, and placed into 5 mL of collagenase type I solution (PanEco, Moscow, Russia) 1 mg/mL in Krebs-Ringer buffer (2 mM D-glucose, 135 mM NaCl, 2.2 mM CaCl2·2H2O, 1.25 mM MgSO4·7H2O, 0.45 mM KH2PO4, 2.17 mM Na2HPO4, 25 mM HEPES, 3.5% BSA, 0.2 mM adenosine) at 37 °C for 35-40 minutes. Krebs-Ringer buffer (37 °C) was ad-

TABLE 1. BASIC CHARACTERISTICS OF PATIENTS

Parameter	
Sex (men/women)	10/1
Age, years	66 (58-70)
Patients with hypertension, n (%)	10 (90,9)
Hypertension duration, years	15 (10-17)
Patients with diabetes mellitus type 2, n (%)	4 (36.4)
Duration of diabetes in patients with diabetes mellitus type 2, years	10.5 (8.5-18)
Atherosclerosis severity (Gensini Score, points)	57.0 (30.5-75.0)
Body mass index, kg/m²	28.7 (24.7-31.0)
Waist circumference, cm	100 (96-111)
Waist-to-hip ratio	0.99 (0.96-1.07)
Statins intake, n (%)	9 (100)

ded to the digested tissue to neutralize collagenase in 1:1 ratio. The suspension of cells was filtered through the nylon mesh (Falcon™Cell strainer, 100 μm), adipocytes were removed, and the suspension of stromal-vascular cells was centrifuged at 400 g for 5 minutes at 4 °C, filtered through the 70 μm strainer (Falcon™Cell strainer) and centrifuged at 400 g for 5 minutes at 4 °C. The pellet was resuspended in RPMI 1640 containing 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin.

The peripheral blood mononuclear cells (PBMC) were isolated 1-2 days prior to the scheduled CABG surgery using Histopaque 1077 (Sigma Aldrich, USA).

Imaging flow cytometry was used to identify FoxP3$^+$Treg cells both in PBMC and stromal vascular fraction of adipose tissue. For this purpose, cells were stained with anti-CD45APC-Cy 7, anti-CD4 FITC, anti-CD25 PE or anti-CD25 APC (BD Pharmingen, USA). The residual erythrocytes were lysed, cells were fixed, permeabilized with a specialized buffer set (BD Pharmingen, USA) and stained with anti-FoxP3 PE or anti-FoxP3 AF647 (BD Pharmingen, USA). After intracellular staining, cells were fixed and stained with DNA dye 7-actinoaminomycin D (7-AAD, BD Pharmingen, USA).

Cells were acquired on Amnis FlowSight (Luminex, USA) equipped with 488 nm and 642 nm lasers in INSPIRE software (Amnis Corporation, Seattle, USA). Brightfield images were acquired in channel 1. Side scatter was evaluated in channel 6 using 785 nm laser. Frequencies of both CD4$^+$CD25^{hi}FoxP3$^+$ and CD4$^+$CD25^{lo}FoxP3$^+$ lymphocytes were evaluated. Nuclear Localization Wizard was used for analysis of FoxP3 nuclear translocation. Cell subset, positive both for 7-AAD and FoxP3 staining, was identified using inbuilt wizard algorithm, and the degree of cross-correlation between 7-AAD and FoxP3 signals was calculated based on the feature Similarity Morphology. As a result, percentages of cells with nuclear and cytoplasmic FoxP3 localization out of all FoxP3-positive cells were obtained separately for CD4$^+$CD25^{hi}FoxP3$^+$ and CD4$^+$CD25^{lo}FoxP3$^+$ lymphocytes.

Data were analyzed using STATISTICA 10.0 (StatSoft, USA). Shapiro-Wilks test was used for evaluation of the type of distribution of variables in the data set. Results were represented as median and interquartile interval (Me ($Q_{0.25}$-$Q_{0.75}$)). Categorical data were described by absolute (n) and relative (%) frequencies. The Mann-Whitney U-test was used to estimate the significance of differences between groups. Spearman's rank correlation coefficient (r_s) was used to estimate relationships between the variables. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

We revealed that EAT had the highest frequency of CD4$^+$CD25^{hi}FoxP3$^+$ cells, which exceeded median values in blood by 3 times approximately, and SAT CD4$^+$CD25^{hi}FoxP3$^+$ cell frequency also tended to increase (Figure 1). As for CD4$^+$CD25^{lo}FoxP3$^+$ cells, their frequency was also the highest in EAT, exceeding peripheral blood frequency by 5 times, followed by the frequency of CD4$^+$CD25^{lo}FoxP3$^+$ cells in SAT, which was 4 times higher than blood median value (Figure 1). Frequencies of both CD4$^+$CD25^{hi}FoxP3$^+$ and CD4$^+$CD25^{lo}FoxP3$^+$ lymphocytes in TAT were comparable to the blood (Figure 1).

Frequency of CD4$^+$CD25^{hi}FoxP3$^+$ cells with FoxP3 nuclear translocation, on the contrary, was lower in all the studied fat depots compared to the blood (Figure 1). Of note, in TAT frequency of CD4$^+$CD25^{hi}FoxP3$^+$ lymphocytes was higher compared to EAT, and tended to increase compared to SAT (Figure 1). Frequency of CD4$^+$CD25^{hi}FoxP3$^+$ lymphocytes with FoxP3 nuclear translocation was also lower in TAT, EAT and SAT compared to blood (Figure 1).

There were no correlations between frequencies of FoxP3$^+$ cells in fat depots and FoxP3$^+$ cells in the blood. Meanwhile, the level of FoxP3 nuclear translocation in EAT correlated with the level of FoxP3 nuclear translocation in TAT in CD4$^+$CD25^{hi}FoxP3$^+$ lymphocytes ($r_s = 0.843$; $p = 0.001$), while the level of FoxP3 nuclear translocation in SAT correlated with the level of FoxP3 nuclear translocation in TAT in CD4$^+$CD25^{lo}FoxP3$^+$ lymphocytes ($r_s = 0.878$; $p < 0.001$); these indicate the common regularities of FoxP3$^+$ cell functioning in adipose tissue, and may be explained by the primarily thymus origin of adipose-resident Tregs [15]. The question remains whether FoxP3$^+$ cells in thymus adipose tissue represent newly

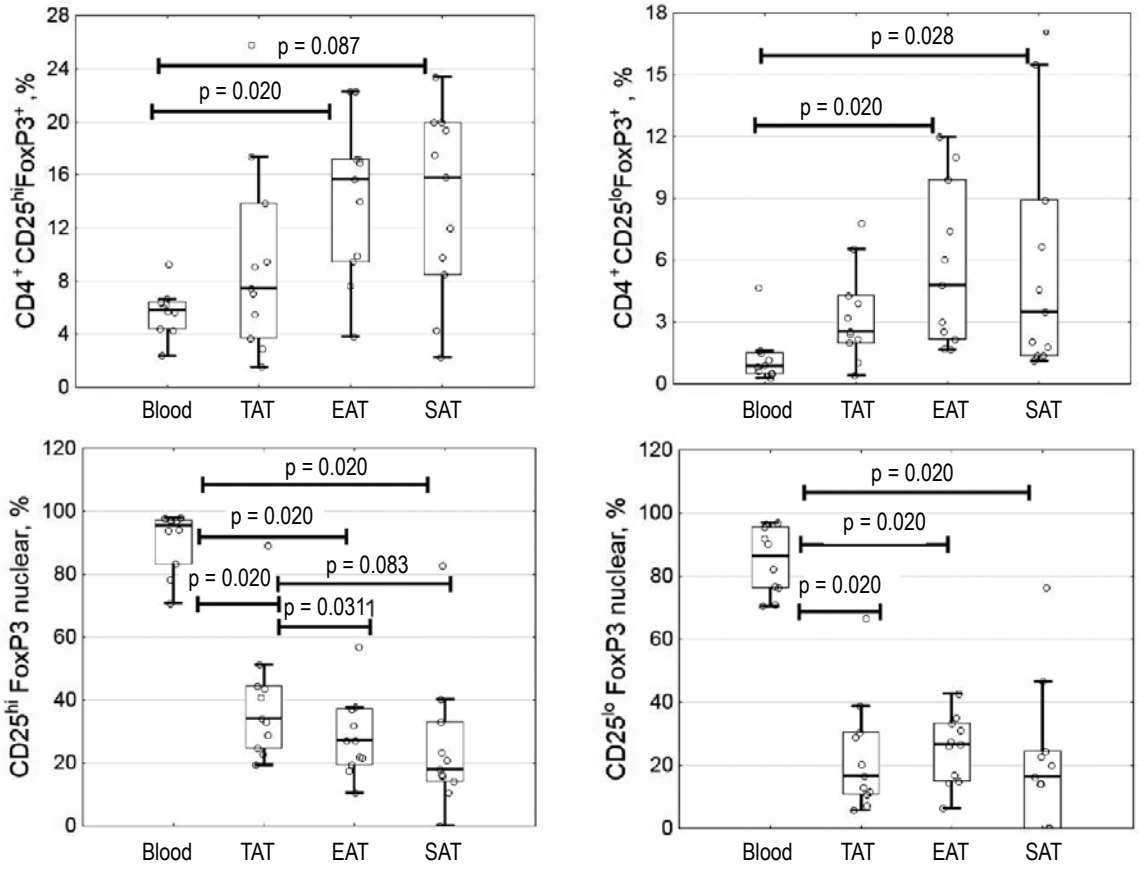

Figure 1. Frequency of T regulatory lymphocytes and frequency of cells with FoxP3 nuclear translocation in peripheral blood and various fat depots

Note. TAT, thymus adipose tissue; EAT, epicardial adipose tissue; SAT, subcutaneous adipose tissue; FoxP3 nuclear, cells with FoxP3 nuclear translocation; indicated p values are after Bonferroni correction.

developed thymic Tregs, or belong to the subset of recirculating Tregs, which could have migrated from the periphery and possess the capacity to suppress *de novo* Treg production [9]. Detection of other cell markers, such as CD31, that was not possible at this stage of work, will be required in future.

Since nuclear translocation of FoxP3 is obligatory for Treg suppressive function, predominant cytoplasmic localization of FoxP3 may display the decreased functional capacity of adipose-resident Treg-cells in CAD patients. However, adipose Tregs have been shown to be highly dependent on activity of PPAR-γ. In adipose tissue, PPAR-γ in conjunction with FoxP3 regulates the transcriptional activity of Treg genes, and mediates Treg responses to the metabolic changes in microenvironment [14]. Hence lower nuclear translocation of FoxP3 in adipose-resident Tregs revealed in our study may be counterbalanced by PPAR-γ activity. This hypothesis requires confirmation in further studies.

Frequency of $CD4^+CD25^{lo}FoxP3^+$ cells was directly related to age in TAT ($r_s = 0.818$; $p = 0.002$), and inversely – in EAT ($r_s = -0.618$; $p = 0.043$). In SAT, we revealed negative correlation between age and frequency of both $CD4^+CD25^{hi}FoxP3^+$ cells and $CD4^+CD25^{lo}FoxP3^+$ cells with FoxP3 nuclear translocation ($r_s = -0.827$; $p = 0.002$ and $r_s = -0.648$; $p = 0.031$, respectively) (Figure 2). The first observation that numbers of Treg cells in adipose tissue depend on age was received in mice. Increase of Tregs was observed between 5 and 25 weeks, then dropped significantly at the age of 40 weeks [2]. According to our data, age influences distribution of human $FoxP3^+$ cells among various fat depots unequivocally. Of note, we did not observe dependence of $CD4^+CD25^{hi}FoxP3^+$ cell numbers upon age, probably due to the small sample size.

Frequency of $CD4^+CD25^{lo}FoxP3^+$ cells with FoxP3 nuclear translocation in TAT negatively correlated with waist-to-hip ratio ($r_s = -0.700$; $p = 0.016$) (Figure 2). Waist-to-hip ratio represents a surrogate marker of visceral adiposity [5]. According to the findings of Yang H. et al., the degree of adiposity in thymus appeared to be inversely related

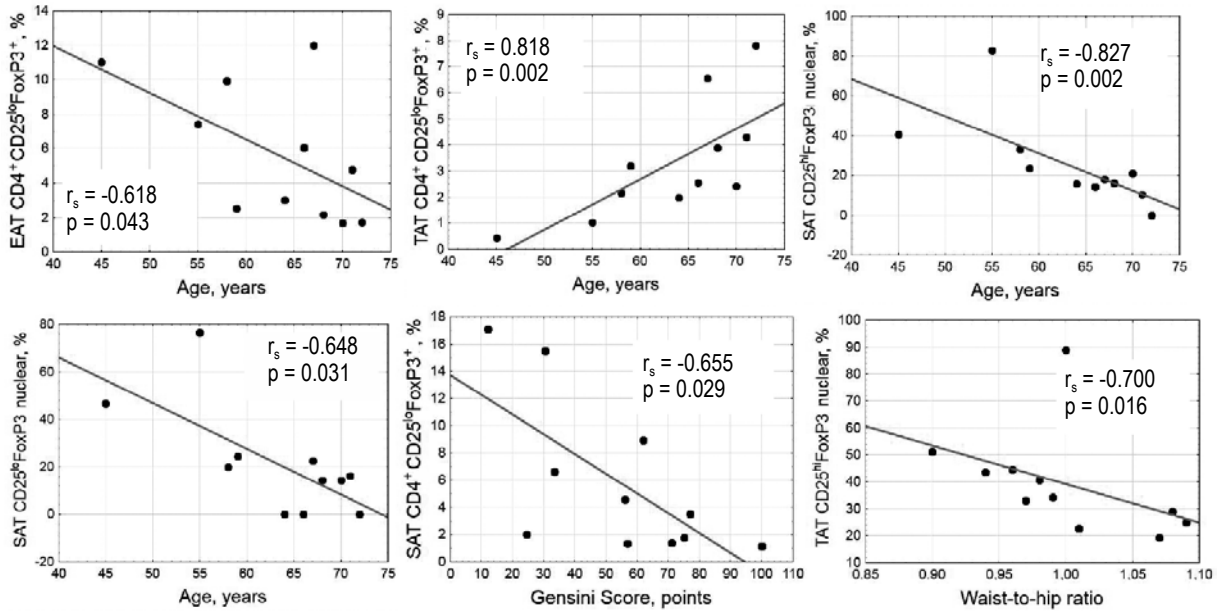

Figure 2. Correlations between basic patient characteristics and frequency of FoxP3⁺ cells in thymus, epicardial and subcutaneous fat depots

Note. TAT, thymus adipose tissue; EAT, epicardial adipose tissue; SAT, subcutaneous adipose tissue; FoxP3 nuclear, cells with FoxP3 nuclear translocation.

to the preservation of thymic immune function [13]. Our results support the idea that thymic regulatory potential may depend on the degree of systemic obesity as well.

The severity of atherosclerosis was related only to the frequency of $CD4^+CD25^{lo}FoxP3^+$ cells in SAT (r_s = -0.655; p = 0.029) (Figure 2). The ability of Tregs to inhibit the development of atherosclerosis have been demonstrated in multiple studies [6]. Usually, visceral or ectopic adipose tissue (such as omental, or, in our case, epicardial adipose tissue) are attributed the major significance in modulation of the cardio-metabolic health compared to subcutaneous fat [8]. Meanwhile, recently it was demonstrated that activation of NLRP3 in SAT is associated with the severity of coronary atherosclerosis and CAD development [1]. Our data indicate that Treg-lymphocytes might be involved in the development of inflammation in SAT and mediate its interconnection with the development of coronary atherosclerosis.

Conclusion

Thus, in our study we demonstrated for the first time, that epicardial and subcutaneous adipose tissues of patients with coronary artery disease are highly enriched with $CD4^+CD25^{hi}FoxP3^+$ and $CD4^+CD25^{lo}FoxP3^+$ lymphocytes, while nuclear translocation of FoxP3 in adipose-resident Tregs is diminished compared to blood. Age is an important factor of modulation of both adipose Treg frequency and the degree of FoxP3 nuclear translocation, while the severity of atherosclerosis is primarily interconnected with FoxP3 nuclear translocation in subcutaneous adipose tissue. The obtained results may further be used for personalized immunomodulatory therapy in patients with atherosclerosis.

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ЛАБОРАТОРНЫЙ БИОМАРКЕР ГАЛЕКТИН-3 В ДИАГНОСТИКЕ ВОСПАЛИТЕЛЬНЫХ ИЗМЕНЕНИЙ МИОКАРДА У ПАЦИЕНТОВ С ФИБРИЛЛЯЦИЕЙ ПРЕДСЕРДИЙ

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Резюме. В настоящее время фибрилляция предсердий (ФП) является одним из наиболее распространенных нарушений сердечного ритма. Многочисленные данные свидетельствуют о значительном вкладе воспалительных изменений миокарда в развитии и прогрессировании ФП. Поиск новых лабораторных биомаркеров, позволяющих оценить активность воспалительных процессов в миокарде, а также изучение их диагностической значимости для неинвазивной диагностики у пациентов с ФП представляется актуальным. В этой связи целью работы явилось изучить особенности сывороточного содержания биомаркера Гал-3 и выявить его взаимосвязь с воспалительными изменениями миокарда у пациентов с ФП. В зависимости от результатов гистологических исследований пациенты были разделены на 2 группы: 1-я группа – с морфологически верифицированным активным лимфоцитарным миокардитом (АЛМ), 2-я – с признаками лимфоцитарной инфильтрации (ЛИ). Анализ частоты выявления и степени выраженности воспалительного процесса в миокарде показал, что активность 4-5 баллов обнаружена только в группе 1, большинство пациентов в группе 2 имели степень активности воспалительного процесса 1 балл. У всех пациентов с ЛИ показано слабое интерстициальное воспаление. В группе с АЛМ регистрировали умеренное и выраженное интерстициальное воспаление, по результатам ИГХ исследования обнаружено высокое количество клеток $CD3^+$ и $CD45^+$ в сравнении с группой 2 ($p < 0,001$). Не выявлено значимых межгрупповых отличий сывороточного уровня Гал-3. При этом в группе 1 показано значимое снижение Гал-3 через 6 месяцев после аблации ($p = 0,028$). У пациентов с АЛМ выявлены положительные корреляции Гал-3 с такими критериями миокардита, как степень выраженности воспалительного процесса, вовлеченность эндокарда. У пациентов группы 1 показана ассоциация сывороточного содержания Гал-3 с уровнем $CD68^+$ ($R = 0,48$ $p = 0,030$). В группе 2 выявлена корреляция между уровнем Гал-3 через 6 месяцев после РЧА с инфильтрацией $CD45^+$ клетками ($R = 0,69$ $p = 0,003$). Таким образом, у пациентов с ФП и признаками активного

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лимфоцитарного миокардита установлены значимые ассоциации между биомаркером Гал-3 и показателями воспалительных изменений в миокарде, что подтверждает важную роль Гал-3 в качестве участника воспалительного процесса.

Ключевые слова: фибрилляция предсердий, воспалительные изменения, миокардит, инфильтрация, лабораторная диагностика, галектин-3

LABORATORY BIOMARKER GALECTIN-3 IN THE DIAGNOSTICS OF MYOCARDIAL INFLAMMATORY CHANGES IN PATIENTS WITH ATRIAL FIBRILLATION

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Abstract. Atrial fibrillation (AF) is one of the most common cardiac arrhythmias. Numerous data indicate a significant contribution of myocardial inflammatory changes in the development and progression of AF. The search for new laboratory biomarkers to assess the activity of myocardial inflammatory processes, and the study of their diagnostic significance for noninvasive diagnosis in patients with AF is relevant. Therefore, the aim was to study the features of the serum level of the biomarker Gal-3 and to identify its relationship with inflammatory changes in the myocardium in patients with AF. Depending on the results of histological studies, the patients were divided into 2 groups: group 1 – with morphologically verified active lymphocytic myocarditis (ALM), group 2 – with lymphocytic infiltration (LI). Analysis of the frequency of detection and severity of the inflammatory process in the myocardium showed that activity of 4-5 scores was detected only in group 1. In 2nd group, activity of the inflammatory process in most patients was 1 score. All patients with LI mild interstitial inflammation were showed. In the ALM group moderate and severe interstitial inflammation was detected. A high number of $CD3^+$ and $CD45^+$ cells were found in 1st group compared to group 2 ($p < 0.001$).

There were no significant intergroup differences in the serum level of Gal-3. At the same time, in 1st group showed a significant decrease in Gal-3 in 6 months after treatment ($p = 0.028$). Positive correlations of Gal-3 with the severity of the inflammatory process and endocardial involvement were revealed in patients with ALM. The association of serum Gal-3 levels with $CD68^+$ levels in 1st group was detected ($R = 0.48$, $p = 0.030$). In 2nd group, a correlation between the level of Gal-3 in 6 months after ablation with infiltration of $CD45^+$ cells was found ($R = 0.69$, $p = 0.003$). Thus, in patients with AF and active lymphocytic myocarditis, significant associations were established between biomarkers of Gal-3 and inflammatory changes in the myocardium. This confirms the important role of Gal-3 as a participant in the inflammatory process.

Keywords: atrial fibrillation, inflammation changes, myocarditis, infiltration, laboratory diagnostics, galectin-3

Introduction

Atrial fibrillation (AF) is one of the most common and clinically significant cardiac arrhythmias. Currently, the prevalence of AF in the adult population ranges from 2 to 4% and increases with age [11]. Despite the rapid development of interventional and pharmacological treatment, the number of arrhythmias is steadily increasing. This increases cardiac mortality, morbidity, duration of hospitalization and overall health system costs.

Currently in population cohort studies have been identified many independent risk factors for AF. Among them, diabetes, arterial hypertension, coronary heart disease, hypertrophic and dilated cardiomyopathy, congestive heart failure and valve defects [15]. However in 3-11% of cases, the etiology of AF cannot be established. In such patients, AF develops in the absence of concomitant diseases, clinical- instrumental, functional and laboratory research methods do not reveal the causes of AF. In these cases they talk about an idiopathic form of AF. The absence of an obvious cause of idiopathic AF can complicate the choice of medical and surgical treatment tactics, reduce the effectiveness

of radiofrequency ablation, and have unpredictable effects on drug therapy [3].

It is known that various pathological processes underlie the occurrence of AF. Numerous clinical and experimental data indicate a significant contribution of inflammatory changes in the myocardium to the initiation, maintenance and progression of AF [7, 10].

Currently various research methods are widely used to diagnose inflammatory changes in the myocardium. Among them instrumental methods of radionuclide imaging and single-photon emission computed tomography with 99mTc-pyrophosphate, laboratory studies of nonspecific markers of inflammation. However, the diagnostic capabilities of these methods in determining signs of myocardial inflammation are limited by insufficient specificity and sensitivity, high cost, and therefore cannot be widely used in everyday clinical practice.

The most accurate diagnostic method is to perform a lifetime endomyocardial biopsy (EMB) [2]. EMB is a reliable method for determining inflammatory myocardial disease based on histological and immunohistochemical studies (IHC) [1]. Despite the obvious advantages of EMB, this method is associated with a number of significant disadvantages, such as limited indications for the procedure and a high risk of complications. In this regard, the search for new methods of noninvasive diagnosis of inflammatory changes in the myocardium is relevant.

An analysis of literature data over the past decade has shown the widespread use of laboratory biomarkers, reflecting various aspects and pathogenetic mechanisms of the development and progression of AF [4, 8]. Recently the role and prognostic value of the biomarker galectin-3 (Gal-3) has been actively studied in AF. Gal-3 is known to induce myocardial fibrosis, enhancing myofibroblast proliferation, extracellular matrix accumulation and macrophage infiltration, stimulating the signaling pathway of transforming growth factor β [13]. Along with this, there is no data on the role of Gal-3 in the diagnosis of myocardial inflammatory changes.

Thus, the search for new laboratory biomarkers for the noninvasive diagnosis of myocardial inflammatory processes and the determination of their diagnostic significance in patients with AF is relevant and necessary for understanding the pathogenetic mechanisms of the development and progression of AF.

Objective: to study the features of the serum level of the Gal-3 and to identify its relationship with myocardial inflammatory changes in patients with idiopathic AF.

Materials and methods

A total of 39 patients with idiopathic AF (41.0±9.2 y. o.) were recruited in the prospective single-center study. All patients underwent radiofrequency ablation (RFA) of the pulmonary veins. Endomyocardial biopsy from the right ventricle with histologic and IHC studies was performed. Histological studies were performed at the light-optical level using an AxioImager M2 Zeiss microscope. Morphological verification of myocarditis was performed in accordance with the modified Dallas criteria. The degree of inflammation activity and the severity of fibrosis were assessed using semi-quantitative histological criteria proposed for assessing morphological changes in inflammatory cardiomyopathy, taking into account the consensus of the European Society of Cardiology for the diagnosis and treatment of myocarditis [10]. An IHC study to determine the immunophenotype of infiltrate cells was performed on paraffin sections. The polyvalent detection system HRP DAB (Spring BioScience) was used to visualize the studied antigens. The calculation of $CD3^+$, $CD45^+$ and $CD68^+$ cells of the infiltrate was carried out taking into account the area of sections in each fragment of the endomyocardium; the number of these cells per 1 mm^2 was calculated.

Venous blood was drawn into sterile vacutainers before surgery (T1) and 6 months after RFA (T2). Obtained blood samples have been stored at room temperature for 30 min and were centrifuged at 3000 rpm for 15 min. The serum samples have been collected and stored at -40 °C until the quantitative analysis was performed. The serum levels of Gal-3 were evaluated using enzyme-linked immunosorbent assay and Human Galectin-3 Platinum ELISA test system (eBioscience, Austria).

The analysis of the obtained data was performed using the STATISTICA 10.0 software. The type of the distribution of the data was evaluated by Kolmogorov–Smirnov test. Results were presented as the median value (Me) and the interquartile range ($Q_{0.25}$-$Q_{0.75}$) in the case of non-normal distribution. The Kruskal–Wallis rank criterion was used for pairwise comparison. Wilcoxon test was used to estimate the significance of differences in the values of the dependent parameters. The Pearson's χ^2-criterion with the Yates correction was used to comparison of results measured in score scales. The Spearman's rank correlation coefficient (R) was calculated to assess the relationship between the parameters. A value of $p < 0.05$ was considered statistically significant in all statistical evaluations.

All patients signed an informed consent forms prior to participation in the study. The protocol of the study was approved by the local ethics committee and it was developed in compliance with the Medical Association Declaration of Helsinki "Ethical principles for medical research involving human subjects" and the "Rules of Clinical Practice in the Russian Federation".

Results and discussion

According to the results of laboratory and instrumental studies, there are no data for organic pathology of the cardiovascular system, as well as inflammatory diseases and conditions that would cause the development of arrhythmia in patients in both groups. Depending on the results of histological and IHC studies the patients were divided into 2 groups: 1 – with active lymphocytic myocarditis (ALM) (n = 22); 2 – with lymphocytic infiltration (IL) (n = 18). Clinical and functional characteristics of the groups were comparable (Table I). A comparative analysis of the frequency of detection and severity of the inflammatory process in the myocardium showed that the number of patients with inflammation activity 0-1 significantly differed between groups (p = 0.035, p = 0.006, respectively). In the 2nd group, activity of the inflammatory process in most patients was 1 score (55.6%). Activity 4-5 scores were detected only in group 1. An equal number of patients in 1st group had a degree of activity of 2 and 3 points (Table 2).

Analysis of the data revealed intergroup differences in the level of interstitial inflammation. All patients with LI mild interstitial inflammation (< 7 T lymphocytes/mm^2) were showed. In the patients with ALM moderate and severe interstitial inflammation was detected. A high number of $CD3^+$ and $CD45^+$ cells were found in the 1st group compared to group 2 ($p < 0.001$). In the 1st group severe (> 14 T lymphocytes/mm^2) interstitial inflammation was recorded in 6 patients (28.6%). In both groups, endocardial involvement was absent in most cases. Also, focal necrosis was registered in most patients: in 9 patients (42.9%) in the 1st group and 11 (61.1%) – in the 2nd group. There were no significant intergroup differences in the level of cardiomyocyte degeneration were not revealed (Table 2).

The results of the IHC study showed significant intergroup differences in infiltration by immunocompetent cells. According to the data obtained, a higher level of $CD3^+$ and $CD45^+$ was detected in the 1st group compared to group 2 ($p < 0.001$) (Table 2).

TABLE 1. CLINICAL AND INSTRUMENTAL CHARACTERISTICS OF PATIENTS DEPENDING ON THE RESULTS OF ENDOMYOCARDIAL BIOPSY

	Group 1 (n = 21)	Group 2 (n = 18)
Age, years	41 (35-49)	41.5 (38-46)
Arrhythmic history, years	4 (2-6)	4.5 (2-9)
Male, n (%) **Female, n (%)**	18 (85.7%) 3 (14.3%)	15 (83.3%) 3 (16.7%)
Arrhythmia form, n (%) **paroxysmal** **persistent** **long-standing persistent**	 5 (23.8%) 7 (33.3%) 9 (42.9%)	 9 (50%) 5 (27.8%) 4 (22.2%)
Left atrium, mm	41 (36-43)	37.5 (34-44)
Right ventricle, mm	25 (21-26)	21.5 (20-26)
End diastolic size, mm	49 (46-54)	50 (48-53)
End systolic size, mm	31 (29-36)	33 (30-37)
Ejection fraction, %	65 (59-67)	65.5 (57-67)
Interventricular septum, mm	10 (9-11)	9 (8-10)
Thickness of the posterior wall of the left ventricle, mm	10 (9-10)	9 (8-10)
End diastolic volume, mL	116 (93-128)	118 (101-128)
End systolic volume, mL	37 (33-50)	42.5 (35-55)
Myocardial mass, g	182 (157-194)	171.5 (135-212)
Myocardial mass index	82 (77-97)	79.5 (69-93)
Stroke volume, mL	70 (61-79)	72 (61-81)
Mean pressure in the right ventricle, mmHg	28 (23.5-31.5)	28.5 (24-31)

TABLE 2. RESULTS OF HISTOLOGICAL, IMMUNOHISTOCHEMICAL AND LABORATORY STUDIES

	Group 1	Group 2	p
Inflammatory process activity, score	n (%)	n (%)	
0	–	5 (27.8)	0.035
1	2 (9.5)	10 (55.6)	0.006
2	5 (23.8)	2 (11.1)	0.541
3	7 (33.3)	1 (5.5)	0.081
4	5 (23.8)	–	0.082
5	2 (9.5)	–	0.538
Interstitial inflammation	n (%)	n (%)	
Weak < 7 T lymphocytes (cells/mm²)	–	18 (100)	0.000
Moderate 7 ≤ 14 T lymphocytes	15 (71.4)	–	0.000
Severe > 14 T lymphocytes	6 (28.6)	–	0.043
Endocardial involvement	n (%)	n (%)	
No detected	13 (61.9)	16 (88.9)	0.119
Availability	8 (38.1)	2 (11.1)	0.119
Necrosis	n (%)	n (%)	
No detected	3 (14.2)	5 (27.8)	0.521
Focal	9 (42.9)	11 (61.1)	0.415
Multifocal	9 (42.9)	2 (11.1)	0.066
CD3⁺	8.0 (5-11)	2.5 (0-5)	< 0.000
CD45⁺	21.0 (14-25)	10.0 (8-14)	0.008
CD68⁺	15.0 (11-22)	14.0 (10-16)	0.496
Galectin-3, ng/mL			
T1	16.8 (14.3-25.7)	17.5 (13.5-22.1)	0.686
T2	15.4 (11.7-18.0)	12.0 (10.1-16.2)	0.141

No intergroup differences were found in infiltration by $CD68^+$.

There were no statistically significant intergroup differences in the serum level of Gal-3 at stages T1 and T2. In the 1st group showed a significant decrease of the Gal-3 level in 6 months after RFA ($p = 0.028$). In the 2nd group the Gal-3level did not differ between stages.

In patients with ALM Gal-3 levels was associated with the severity of myocardial inflammatory processes ($R_{T1} = 0.52$, $p = 0.016$; $R_{T2} = 0.48$, $p = 0.031$) and interstitial inflammation ($R_{T1} = -0.53$, $p = 0.015$). In 1st group Gal-3 levels were significantly higher in patients with the identified criterion of endocardial involvement compared with patients without sign ($p_{T1} = 0.022$, $p_{T2} = 0.049$, respectively). The association of serum levels of Gal-3 with $CD68^+$ levels in 1st group was shown ($R = 0.48$, $p = 0.030$). In patients with LI, a correlation between the level of Gal-3 in 6 months after RFA and the $CD45^+$ cells level was found ($R = 0.69$, $p = 0.003$). There were no correlations between Gal-3 expression and histological criteria in 2nd group. However, positive correlations of infiltration by $CD3^+$ and $CD45^+$ cells with the activity of the inflammatory process and necrosis were revealed ($R = 0.55$, $p = 0.018$). In patients with ALM, there were no correlations between IHC and histological criteria of inflammatory processes in the myocardium.

Numerous clinical and fundamental studies have been devoted to the study of the role of inflammation in the initiation, maintenance and progression of AF. It is believed that the presence of inflammation in the atrial tissue is crucial in the mechanisms of electrical remodeling and structural adjustment, such as a decrease in the conduction velocity, a reduction in the duration of the action potential, as well as an increase in the size of the atrium [8].

It is known that the frequency of myocarditis is from 20 to 30% of all non-coronary heart diseases [1]. Analysis of the literature data showed that, despite the small number of EMB results in patients with AF, all of them indicate a high prevalence of myocarditis. A number of studies have revealed infiltration by immunocompetent cells in the atrial myocardium of patients with isolated AF was detected, and the number of patients with active lymphocytic myocarditis reached 80% [10]. According to our results, histological signs of ALM were detected in 21 patients (53.8%) with idiopathic AF, in other cases LI was detected.

It is known that activation of T lymphocytes in peripheral blood can play an important role in the

pathogenesis of AF. In patients with AF, endocardial infiltrates containing $CD45^+$, $CD3^+$T lymphocytes, $CD68^+$ macrophages were detected [9]. According to the authors, the T cell response mediated by a chronic inflammatory process may be part of the pathogenetic process leading to the development of cardiac arrhythmias. In our study, patients with ALM showed moderate (≥ 7 and < 14 T lymphocytes/mm^2) and severe interstitial inflammation (≥ 14 T lymphocytes/mm^2). In addition, according to the results of IHC studies, it was revealed that the expression of the membrane protein CD3, which is one of the main components of the T cell receptor, was significantly higher in ALM. The expression level of CD45, which is a common leukocyte antigen, was also significantly higher than in the group with LI.

According to our results, in patients with AF, infiltration of a large number of $CD68^+$ macrophages into the atrial tissue was observed without significant intergroup differences. It is known that macrophages ($CD68^+$ cells) regulate the development of inflammatory response and fibrosis, and can support chronic subclinical inflammation, and are involved in the development of autoimmune reactions. CD45 is a common leukocyte antigen. The expression level $CD45^+$ was also significantly higher than in the group with LI. According to our results, patients with AF had infiltration of a large number of $CD68^+$ macrophages into the atrial tissue without significant intergroup differences. As is known, macrophages ($CD68^+$ cells) regulate the development of inflammatory response and fibrosis, can support chronic subclinical inflammation, and are involved in the development of autoimmune reactions.

The histological criterion of endocardial involvement characterizes how deep the infiltration of immunocompetent cells occurs outside the myocardium. In our study, in the group with ALM, signs of endocardial involvement, as well as multifocal necrosis, were detected more often. The presence of individual signs of myocarditis in the 2nd group may be due to the fact that AF itself is a factor provoking the development of inflammation in the atrial myocardium.

It is likely that the identified myocardial inflammatory changes are signs of an ongoing inflammatory process associated with infiltration by immunocompetent cells, and can further lead to structural restructuring, fibrosis, and the formation of new multiple diffusely located arrhythmogenic foci in the left atrium.

The possibilities of diagnosing inflammatory changes in the myocardium are currently limited. Therefore, the search for new laboratory biomarkers that will allow indirectly assess the activity of myocardial inflammatory processes, as well as the study of their clinical and diagnostic significance in patients with AF seems relevant.

To date, there is no information on randomized trials to assess the significance and informativeness of determining biomarkers for myocarditis. According to the clinical guidelines for the diagnosis and treatment of myocarditis [1], in the absence of specific biomarkers, it is recommended to study of C-reactive protein, troponin T and I, the N-terminal fragment of the natriuretic propeptide, cardiac autoantibodies specific to myocardial tissue in the blood serum.

Gal-3 plays an important role in immune and inflammatory responses, regulating homeostasis and immune cells function. Depending on the intracellular or extracellular localization, Gal-3 can play a damaging or protective role. As a regulator of the immune response and T cell activity, Gal-3 participates in the development of autoimmunity mediated T cell [12]. Gal-3 is a well-studied biomarker associated with cardiac remodeling and unfavorable prognosis in heart failure of various etiologies. Ho et al. (2014) have demonstrated that a high level of circulating Gal-3 is associated with an increased risk of AF [6]. Along with this, there are no data on the role of Gal-3 in the diagnosis of inflammatory changes in the myocardium in patients with various forms of rhythm and conduction disturbances.

A number of studies have shown that the level of the Gal-3 in patients with AF is higher than in healthy volunteers [5, 14]. The results of our study did not reveal any differences in the Gal-3 level between patients with ALM and LI. At the same time, only in 1st group a significant decrease in Gal-3 in 6 months after RFA was shown.

According to the literature, there is information about the relationship between the level of Gal-3 in patients with AF and the severity of left atrial fibrosis, established by magnetic resonance imaging [14]. In our work in patients with ALM, positive correlations of Gal-3 with criteria of myocarditis (as the degree of severity of the inflammatory process and interstitial inflammation) were revealed. For the first time in patients with ALM was established a relationship between the serum levels of Gal-3 and the criterion of endocardial involvement.

In an experimental mouse model of acute and chronic myocarditis induced by Coxsackievirus B3, high expression of Gal-3 in macrophages, T cells and fibroblasts was shown using flow cytometry analysis [12]. The positive correlations between the level of Gal-3 and infiltration by immunocompetent cells revealed in our work and confirm the important role of Gal-3 as an active participant in the inflammatory process.

The comparatively small number of the recruited patients may be regarded as the major limitation of

our study. However, all the patients included in our study, underwent rigorous screening to correspond to the strict inclusion and exclusion criteria.

Conclusion

Thus, in patients with AF and signs of active lymphocytic myocarditis, significant associations were established between the galectin-3 biomarker and indicators of inflammatory changes in the myocardium. Further studies are needed to assess the prognostic value of the level of circulating galectin-3 in the diagnosis of myocardial inflammatory changes in AF.

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ЭОТАКСИН И СЕРДЕЧНО-ЛОДЫЖЕЧНЫЙ СОСУДИСТЫЙ ИНДЕКС У ПАЦИЕНТОВ ВЫСОКОГО И ОЧЕНЬ ВЫСОКОГО СЕРДЕЧНО-СОСУДИСТОГО РИСКА

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Резюме. Эотаксин – хемокин, который является хемоаттрактантом преимущественно для эозинофилов, а также базофилов и Th2-лимфоцитов. По данным исследований, сверхэкспрессия эотаксина обнаружена в эндотелиальных и гладкомышечных клетках сосудов в области атеросклеротической бляшки. В клинической медицине широко используется сердечно-лодыжечный сосудистый индекс (CAVI) как индикатор артериосклероза и предиктор сердечно-сосудистых событий. В немногочисленных исследованиях показана взаимосвязь содержания эотаксина с наличием коронарного атеросклероза, тогда как в других исследованиях не было установлено ассоциации содержания в крови эотаксина с атеросклерозом, инфарктом миокарда и скоростью пульсовой волны. Целью настоящего исследования является оценка уровня эотаксина в крови и сердечно-лодыжечного сосудистого индекса и их ассоциация с основными кардиоваскулярными факторами риска у пациентов высокого и очень высокого сердечно-сосудистого риска. Было обследовано 65 пациентов высокого и очень высокого сердечно-сосудистого риска, обусловленного документированной ишемической болезнью сердца, сахарным диабетом 2-го типа или сочетанием кардиоваскулярных факторов риска, и находящихся на общепринятой кардиоактивной, сахароснижающей и липидснижающей терапии. Всем пациентам выполнено исследование эластических свойств сосудистой стенки методом объемной сфигмографии с оценкой индекса CAVI. В крови определяли концентрацию эотаксина, высокочувствительного С-реактивного белка, гликозилированного гемоглобина и показателей липидного спектра. Все обследованные были разделены на две группы: с нормальным значением CAVI (менее 8) и повышенным. Пациенты с повышенным CAVI имели более высокие концентрации эотаксина ($p = 0,013$), общего холестерина ($p = 0,009$), холестерина липопротеинов низкой плотности ($p = 0,016$), а также были старше ($p < 0,0001$) и реже принимали статины ($p = 0,002$). У всех обследованных были выявлены корреляции между концентрацией эотаксина в сыворотке крови и CAVI ($r_s = 0,34$; $p = 0,005$), а также возрастом ($r_s = 0,32$; $p = 0,006$). Возраст пациентов коррелировал с CAVI ($r_s = 0,35$; $p = 0,007$). Таким образом, в нашем исследовании мы впервые показали взаимосвязь высоких концентраций эотаксина с повышенным сердечно-лодыжечным сосудистым индексом у пациентов высокого и очень высокого сердечно-сосудистого риска. Сердечно-лодыжечный сосудистый индекс был ассоциирован

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с возрастом, с показателями липидного обмена и наличием липиднижающей терапии. Полученные результаты позволяют рассматривать эотаксин как фактор, связанный с атерогенезом и артериальной жесткостью.

Ключевые слова: эотаксин, хемокины, артериальная жесткость, сердечно-лодыжечный сосудистый индекс, атеросклероз, факторы риска сердечно-сосудистых заболеваний

EOTAXIN AND CARDIO-ANKLE VASCULAR INDEX IN PATIENTS WITH HIGH AND VERY HIGH CARDIOVASCULAR RISK

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Abstract. Eotaxin is a chemokine, which is a chemoattractant mainly to eosinophils, as well as basophils and Th2 lymphocytes. According to studies, overexpression of eotaxin is found in endothelial and smooth muscle cells of blood vessels in the area of atherosclerotic plaque. In clinical medicine, cardio-ankle vascular index (CAVI) is widely used as an indicator of arteriosclerosis and a predictor of cardiovascular events. Few studies have shown the relationship of eotaxin with coronary atherosclerosis; in other studies, the relationship of eotaxin with atherosclerosis, myocardial infarction and pulse wave velocity was not revealed. The aim of the present study was to assess blood level of eotaxin and cardio-ankle vascular index and their association with major cardiovascular risk factors in patients with high and very high cardiovascular risk. We examined 65 patients with high and very high cardiovascular risk, due to documented coronary artery disease, type 2 diabetes mellitus, or combination of cardiovascular risk factors and who were undergoing generally accepted cardioactive, hypoglycemic therapy and lipid-lowering therapy. All patients were examined for the elastic properties of the vascular wall by volumetric sphygmography with assessment of CAVI. In the blood, the concentrations of eotaxin, high-sensitivity C-reactive protein, glycosylated hemoglobin and lipid spectrum indicators were determined. All examined were divided into two groups: with a normal value of CAVI (less than 8) and elevated. Patients with elevated CAVI had higher concentrations of eotaxin ($p = 0.013$), total cholesterol ($p = 0.009$), low-density lipoprotein cholesterol ($p = 0.016$), were older ($p < 0.0001$) and less likely to take statins ($p = 0.002$). In all those examined, correlations were found between serum eotaxin concentration and CAVI ($r_s = 0.34$; $p = 0.005$), as well as age ($r_s = 0.32$; $p = 0.006$). The age of the patients correlated with CAVI ($r_s = 0.35$; $p = 0.007$). Thus, in our study, we for the first time showed the relationship between higher concentrations of eotaxin and an increased cardio-ankle vascular index in patients with high and very high cardiovascular risk. Cardio-ankle vascular index was associated with age, lipid metabolism and lipid-lowering therapy. The obtained results allow us to consider eotaxin as a factor associated with atherogenesis and arterial stiffness.

Keywords: eotaxin, chemokines, arterial stiffness, cardio-ankle vascular index, atherosclerosis, risk factors for cardiovascular disease

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Introduction

Chemokines are a class of low-molecular cytokines that can induce directed chemotaxis of immune system cells, smooth muscle cells, fibroblasts, and other body cells in response to the activation of receptors associated with G-protein. However, the biological activity of chemokines is not limited to the stimulation of chemotaxis. Stimulation of chemokine receptors can affect proliferation, differentiation, degranulation, respiratory burst in cells; has an effect on vascular permeability and angiogenesis [5]. Eotaxin (CC chemokine ligand 11, CCL11) is a chemokine of class CC, which is a chemoattractant predominantly to eosinophils, as well as basophils and Th2 lymphocytes, activating CCR3 receptors [2, 7]. The detected overexpression of CCR3 and eotaxin mRNA in human atherosclerotic plaques indicates the role of this chemokine in vascular inflammation and atherosclerotic process [4].

In mouse smooth muscle cell culture, eotaxin has been shown to be a potent chemotactic factor for smooth muscle cells, capable of regulating their migration to the atherosclerotic plaque region [7]. Eotaxin has also been found to stimulate the calcification of smooth muscle cells [9]. On the other hand, other studies have found no association between eotaxin levels and the presence of coronary atherosclerosis or prior myocardial infarction [1, 8]. The relationship of eotaxin with such risk factors for cardiovascular diseases as obesity and smoking has been established [3, 10, 14]. Cell culture studies show that increased expression of eotaxin plays a role in vascular inflammation and atherosclerotic process by increasing endothelial permeability, migration and calcification of smooth muscle cells in the presence of reactive oxygen intermediates [10].

At the present time, the importance of assessing vascular stiffness as an indicator of arteriosclerosis and a predictor of cardiovascular events has been demonstrated. For the past fifteen years, cardio-ankle vascular index (CAVI) has been widely used in clinical medicine to assess the risk of cardiovascular disease. As a marker of arterial stiffness, CAVI has a number of advantages over other methods, namely, it is easy to measure, has high reproducibility, reflects the stiffness of the entire aorta, femoral, popliteal and tibial arteries, allows you to assess the vascular age, to control the dynamics of treatment and the effectiveness of lifestyle changes, and to evaluate the severity of atherosclerotic process, and it is less dependent on blood pressure than pulse wave velocity [6, 12, 13].

Few studies have shown the relationship of eotaxin with coronary atherosclerosis; in the other studies, the relationship of eotaxin with atherosclerosis, myocardial infarction and such a marker of vascular stiffness as pulse wave velocity was not revealed.

The aim of the present study was to assess blood level of eotaxin and cardio-ankle vascular index and their association with major cardiovascular risk factors in patients with high and very high cardiovascular risk.

Materials and methods

We examined 65 patients aged 41-70 years, 29 men and 36 women. 86% of the surveyed were diagnosed with coronary artery disease, 97% were diagnosed with arterial hypertension, and 54% were diagnosed with type 2 diabetes mellitus. At the time of the study, all patients were undergoing generally accepted cardioactive and hypoglycemic therapy. Statins were taken by 62% of those examined. The study did not include patients with acute coronary syndrome, persistent atrial fibrillation, acute infectious diseases, allergic and autoimmune diseases, or oncological diseases. All the procedures and tests were conducted in accordance with the guidelines of the Declaration of Helsinki and "Rules of Clinical Practice in the Russian Federation", approved by the Order of the Ministry of Health of the Russian Federation. The study's protocol was approved by the Biomedical Ethics Committee of Cardiology Research Institute, Tomsk NRMC (protocol No. 210 from February 18, 2021). All the patients recruited into the study signed an informed consent.

All patients underwent a study of the elastic properties of the vascular wall by volumetric sphygmography on the VaSera VS-1000 device (Fukuda Denshi, Japan) with an assessment of cardio-ankle vascular index on the right and on the left and calculation of the average cardio-ankle index. In whole blood, the concentration of glycosylated hemoglobin A_{1C} (HbA_{1C}) was determined by the immunoturbidimetric method (DiaSys, Germany). The concentration of total cholesterol (TCH) and triglycerides (TG) was determined by the colorimetric enzymatic method (DiaSys, Germany). To determine the cholesterol of high-density lipoproteins (HDL), a combined method without precipitation was used (DiaSys, Germany); the concentration of low-density lipoprotein (LDL) cholesterol was calculated using Friedwald's formula. High-sensitivity C-reactive protein (hsCRP) was determined in the blood serum by enzyme-linked immunoassay (Vector-Best, Russia).

Eotaxin concentration was measured with Human Cytokines/Chemokines-38 kit using multiplex instrument FLEXMAP 3D (Luminex Corporation) and MILLIPLEX Analyst 5.1 software (Merck KGaA, Milliplex; Darmshdadt), the Core Facility "Medical genomics", Tomsk NRMC.

All examined were divided into two groups: with a normal value of cardio-ankle vascular index (less than 8) and elevated (more than 8).

The results were statistically processed using the STATISTICA 10.0 software package (StatSoft Inc., USA). The compliance of the law of distribution of variables with the normal one was checked using the Shapiro-Wilk test. Since the distributions of all variables were non-normal, the results are presented as median and interquartile interval: Me ($Q_{0.25}$-$Q_{0.75}$). Qualitative variables are presented as absolute and relative frequencies. The Mann–Whitney test was used to compare the groups. Qualitative variables were compared using Fisher's exact test. Correlation relationships were assessed using the Spearman rank coefficient (r_s). The critical significance level (p) was assumed to be 0.05.

Results and discussion

Patients with elevated CAVI were older. Higher concentrations of total cholesterol and low-density lipoprotein cholesterol in patients with elevated CAVI were consistent with rarer statin intake (Table 1).

TABLE 1. CHARACTERISTICS OF THE EXAMINED PATIENTS DEPENDING ON THE LEVEL OF CARDIO-ANKLE VASCULAR INDEX

Indicator	CAVI < 8 n = 23	CAVI ≥ 8 n = 42	p
CAVI	7.3 (6.90-7.45)	9.1 (8.6-9.8)	0.000
Eotaxin, pg/mL	60.84 (46.28-98.66)	93.09 (63.19-139.43)	0.013
Age, years	54 (50-59)	65 (56-67)	0.000
Men, n (%)	10 (43.5)	19 (45.2)	0.550
Smoking, n (%)	7 (30.4)	14 (33.3)	0.519
Patients with coronary artery disease, n (%)	19 (82.6)	37 (88.1)	0.397
Coronary artery disease, years	1 (0.5-4.0)	2 (0.5-5.0)	0.169
Patients with hypertension, n (%)	22 (95.7)	41 (97.6)	0.586
Hypertension duration, years	10 (5-20)	10 (5-17)	0.951
Patients with diabetes mellitus type 2, n (%)	12 (52.2)	23 (54.8)	0.523
Duration of diabetes mellitus type 2, years	0.5 (0-9)	1 (0-10)	0.995
Body mass index, kg/m²	30.4 (29.1-34.7)	30.9 (28.3-33.3)	0.600
Waist-to-hip ratio	0.95 (0.88-0.98)	0.97 (0.93-1.02)	0.259
Systolic blood pressure, mm Hg	120 (110-130)	129 (120-140)	0.061
Diastolic blood pressure, mm Hg	80 (75-80)	80 (70-82)	0.433
Statin intake, n (%)	20 (86.9)	20 (47.6)	0.002
HbA_{1C}, %	6.0 (5.2-7.7)	6.8 (5.7-7.7)	0.195
TCH, mmol/L	3.85 (3.24-5.03)	5.08 (4.33-5.44)	0.009
TG, mmol/L	1.40 (1.03-1.96)	1.37 (1.13-1.92)	0.908
LDL, mmol/L	2.21 (1.77-2.90)	3.06 (2.29-3.74)	0.016
hsCRP, mg/L	2.37 (1.35-3.61)	1.92 (1.01-3.94)	0.802

In all those examined, correlations were found between serum eotaxin concentration and CAVI, as well as age (Figure 1). The age of the patients correlated with CAVI ($r_s = 0.35$; $p = 0.007$).

Of all the cardiovascular risk factors studied, the eotaxin in the present study was associated only with age and the arterial stiffness score, CAVI. CAVI, in turn, was associated with age, the state of lipid metabolism and lipid-lowering therapy, which is consistent with other studies [6, 11, 12, 13].

Age is a significant predictor of cardiovascular risk. Depending on the ratio of chronological age and biological age, the concept of early vascular aging (EVA) and normal (healthy) vascular aging was proposed [12]. In 2019, leading experts in the study of vascular stiffness confirmed the hypothesis that

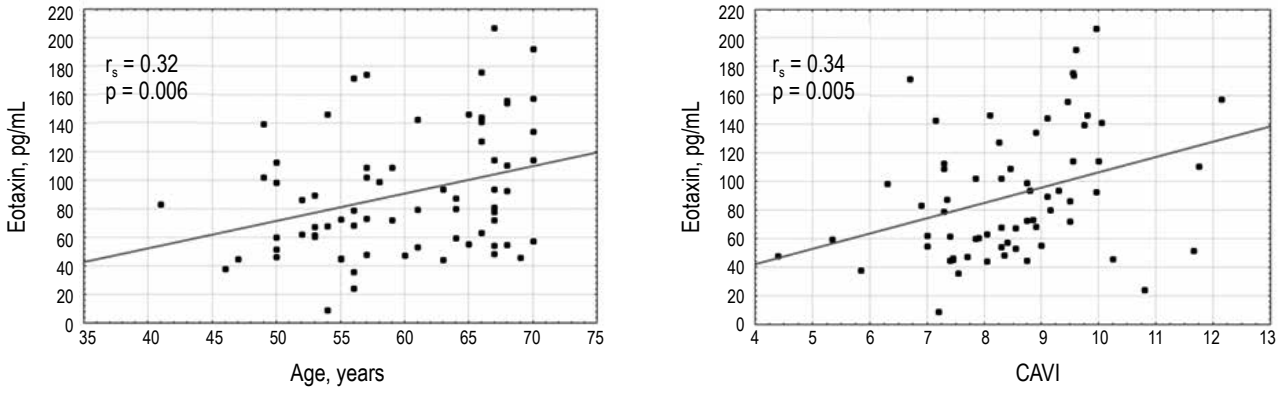

Figure 1. Correlations of eotaxin concentration in the blood with age and cardio-ankle vascular index

arterial stiffness is the best indicator of the combined action of known and unknown risk factors for damage to the arterial wall, and proposed to express very high and very low arterial stiffness in terms of EVA and SUPERNOVA (supernormal vascular aging). Patients with the SUPERNOVA phenotype have extremely low vascular stiffness for their age and sex. The plasticity of vascular smooth muscle cells plays an important role in increasing blood pressure not only by regulating the interaction of actomyosin for contraction, but also by participating in the homeostasis of the cell-extracellular matrix and is very important for the physiology of normal and early vascular aging [11].

The activation of CCR3 receptors and increased expression of eotaxin in case of injury can stimulate the migration of vascular smooth muscle cells from the media of the artery to the intima. This migration and subsequent proliferation of smooth muscle cells in the intima leads to intimal hyperplasia and narrowing of the clearance [7]. Eotaxin promotes the formation of reactive oxygen intermediates through the activation of NADPH oxidase, which leads to transdifferentiation of vascular smooth muscle cells and an increase in the rate of calcification [9]. As chemokine, eotaxin can not only promote inflammation, but also maintain it, due to its chemotactic effect on the endothelial cells of human vessels [2]. As a result, influencing the cells of all layers of the artery wall, eotaxin can affect arterial stiffness.

Conclusion

Thus, in our study, we showed the relationship between higher concentrations of eotaxin and an increased cardio-ankle vascular index in patients with high and very high cardiovascular risk. Cardio-ankle vascular index was associated with age, lipid metabolism and lipid-lowering therapy. The obtained results allow us to consider eotaxin as a factor associated with atherogenesis and arterial stiffness.

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ВЛИЯНИЕ ТЕРАПИИ ДЕКСАМЕТАЗОНОМ НА ФАКТОРЫ АДГЕЗИВНОСТИ И КОАГУЛЯЦИИ ПРИ ОСТРОЙ ИШЕМИИ НИЖНИХ КОНЕЧНОСТЕЙ

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Резюме. Лейкоцитарно-тромбоцитарная адгезия при гипоксии, повреждении тканей, активации воспаления и коагуляции ассоциирована с экспрессией мембранных молекул ICAM-1 и интегринов клетками крови и тканей. Одновременно адгезионные рецепторы тромбоцитов обусловливают их адгезию к эндотелию и к рекрутированным лимфоцитам. Роль тромбоцитов в патогенезе ишемических сердечно-сосудистых заболеваний также состоит в их способности модулировать как реакции гемостаза, так и воспалительные реакции, что сопровождается секрецией воспалительных медиаторов и факторов, способствующих рекрутированию лейкоцитов в места повреждения тканей. Цель исследования – изучить влияние синтетического глюкокортикоида дексаметазона на экспрессию адгезионных рецепторов $CD18^+$ и $CD54^+$ на лейкоцитах, содержание тромбоцитов и фибриногена в крови пациентов с ОИНК, связь этих показателей с тяжестью течения и исходом заболевания.

Для изучения влияния противовоспалительной терапии сформирована группа из 32 пациентов с терапией дексаметазоном; группа сравнения представлена 71 пациентом с базисной терапией, контрольную группу составили 15 волонтеров. После операции реваскуляризации все больные получали дезагрегантную и антикоагулянтую терапию. Инфузии дексаметазона проводили курсом от 4 до 6 дней после реконструктивной операции. У всех пациентов определяли содержание С-реактивного белка в крови, содержание тромбоцитов и фибриногена. С помощью иммуноцитохимического метода подсчитывали число лимфоцитов, экспрессирующих молекулы адгезии ICAM-1 ($CD54^+$) и интегрины ($CD18^+$). Исследования выполняли до операции и на 1-е, 3-и, 7-е, 10-е сутки после операции.

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При обострении ишемии и повреждении эндотелия, накоплении продуктов цитолиза усиливается экспрессия молекул адгезии как на эндотелиоцитах, так и на клетках-эффекторах воспаления – лейкоцитах и тромбоцитах. Молекулы адгезии проводят активационный сигнал внутрь клетки, что способствует адгезии лейкоцитов и тромбоцитов к эндотелию, лимфоцитарно-тромбоцитарной адгезии, образованию пристеночного тромба и возможной окклюзии поврежденных сосудов. Усиление экспрессии молекул адгезии связано с активацией метаболизма, воспаления, коагуляции и оксидативного стресса, стимулирует все ростки кроветворения, в том числе тромбоцитарный. Уровень вовлечения клеточных реакций в патогенез заболевания влияет на эффективность и продолжительность лечения, риск рецидивов тромбоза и летального исхода. Противовоспалительная терапия с дексаметазоном способствовала более ранней ремиссии, снижению доли инфекционных осложнений, таких как нагноение ран с 10% до 6%, количества необходимых ампутаций с 32% до 16%, частоты летальных исходов с 31% до 6%, сокращению сроков пребывания в стационаре с 13 дней до 10.

Воспаление, адгезивность клеток-эффекторов и тромбоз являются важными факторами патогенеза острой ишемии нижних конечностей. Терапия дексаметазоном способствует снижению уровня системного воспалительного ответа, количества необходимых ампутаций, числа осложнений и неблагоприятных исходов при лечении ОИНК, сокращению сроков пребывания в стационаре.

Ключевые слова: острая ишемия нижних конечностей, молекулы адгезии, фибриноген, тромбообразование, дексаметазон

EFFECT OF DEXAMETHASONE THERAPY ON FACTORS OF ADHESIVENESS AND COAGULATION IN ACUTE LOWER LIMB ISCHEMIA

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Abstract. Leukocyte-platelet adhesion during hypoxia, tissue damage, activation of inflammation and coagulation is associated with the expression of ICAM-1 membrane molecules and integrins by blood and tissue cells. At the same time, platelet adhesion receptors determine their adhesion to the endothelium and recruited lymphocytes. The role of platelets in the pathogenesis of ischemic cardiovascular diseases also consists in their ability to modulate both hemostasis and inflammatory reactions, which is accompanied by the secretion of inflammatory mediators and factors that promote the recruitment of leukocytes to tissue damage sites. Purpose of the study: to study the effect of the synthetic glucocorticoid dexamethasone on the expression of adhesion receptors $CD18^+$ and $CD54^+$ on leukocytes, the content of platelets and fibrinogen in the blood of patients with ALLI, the relationship of these indicators with the severity and outcome of the disease.

To study the effect of anti-inflammatory therapy, a group of 32 patients treated with dexamethasone was formed; the comparison group was represented by 71 patients with basic therapy, the control group consisted of 15 volunteers. After revascularization, all patients received antiplatelet and anticoagulant therapy. Dexamethasone infusions were carried out in a course of 4 to 6 days after reconstructive surgery. In all patients, the content of C-reactive protein in the blood, the content of platelets and fibrinogen were determined. The number of lymphocytes expressing adhesion molecules ICAM-1 ($CD54^+$) and integrins ($CD18^+$) was counted using the immunocytochemical method. Studies were performed before surgery and on days 1, 3, 7, and 10 after surgery.

With exacerbation of ischemia and damage to the endothelium, the accumulation of cytolysis products, the expression of adhesion molecules increases both on endotheliocytes and on inflammatory effector cells – leukocytes and platelets. Adhesion molecules conduct an activation signal inside the cell, which promotes adhesion of leukocytes and platelets to the endothelium, lymphocytic-platelet adhesion, the formation of a

parietal thrombus, and possible occlusion of damaged vessels. Increased expression of adhesion molecules is associated with the activation of metabolism, inflammation, coagulation and oxidative stress, stimulates all hematopoietic lineages, including platelets. The level of involvement of cellular reactions in the pathogenesis of the disease affects the effectiveness and duration of treatment, the risk of recurrent thrombosis and death. Anti-inflammatory therapy with dexamethasone contributed to earlier remission, a decrease in the proportion of infectious complications, such as wound suppuration from 10% to 6%, the number of necessary amputations from 32% to 16%, the frequency of deaths from 31% to 6%, and a reduction in hospital stay from 13 days to 10.

Inflammation, adhesiveness of effector cells and thrombosis are important factors in the pathogenesis of acute lower limb ischemia. Therapy with dexamethasone helps to reduce the level of systemic inflammatory response, the number of necessary amputations, the number of complications and adverse outcomes in the treatment of ALLI, and reduce the length of stay in the hospital.

Keywords: acute lower limb ischemia, adhesion molecules, fibrinogen, thrombus formation, dexamethasone

Introduction

Thrombogenesis underlies a number of diseases of the cardiovascular system (cerebral stroke, myocardial infarction, damage to the vessels of the extremities, kidneys, etc.) and is currently the main cause of death in industrialized countries. The leading component of the pathogenesis of vascular diseases is endothelial dysfunction, and thrombosis in arterial vessels is mainly based on activation of the vascular-platelet link of hemostasis. The cessation of blood flow due to blockage of the vessel by a thrombus leads to the development of acute lower limb ischemia (ALLI). Currently, the most effective method of revascularization in patients with ALLI is the surgical method, however, this does not eliminate the main cause of the disease [14]. One of the most common postoperative complications are retromboses and restenoses in the area of vascular reconstruction due to neointimal hyperplasia, as well as general hemodynamic disorders and disorders of the blood coagulation system [12].

In patients with ALLI, obviously, vascular occlusion is the result of activation of chronic inflammation of the vascular wall, increased adhesive properties of blood leukocytes and endothelium, tissue ischemia. Recirculation and recruitment of leukocytes in the area of inflammation is mediated by a specific ligand-receptor interaction between adhesion molecules of endotheliocytes, platelets and leukocytes, the expression of which is regulated by inflammatory mediators and cytokines. Membrane molecules involved in lymphocytic-platelet adhesion are ICAM-1 (Intercellular adhesion molecule, $CD54^+$), expressed by blood and tissue cells [12].

Adhesion molecules that mediate leukocyte-endothelial interactions undergo complex changes in patients treated for ALLI. Both postischemic reperfusion and the particular treatment chosen seem to influence the expression of adhesion molecules, which include ICAM-1, selectins, and integrins.

Integrins are a large family of cell surface molecules found on cells of various tissues. Integrins mediate the interaction of cells with their microenvironment, providing cell-to-cell and cell-to-matrix adhesion. Integrins are heterodimers of glycoproteins, consisting of various combinations of a- and J-chains. Expressed on leukocytes: CD18 as part of lymphocyte function associated antigen-1 (LFA-1, CD11a), macrophage antigen-1 (Mac-l, CD11b), pl50.95. Ligands for LFA-1 are: ICAM-1 ($CD54^+$), ICAM-2, ICAM-3, for Mac-l ICAM-1. These integrins mediate adhesion to the endothelium of neutrophils, basophils, eosinophils, monocytes, and lymphocytes.

The αMβ2 leukocyte integrin (CD11b/CD18, Mac-1) is also a high-affinity fibrinogen receptor on stimulated macrophages, monocytes, and neutrophils.

The function of inflammatory CAMs can be modulated by several mechanisms, including competitive blockade, altered cell surface expression, and, for integrins, interference with receptor activation. The ultimate therapeutic goal of each is to interrupt the multi-step recruiting cascade. In clinical practice, several groups of pharmaceuticals are used that directly or indirectly affect the function of CAMs [13]. For example, inhibition of IL-1β or TNFα by antibodies or soluble receptors has a powerful effect on the expression of CAMs on endothelial cells. Moreover, corticosteroids, non-steroidal anti-inflammatory drugs, and antioxidants also reduce the expression of inflammatory CAMs and chemokines, at least in part, by blocking the function of the inflammatory nuclear transcription factor κB (NF-κB) [5, 10].

In an experimental model, Mac-1 and CD18 knockout mice show reduced infarct volume and lower mortality after cerebral ischemia/reperfusion [6]. Immunoblockade of CD11b, CD18 or Mac-1 also protects the brain from ischemic damage [5]. In addition, CD18 immunoblockade reduces leukocyte recruitment while reducing cerebral edema and infarct size [4, 6].

Blocking CAMs that mediate leukocyte accumulation during inflammation is considered an effective strategy for the treatment of clinical

inflammatory diseases. However, despite promising preclinical results, results from clinical trials have been inconsistent. With the exception of some positive effects in psoriasis and asthma, prevention of either selectin or CD18 β2-integrin activity has had a limited effect, especially in the treatment of ischemia-reperfusion injury [2].

The common chain β2 integrin (CD18) is a major target for modulating innate immunity, and blocking this pathway has a profound effect on neutrophil adhesion and accumulation in acute inflammation [6]. However, clinical trials of CD18-blocking monoclonal antibodies (mAb) Rovelizumab and Erlizumab (LeukArrest™) were unsuccessful in reducing myocardial or brain ischemic injury [1, 4]. Moreover, a mAb against ICAM-1, CD54 (Enlimomab), the main counter-receptor for CD18 on leukocyte and endothelial cells, even had a negative effect in a phase II study in stroke [11]. The study highlights that blocking the effects of inflammatory CAMs may be of limited use in the treatment of ischemic injury. The use of monoclonal antibodies to prevent thrombosis did not lead to the desired clinical result, because radical inhibition of leukocyte adhesion to the endothelium led to undesirable side effects (development of infections, death [1, 11]).

The aim of the study was to study the effect of the synthetic glucocorticoid dexamethasone on the expression of adhesion receptors $CD18^+$ and $CD54^+$ on leukocytes, the content of platelets and fibrinogen in the blood of patients with ALLI, the relationship of these parameters with the severity of the course and outcome of the disease.

Materials and methods

To achieve the goal, 2 groups of patients were formed: the main one, in which, against the background of basic therapy, dexamethasone (DM) was administered intravenously at a dose of 8 mg in 200 ml of isotonic sodium chloride solution for 4-6 days after reconstructive surgery, 32 patients, age 76 (70-81) years; comparison group – 71 patients with basic therapy, age 70 (64-83) years. Basic therapy (BT) included painkillers, antibacterial, antiplatelet, anticoagulant agents. Patients with uncompensated ischemia underwent revascularization; patients with irreversible ischemia underwent revascularization to reduce the level of amputation; the volume of surgical intervention was to perform an embolectomy followed by a large amputation. The control group is represented by practically healthy volunteers, aged 70 (55-80) years.

The studies were performed before surgery upon admission to the hospital and on days 1, 3, 7, and 10 after surgery. In all patients, the blood levels of C-reactive protein (CRP) as an inflammation marker, creatine kinase activity (Cobas 6000 C501, Switzerland), the number of platelets – PLT and large platelets – P-LCR – (SYSMEX XT4000i, Japan), the relative content of $CD18^+$ and $CD54^+$ mononuclear leukocytes by immunocytochemical method (Novocastra, UK), fibrinogen content in blood (StaCompact Plus, France). To assess comorbidity, the Charlson M.E. index was used. [3]. The level of systemic inflammatory response (SIRS) was determined using the criteria adopted at the ACCP/SCCM consensus conference in 1992 (USA). The result of treatment was determined by the length of stay in the hospital and the outcome of the disease: good (4) – discharge after 7 days and earlier; satisfactory (3) – treatment for more than 7 but less than 14 days; unsatisfactory (2) – treatment for more than 14 days; bad (1) – death. Statistical processing was performed by the methods of variation statistics (Statistica 6.0): the median (Me) and percentiles ($Q_{0.25}$-$Q_{0.75}$), Spearman's correlation coefficient, Student's t-test were determined. The critical significance level (p) of statistical hypotheses was taken as 0.05.

Results and discussion

In the period of acute ischemia (before surgery), 51% of patients with basic therapy developed SIRS (2 or more signs), in the reperfusion period, SIRS was registered in 53% of patients, and in 10% the degree of SIRS increased to 4 signs (Table 1). In patients treated with DM, SIRS was noted in 47% of patients on admission; in the reperfusion period, SIRS decreased to 27%. Thus, the reduction in the degree of systemic inflammation is more pronounced in the treatment of DM. The level of systemic inflammatory response in the acute period (at admission before surgery) had a high predictive value for the outcome of the disease (AUC = 0.88; sensitivity 88%, specificity 75%); during the reperfusion period, an increase in the systemic inflammatory response leads to an unfavorable outcome (predictive value AUC = 0.93; sensitivity 89%, specificity 92%).

In all patients, chronic cardiovascular diseases prevailed in the structure of comorbidity. The mean Charlson comorbidity index in patients treated with BT + DM was 10.0 points; in patients with BT – 9.9 points, in volunteers of the control group – 6.7 points. The concentration of CRP in the blood of all patients upon admission to the hospital exceeded normal values by 11-14 times and reached a maximum in the period of reperfusion, that is, on days 3-7 after surgery (Table 2). In patients treated with DM, the concentration of CRP decreased by the 7th day of observation and was 2 times lower than in the comparison group.

Receptors involved in lymphocyte-platelet adhesion are ICAM-1 ligands for β2-integrins (CD11a/CD18, CD11b/CD18), LFA-1, Mac-1, and CD43 [9]. The content of cells carrying integrins was increased in all patients during the period of acute

ischemia, after thrombectomy, the number of $CD18^+$ and $CD54^+$ began to gradually decrease, but only in patients receiving dexamethasone infusions, these parameters normalized by day 7 of the disease (Table 2).

In all patients with ALLI, the content of $CD54^+$ lymphocytes correlated with the outcome of ALLI before thrombectomy ($r = -0.652$, $p < 0.05$) and during reperfusion ($r = -0.956$, $p < 0.05$). The number of these cells is associated with the activity of inflammation, as evidenced by the correlation with the concentration of CRP ($r = 0.952$; $p < 0.01$) and the number of large (immature) platelets ($r = -0.845$; $p < 0.001$). An increase in the content of large platelets is also associated with the number of $CD18^+$ mononuclear cells ($r = 0.563$; $p < 0.001$).

The content of fibrinogen in the blood of patients with ALLI at admission increased 1,3 times compared with the concentration in practically healthy people. In the period of reperfusion, the products of necrosis and cytolysis accumulated as a result of vessel occlusion are washed into the bloodstream and contribute to the activation of inflammation, oxidative stress [7, 8], and coagulation. Strengthening the coagulant properties of blood is accompanied by the risk of thrombosis, which can also occur in other problematic vascular areas (myocardial infarction, acute cerebrovascular accident). For this period, patients with ALLI are characterized by a continuing increase in the concentration of fibrinogen in the blood (Table 2), which leads to an increase in viscosity, and, consequently, a slowdown in blood flow and the risk of rethrombosis.

TABLE 1. RATIO OF THE NUMBER OF SIGNS OF SIRS IN PATIENTS WITH DIFFERENT THERAPIES IN THE ACUTE PERIOD AND THE PERIOD OF REPERFUSION

Type of therapy	Disease period	Number of SIRS signs in the group (%)				
		0	1	2	3	4
Basic therapy	Acute ischemia	13	36	31	20	0
	Reperfusion period	19	28	23	20	10
Dexamethasone therapy	Acute ischemia	13	40	29	18	0
	Reperfusion period	33	40	20	7	0

TABLE 2. FACTORS OF ADHESIVENESS AND THROMBOSIS IN PATIENTS WITH ACUTE ISCHEMIA OF THE LOWER EXTREMITIES WITH DIFFERENT THERAPY

Terms of observation	Patients with basic therapy					Patients with therapy dexamethasone				
	CRP (mg/L)	$CD18^+$ (%)	$CD54^+$ (%)	Plt (10^9/L)	fibrinogen (g/L)	CRP (mg/L)	$CD18^+$ (%)	$CD54^+$ (%)	Plt (10^9/L)	fibrinogen (g/L)
Before surgery	41* (9-86)	59 (48-62)	32* (23-41)	246 (171-283)	4.9* (4.3-5.8)	35* (8-52)	65* (55-72)	29* (25-40)	255 (180-329)	4.4* (4.3-5.2)
After surgery	67* (31-156)	57* (54-68)	25* (21-34)	229 (154-279)	4.8* (4.2-5.3)	62* (19-86)	56 (50-71)	24 (14-31)	241 (179-322)	5.2* (3.4-6.1)
3rd day	108* (52-157)	66* (53-73)	24 (19-27)	350* (298-409)	6.1* (5.2-7.1)	71* (13-81)	60 (34-73)	20 (14-24)	295 (202-351)	5.9* (5.2-6.0)
7th day	62* (21-135)	78* (76-80)	15* (9-18)	421* (341-516)	7.2* (6.1-8.3)	28* (15-34)	42# (34-52)	9* (5-16)	299# (234-362)	6.2* (4.4-8.5)
10th day	74* (51-141)	66 (56-69)	26* (22-31)	324 (257-340)	7.2* (6.0-7.3)	31*# (22-39)	42# (34-49)	17# (14-23)	282 (202-360)	5.1# (5.0-6.0)
Control	3 (1-4)	49 (46-53)	19 (15-22)	227 (189-258)	3.7 (3.5-4.0)	3 (1-4)	49 (46-53)	19 (15-22)	227 (189-258)	3.7 (3.5-4.0)

Note. *, differences from the control group; #, differences between groups of patients; $p < 0.05$.

When using DM infusions, we observed a decrease in the concentration of fibrinogen by the 10th day of observation to the reference values. In patients receiving basic therapy, the fibrinogen level increased from the 3rd day after the operation and remained elevated up to 10 days despite the ongoing anticoagulant therapy (Table 2). That is, with standard therapy in patients with ALLI, along with persistent clinical and laboratory signs of inflammation activity, the level of fibrinogen and platelets remains elevated, which indicates the importance of limiting inflammation in the treatment of ALLI. The use of additional anti-inflammatory therapy contributed both to the onset of remission of ALLI, combined with a significant decrease in the level of fibrinogen and platelets, the number of leukocytes expressing adhesion receptors.

The activity of creatine kinase as a marker of cytolysis in ischemic tissues also significantly increased upon admission of patients and reached 1600-2500 U/L (in healthy volunteers 76 (65-97) U/L). In patients with DM therapy, the activity of this enzyme returned to normal by day 5, in patients with BT, only by day 7 after surgery.

Correlation analysis showed that the concentration of fibrinogen in the blood in the acute period is associated with the level of inflammation (leukocyte level: $r = 0.952$, $p < 0.05$, SIRS: $r = 0.910$, $p < 0.05$, CRP: $r = 0.995$, $p < 0.05$), cytolysis ($r = 0.985$, $p < 0.05$), chemiluminescence of blood leukocytes ($r = 0.909$, $p < 0.05$), ALT activity $r = 0.943$, $p < 0.05$. Also, a high concentration of fibrinogen creates a risk of a lethal outcome of the disease ($r = -0.914$, $p < 0.05$). In the reperfusion period, the fibrinogen level also maintained a correlation with the CRP level ($r = 0.651$, $p < 0.05$), platelet count ($r = 0.586$, $p < 0.05$), leukocyte chemiluminescence ($r = 0.921$, $p < 0.05$). On the 10th day of the disease, the correlation with the above indicators remained. In the acute period and in the reperfusion period, the platelet count in the blood correlated with creatine kinase activity ($r = 0.634$, $p < 0.01$) and creatinine level ($r = -0.465$, $p < 0.05$), which demonstrated the importance of cytolysis for the involvement of the renal glomeruli in the pathological process with ALLI. $CD18^+$ correlates with the content of large platelets ($r = 0.719$, $p < 0.001$), which also increases the risk of acute renal failure, since, due to their size, large platelets do not easily cross the renal tubules.

Evaluation of treatment outcomes is shown in Picture1. The method of treatment using dexamethasone allowed not only to improve the results of postoperative treatment of patients with acute ischemia of the lower extremities, but also to reduce mortality from 31% with basic therapy to 6%, the incidence of complications: gangrene of limb tissues from 30% with BT to 6%; sepsis from 10% to 6%; wound suppuration from 10% to 6%, respectively. Treatment with dexamethasone reduces the number of amputations from 32% in patients with basic therapy to 16%, and reduces the duration of treatment for patients discharged from the hospital from an average of 13 to 10 bed-days.

1. Predictors of early postoperative mortality are: an increase in the content of leukocytes expressing $CD18^+$% adhesion molecules (according to ROC analysis, predictive value: AUC = 0.82; sensitivity 70%, specificity 70%); development of a systemic inflammatory response (predictive value: AUC = 0.88; sensitivity 88%, specificity 75%); comorbid status (AUC = 0.80; sensitivity 78%, specificity 72%).

2. In the reperfusion period, the predictors of an unfavorable outcome are an increase in the content of leukocytes expressing $CD54^+$% adhesion molecules ($r = -0.956$, $p < 0.05$), an increase in the systemic inflammatory response (AUC = 0.93, sensitivity 89%, specificity 92%) and comorbid status ($r = -0.361$, $p < 0.001$).

Conclusions

Therapy with dexamethasone helps to reduce the level of systemic inflammatory response, the number of required amputations (from 32% to 16%), the number of complications and adverse outcomes in the treatment of ALLI (from 32% to 6%).

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РОЛЬ МОРФОГЕННЫХ БЕЛКОВ WNT-СИГНАЛЬНОГО ПУТИ ПРИ ИШЕМИЧЕСКОЙ БОЛЕЗНИ СЕРДЦА

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Резюме. Исследования последних лет доказывают сложность патофизиологических процессов, участвующих в развитии острых форм ишемической болезни сердца и патологического ремоделирования миокарда. В последние годы внимание исследователей направлено на изучение WNT-сигнального пути, регулирующего процессы эмбриогенеза и участвующего в развитии патологических состояний. При этом роль морфогенных белков WNT-сигнального пути в генезе кардиоваскулярной патологии практически не выяснена. Целью исследования явилось комплексное изучение основных белков WNT-сигнального пути (β-катенина, склеростина, GSK-3α, GSK-3β, WIF-1 и DVL-1) сыворотки крови 353 больных острыми формами ишемической болезни сердца, находившихся на лечении в региональном сосудистом центре Орловской области с 2019 по 2021 гг., и 50 здоровых лиц. Комплексный анализ включал оценку клинико-лабораторных и инструментальных показателей в рамках действующих клинических рекомендаций, а также иммунологическое обследование по определению морфогенных белков WNT-сигналинга методом иммуноферментного анализа. Результаты исследований показали широкую вариабельность значений морфогенных белков WNT-сигнального пути в сыворотке крови больных. При этом уровень β-катенина, WIF-1 и DVL-1 значительно превышал аналогичные показатели, полученные у здоровых лиц, а концентрации склеростина и GSK-3β не имели с ними достоверных отличий. Наряду с этим уровень GSK-3α в сыворотке крови пациентов был в 2 раза ниже, чем у здоровых лиц. Максимально высокие концентрации склеростина были выявлены у пациентов с имеющимся кальцинозом створок аортального клапана и стенок аорты. Неблагоприятное течение острого коронарного синдрома наблюдалось у пациентов на фоне как крайне высоких, так и максимально низких показателей WIF-1 сыворотки крови. Установлены значимые корреляционные зависимости между уровнем морфогенных белков WNT-сигнального пути и показателями липидного обмена, а также ремоделирования миокарда. Полученные данные об изменении продукции агонистов и антагонистов WNT-сигнального пути позволяют расширить представления о молекулярных аспектах иммунопатогенеза миокардиального ремоделирования при ишемической болезни сердца, повышают предиктивный потенциал диа-

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гностики сердечно-сосудистых заболеваний и определяют вектор дальнейшего развития кардиоиммунологии.

Ключевые слова: WNT-сигнальный путь, β-катенин, склеростин, GSK3α, GSK3β, WIF-1, DVL-1, инфаркт миокарда, ишемическая болезнь сердца

ROLE OF MORPHOGENIC PROTEINS OF THE WNT SIGNALING PATHWAY IN CORONARY ARTERY DISEASE

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Abstract. In recent years, researchers' attention has been directed to the WNT signaling pathway study, which regulates embryogenesis processes and is involved in pathological condition development. The role of morphogenic proteins of WNT signaling pathway in the cardiovascular pathology genesis is practically not clear. The research aim was a comprehensive study of the main proteins of WNT signaling pathway (β-catenin, sclerostin, GSK-3α, GSK-3β, WIF-1 and DVL-1) in the blood serum of 353 patients with coronary artery disease acute forms who were treated at the Orel regional vascular center from 2019 to 2021, and 50 healthy individuals. A comprehensive analysis included an assessment of clinical, laboratory and instrumental parameters in the framework of current clinical guidelines, as well as an immunological examination to determine the morphogenic proteins of WNT signaling by enzyme immunoassay. The results showed a wide variability in the values of morphogenic proteins of WNT signaling pathway in the patient's blood serum. The levels of β-catenin, WIF-1 and DVL-1 significantly exceeded those obtained in healthy individuals, while the concentrations of sclerostin and GSK-3β did not differ significantly from them. The level of GSK-3α of patients was twice lower than in healthy individuals. The highest sclerostin concentrations were found in patients with existing calcification of the aortic valve leaflets and aortic walls. Acute coronary syndrome unfavorable course was observed in patients with both extremely high and extremely low WIF-1 levels. Significant correlations were established between the level of morphogenic proteins of WNT signaling pathway and lipid metabolism, as well as myocardial remodeling. The obtained data on changes in the protein production of WNT signaling pathway allow us to expand our understanding of the molecular aspects of the immunopathogenesis of myocardial remodeling in coronary artery disease, increase the predictive potential for cardiovascular disease diagnosis and determine the vector for further development of cardioimmunology determination.

Keywords: WNT signaling pathway, β-catenin, sclerostin, GSK3α, GSK3β, WIF-1, DVL-1, myocardial infarction, coronary heart disease

Introduction

Cardiovascular diseases remain one of the most acute problems of modern medicine, due to their high prevalence and high mortality among young and middle-aged people. Recent studies prove the complexity of pathophysiological processes involved in the development of acute forms of coronary artery disease (CAD) and pathological myocardial remodeling, among the chief causes of which are immune dysfunction, activation of signaling pathways, oxidative stress, and mitochondrial disorders [2, 3, 4, 10].

At the same time, the molecular mechanisms associated with inflammation and reflecting various aspects of the pathological process remain the subject of discussion and need to be clarified. Over recent years, the attention of researchers has been focused on the study of the WNT signaling pathway, which regulates the processes of embryogenesis and is involved in the development of various pathological conditions. At the same time, the role of morphogenic proteins of

the WNT signaling pathway in the pathogenesis of cardiovascular pathology is practically not clear.

In this regard, **the aim of the study** was a comprehensive study of the main proteins of the WNT signaling pathway (β-catenin, sclerostin, GSK-3α, GSK-3β, WIF-1 and DVL-1) in the blood serum of patients with acute forms of coronary artery disease and healthy individuals (HI).

Materials and methods

The study included 353 young and middle-aged patients (from 18 to 59 years old) with acute forms of CAD who were treated in the cardiology departments of the regional vascular center of the Orel Regional Clinical Hospital in the period from 2019 to 2021. All patients with coronary artery disease were divided into 2 groups: group I consisted of patients with myocardial infarction (MI) (165 people), group II included patients with unstable angina (UA) (188 people). The average age of patients with CAD and myocardial infarction was 50.8±7.4 years and with unstable angina 53.2±5.7 years. There were no statistical differences in age ($p > 0.05$). In both groups, the number of men was predominant and amounted to 79.4% of all subjects. A comprehensive analysis included an assessment of the anamnesis and objective status of patients, a general clinical laboratory examination, echocardiography, within the current clinical guidelines for the management of an exacerbation of CAD, as well as an immunological examination to determine morphogenic WNT signaling proteins. Blood sampling for research from a peripheral vein was carried out in the first 24 hours after hospitalization. The concentration of morphogenic WNT proteins (β-catenin, sclerostin, WIF-1, GSK-3α and GSK-3β, DVL-1) in blood serum was determined by enzyme-linked immunosorbent assay (ELISA) on a STAT FAX 2100 photometer using reagent kits Sunlong Biotech Co (China) in the Laboratory of Clinical Immunology of the Medical Institute of the "Orel State University named after I.S. Turgenev".

To determine the values of the immunological parameters of the WNT signaling pathway, taken as the physiological norm, we conducted a survey of 50 healthy individuals who did not have CAD, were comparable in age and gender with patients in the research groups.

The research was performed in accordance with the standards of clinical practice (Good Clinical Practice) and the principles of the Declaration of Helsinki, the study protocol was approved by the Ethics Committee of the Orel State University.

Inclusion Criteria: young (18-44 years) and middle-aged (45-59 years) people with a clinically established diagnosis of acute forms of CAD, the patient's consent to participate in the research.

Exclusion Criteria: age younger than 18 years and older than 59 years, cardiogenic shock, hypertension above 2 stage, chronic heart failure above I stage, verified oncological, autoimmune, neuropsychiatric diseases, the presence of a pathology affecting lipid metabolism, decompensated diabetes mellitus, exacerbation of chronic diseases, pathologies of hemostasis, pregnancy and lactation, acute infectious diseases, refusal of the patient to participate in the research.

Results and discussion

It is known that the WNT signaling pathway is traditionally divided into two types: canonical and non-canonical. Although the modalities of WNT signaling in the embryonic stages of heart development are fairly well understood and experimental evidence identifies canonical WNT/β-catenin signaling as a "key factor" in the regulation of cardiac function and dysfunction, however, in a few different studies conflicting data have been obtained on the involvement of morphogenic proteins of the canonical and non-canonical WNT signaling pathway in the pathogenesis of cardiovascular diseases, including coronary heart disease [4].

There is growing evidence that reactivation of the canonical WNT pathway negatively affects myocardial healing after ischemic exposure, causing death of cardiomyocytes and the development of the heart muscle fibrosis [10].

In addition, the clinical significance of serum concentrations of morphogenic proteins of the WNT signaling pathway remains debatable and needs to be clarified in order to determine them as possible potential predictors in the pathology of the circulatory system and to develop new approaches to targeted therapy.

Taking this into account, it was of interest to study the features of the production of the main proteins of the WNT signaling pathway (β-catenin, sclerostin, GSK3α, GSK3β, WIF-1 and DVL-1) in acute forms of CAD, as well as to establish the relationship of the studied morphogenic proteins with clinical and laboratory data and indicators of the structural and functional state of the myocardium in patients with MI and UA.

According to current data, β-catenin is an integral structural component and the main effector

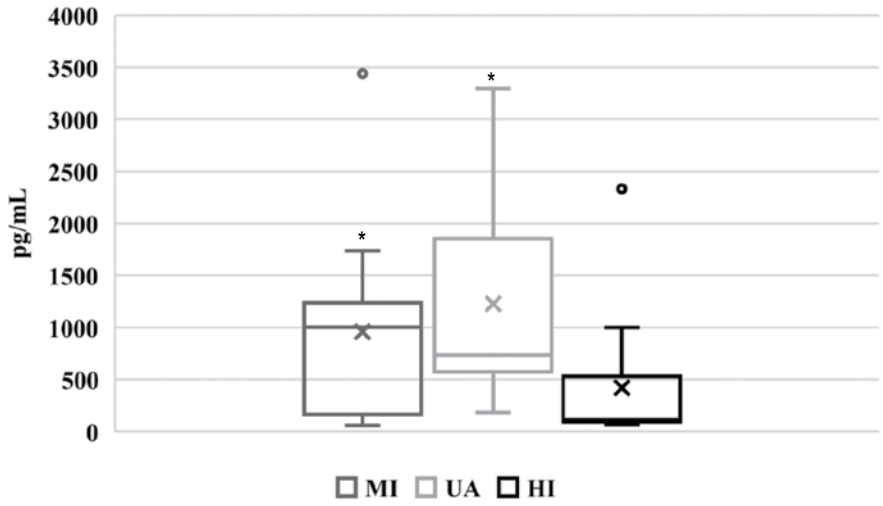

Figure 1. Level of DVL-1 in blood serum, pg/mL

Note. For distributions that differ from normal in Figures 1 Me (median) is given; 25-75 percentiles (upper and lower quartiles $Q_{0.25}$-$Q_{0.75}$); minimum and maximum sample values; outlier. *, $p < 0.05$, compared with HI.

of canonical WNT signaling involved in tissue homeostasis, myocardial remodeling, and control of the proliferative capacity of cardiomyocytes [10].

The results of our studies have shown a wide variability in the values of β-catenin in the blood serum of patients with coronary artery disease. At the same time, its level in patients with MI and unstable angina was 418 (293-579) pg/mL and 462 (384-588) pg/mL, respectively, which was several times higher than similar indicators obtained in HI 63.5 (57.25-86) pg/mL, $p < 0.001$. It is worth noting the revealed data on a significant increase in serum β-catenin in patients with coronary heart disease with concomitant hyperlipidemia ($p = 0.007$), which was also confirmed by the results of correlation analysis: a moderate direct correlation between the level of β-catenin and total cholesterol, low density lipoprotein (LDL) and a noticeable direct correlation with high-density lipoprotein, which is consistent with literature data indicating a correlation between impaired cholesterol metabolism and WNT/β-catenin signaling [7].

Given the important role of β-catenin in the processes of myocardial remodeling, its relationship with the parameters of intracardiac hemodynamics and the structural and functional state of the myocardium was evaluated. The most significant inverse correlations were found between the level of β-catenin and end-diastolic and systolic heart sizes ($p = 0.015$, $p = 0.018$, respectively).

The search for possible early markers of the development of cardiovascular complications led to the interest in the study of sclerostin, on the one hand, as the main inhibitor of the WNT signaling pathway, and on the other hand, as a potential participant in extraosseous calcification [5]. Taking this into account, we carried out the determination of the level of sclerostin in the blood serum of young and middle age patients with coronary artery disease and in HI.

According to the results of the research, the level of sclerostin in patients with CAD did not differ significantly from HI ($p > 0.05$). However, the analysis of the data obtained showed that the highest concentrations of sclerostin (above 215 pg/mL) were detected in patients with calcification of the aortic valve leaflets and aortic walls according to ECHO-CG data ($p = 0.002$; $p = 0.004$, respectively). It should also be noted that there was a direct statistically significant correlation between the level of sclerostin and the indicator of cardiovascular conjugation ($r = 0.7$; $p \leq 0.01$).

In recent years, data have appeared that the extracellular antagonist of the WNT signaling pathway WIF-1 is an important modulator of an adequate inflammatory process after myocardial injury, and the absence of this factor can lead to an increase in the inflammatory response and the development of pathological myocardial remodeling [8].

Analysis of the level of WIF-1 in the blood serum showed that in patients with acute forms of coronary artery disease, the concentration of WIF-1, on average, was 15.5 times higher than in healthy individuals ($p < 0.001$), and MI was characterized

by an even higher content of WIF-1 in blood serum. At the same time, in patients with a history of postinfarction cardiosclerosis, the ischemic process proceeded against the background of both extremely high (more than 3000 pg/mL) and extremely low (less than 1400 pg/mL) WIF-1 serum levels, which was combined with an unfavorable course of acute coronary syndrome.

Noteworthy are the results of a statistically significant high correlation between the concentration of WIF-1, the level of leukocytes and erythrocyte sedimentation rate ($r = -0.81$, $r = -0.70$, $p < 0.001$, respectively), as well as the content of β-catenin ($r = 0.743$; $p < 0.001$).

The study of DVL-1, which is involved in both canonical and non-canonical transmission of WNT signals, showed that in patients with acute forms of coronary artery disease, the level of DVL-1 significantly exceeded (8 times) the level of HI ($p = 0.009$), however, no statistically significant intergroup differences were found. In patients with transmural MI, maximum DVL-1 values were recorded at the level of 3400-3440 pg/mL (Figure 1). The analysis of the serum level of DVL-1 with clinical and laboratory data in patients with coronary artery disease made it possible to establish statistically significant direct correlations between DVL-1, total cholesterol ($p = 0.008$) and LDL ($p = 0.006$), as well as the presence of a direct relationship with levels of β-catenin ($p < 0.001$) and WIF-1 ($p < 0.001$), indicating the important role of DVL-1 as an integrator of canonical and non-canonical WNT signaling [11].

Recently it has been shown that glycogen synthase kinase-3 (GSK-3α and GSK-3β) plays an important role in the regulation of cell proliferation processes, including cardiomyocytes [1].

Considering the involvement of GSK-3α in the pathophysiology of cardiometabolic diseases [6, 9], in this study, we analyzed the obtained data on the content of GSK-3α in the blood serum of patients with acute forms of coronary artery disease, the level of which was 2 times lower than in healthy people (277 (238.25-875) pg/mL; $p = 0.021$); no statistically significant differences were found in patients of groups I and II. It should be noted that at normal levels of LDL and cholesterol, lower concentrations of GSK-3α were recorded, and patients with atherosclerosis had higher GSK-3α values, the highest level of GSK-3α was noted in patients with coronary artery disease and grade 3 obesity. The study of the content of GSK-3β in blood serum, which has an antifibrotic effect, did not reveal statistically significant differences in patients of the research groups and healthy individuals.

The absence of statistically significant differences in the concentrations of GSK-3α and β between the studied groups, along with the revealed changes in the level of other morphogenic proteins of the WNT signaling pathway, apparently, may be due to dysregulation of other signaling pathways in which GSK-3 plays a key role [9].

Conclusions

Thus, the obtained data on changes in the production of agonists and antagonists of the WNT signaling pathway (β-catenin, sclerostin GSK-3α, GSK-3β, WIF-1 and DVL-1) allow us to expand our understanding of the molecular aspects of the immunopathogenesis of myocardial remodeling in CAD, increase the predictive potential of diagnosing cardiovascular diseases and determine the vector of further development of cardioimmunology.

There is no conflict of interest.

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ГИПОКСИЕЙ ИНДУЦИРОВАННЫЙ ФАКТОР-1α И МАРКЕРЫ ВОСПАЛЕНИЯ У ПАЦИЕНТОВ С ИШЕМИЧЕСКИМ ИНСУЛЬТОМ

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Резюме. Ишемический инсульт (ИИ) возникает в результате локального нарушения гемоциркуляции и гипоксии в ткани головного мозга. Индуцируемый гипоксией фактор-1α (HIF-1α), участвующий в регуляции уровня кислорода в тканях, играет важную роль в патофизиологии инсульта, включая выживаемость нейронов, нейровоспаление, ангиогенез, метаболизм глюкозы, проницаемость гематоэнцефалического барьера (ГЭБ), и имеет значение в исходах ишемического инсульта. Сведения о роли HIF-1α в развитии инсульта разноречивы. Эффекты влияния HIF-1α связаны с длительностью и тяжестью ишемии. Известно о роли HIF-1α в ишемическом повреждении головного мозга, включая воспалительную реакцию и потерю целостности ГЭБ после инсульта. Цель исследования – определение связи содержания гипоксией индуцированного фактора-1α в крови пациентов со степенью неврологического дефицита в остром периоде ишемического инсульта и исходом заболевания. Обследованы 58 человек с ишемическим инсультом в возрасте 73 (67-81) лет. Пациенты были разделены на две группы – выписанные и умершие. Определяли тяжесть инсульта (NIHSS), неврологический дефицит, индекс коморбидности, содержание в крови HIF-1α, белка p53, интерлейкина-6, цистатина С, СРБ, креатинина, гематологические показатели при поступлении, на 3-и и 10-е сутки заболевания. Содержание HIF-1α в крови пациентов с ИИ при поступлении было ниже, чем в группе сравнения и оставалось сниженным до 10-го дня наблюдения. На 10-е сутки определялась связь HIF-1α с NIHSS, неврологическим дефицитом, индексом коморбидности и исходом заболевания. Наблюдали обратную связь HIF-1α с содержанием эритроцитов, гемоглобина и гематокрита что можно расценивать как отражение гемической составляющей смешанной гипоксии. Также у пациентов с неблагоприятным исходом ИИ выявлено повышенное содержание цистатина С в крови, которое было связано с концентраций HIF-1α. Во все сроки наблюдения церебральной катастрофы была отмечена корреляционная связь цистатина С с содержанием креатинина и СРБ. Эти результаты могут свидетельствовать о дисфункции эндотелиоцитов, нарушении клубочковой фильтрации, воспалении, ассоциированными с гипоксией при ИИ. Прогностическая значимость уровня HIF-1α в

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крови на 10-е сутки заболевания для исхода ИИ составила AUC = 0,900. Содержание HIF-1α в крови в остром периоде связано с тяжестью ишемического инсульта и исходом заболевания.

Ключевые слова: гипоксией индуцированный фактор-1α, апоптоз, маркеры воспаления, цистатин С, ишемический инсульт, исход

HYPOXIA-INDUCED FACTOR-1α AND MARKERS OF INFLAMMATION IN PATIENTS WITH ISCHEMIC STROKE

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Abstract. Ischemic stroke (IS) occurs as a result of local disturbance of hemocirculation and hypoxia in the brain tissue. Hypoxia-inducible factor-1α (HIF-1α), which is involved in the regulation of tissue oxygen levels, plays an important role in the pathophysiology of stroke, including neuronal survival, neuroinflammation, angiogenesis, glucose metabolism, blood-brain barrier permeability, and is important in IS outcomes. The purpose of the study was to determine the relationship between blood levels of HIF-1α and the degree of neurological deficit in the acute period of IS and the outcome of the disease. We examined 58 people with IS aged 73 (67-81) years. Patients were divided into two groups – discharged and dead. The severity of stroke (NIHSS), neurological deficit, comorbidity index, blood levels of HIF-1α, p53 protein, interleukin-6, cystatin C, CRP, creatinine, hematological parameters were determined at admission, on days 3 and 10 of the disease. At admission the blood levels of HIF-1α was lower than in the comparison group and remained reduced until the 10th day. On day 10 the association of HIF-1α with neurological deficit, comorbidity index and disease outcome was determined. We observed a feedback of HIF-1α with the content of erythrocytes, hemoglobin and hematocrit, which can be regarded as a reflection of the hemic component of mixed hypoxia. In dead patients, an increased blood level of cystatin C was detected, which was associated with HIF-1α concentrations. In all periods of observation of IS, a correlation between cystatin C and creatinine and CRP levels was noted. These results may indicate dysfunction of endotheliocytes, inflammation associated with hypoxia in IS. The prognostic significance of the blood level of HIF-1α on the 10th day for the outcome of IS was AUC = 0.900. Blood levels of HIF-1α in the acute period was associated with the severity of IS and the outcome of the disease.

Keywords: hypoxia-induced factor-1α, apoptosis, markers of inflammation, cystatin C, ischemic stroke, outcome

Introduction

Ischemic stroke (IS) occurs as a result of a local disturbance of hemocirculation in the brain tissue, which causes the development of hypoxia. Hypoxia-inducible factor 1α (HIF-1α), which is involved in the regulation of tissue oxygen levels, has been shown to play an important role in the pathophysiology of stroke, including neuroinflammation, regulation of blood-brain barrier permeability, glucose metabolism, neuronal survival, and angiogenesis [4].

HIF-1 (hypoxia inducible factor) is a transcription factor that allows the cell to survive in hypoxia; it was discovered more than 20 years ago when studying the mechanisms of adaptation of living organisms to changes in the oxygen content in the environment; found in various cells and tissues subject to hypoxia.

HIF is a heterodimer containing two protein subunits, HIF-1α (73-120 kDa) and HIF-1β (91-94 kDa) [11]. HIF-1α is a DNA-binding protein that undergoes hydroxylation in the presence of oxygen and binds to the conservative protein ubiquitin, which is involved in the regulation of intracellular proteasome degradation of other proteins [5]. With a decrease in the level of oxygen in the cell, hydroxylation does not occur, HIF-1α stabilizes and dimerizes with the second subunit of the HIF transcription factor, HIFβ, which is insensitive to oxygen. The resulting complex is translocated to the nucleus, where it binds to hypoxia response elements, HREs, thus launching the genetic program for cell survival under conditions of oxygen deficiency.

HIF-1α mediates the expression of many genes that are involved in neurogenesis, angiogenesis, cell proliferation, erythropoiesis, and cell metabolism, increasing the adaptation of nervous tissue to ischemic stress and, therefore, exhibiting a neuroprotective role [5]. Other studies have reported a detrimental role for HIF-1α in ischemic brain injury, including the inflammatory response and loss of blood-brain barrier (BBB) integrity following ischemic stroke. This indicates that HIF-1α is likely to be a mediator of neuroinflammation or a factor that determines BBB permeability [4]. Pan Z. et al. (2021) described

the mechanisms of regulation of HIF-1 production in stroke, when an elevated level of reactive oxygen species promotes HIF-1 expression by inhibiting prolyl hydrolase (PHD) activity and the NF-κB pathway. HIF-1 increases glucose uptake by regulating glucose transport proteins and glycolytic enzymes. Also, HIF-1 induces the production of pro-inflammatory cytokines IL-6, TNF, IL-20, MCP-1.

HIF-1 is involved in the regulation of expression of BNIP3, a pro-apoptotic member of the Bcl-2 and p53 family, to activate autophagy and inhibit the anti-apoptotic Bcl-2 protein, thus activating apoptotic signaling pathways and apoptosis. Increased production of erythropoietin (EPO) and vascular endothelial growth factor (VEGF) under the influence of HIF-1 contributes to the weakening of apoptosis. At the same time, the production of VEGF leads to disruption of the BBB [15]. The main structural component of the BBB is the endothelial cells of the walls of blood vessels that provide exchange in the blood-CNS system. Proliferation and migration of endothelial cells are largely dependent on VEGF. In addition, VEGF increases vascular permeability by initiating angiogenesis and neovascularization in ischemia-damaged tissue. After a stroke, the formation of a new vasculature is vital to compensate for the function of an occluded vessel in the penumbra. However, structural changes and an increase in vascular basement membrane permeability during activation of angiogenesis can lead to cerebral edema or hemorrhagic transformation in the acute phase of a stroke.

Information about the role of HIF-1 in the development of stroke is contradictory. In a mouse stroke model deficient in HIF1α/HIF2α, early neuronal death and neurological damage were reduced. At 24 h after stroke, cell death and edema were significantly reduced, but after 72 h, cerebral edema increased, accompanied by activation of apoptosis and a decrease in angiogenesis [1].

In another study, suppression of HIF-1α production after 0.5 h of ischemia in rats attenuated cerebral edema and apoptosis, while after 8 h of ischemia, neuronal damage increased and VEGF expression decreased [14]. It is likely that the effects of HIF-1 are associated with the duration and severity of ischemia. Chen C.H. et al. (2010) reported that HIF-1 inhibition can reduce BBB damage after stroke in adult and neonatal rats, which may be due in part to blocking the HIF-1α signaling pathway in neuronal apoptosis and suppressing VEGF activity to protect the BBB. Baranova O. et al. (2007) in an experiment showed that the expression of HIF-1 α is associated with the outcome of ischemic stroke.

Purpose of the study – to determine the relationship between the content of hypoxia-induced factor (HIF-1α) in the blood of patients with the degree of neurological deficit in the acute period of ischemic stroke and the outcome of the disease.

Materials and methods

We examined 58 people with ischemic stroke (IS), aged 73 (67-81) years, including 34 women, 24 men. The examination was carried out in accordance with the Procedure for the provision of medical care to patients with stroke of the Ministry of Health of the Russian Federation No. 928n, 2012. The condition of patients was assessed using the National Institutes of Health Stroke Scale (NIHSS), a modified Rankin scale for assessing the degree of disability and functional patient independence, Rivermead mobility index, Charlson comorbidity index (Table 1). The comparison group consisted of 25 volunteers aged 65.0 (62.0-67.0) years.

Blood levels of hypoxia-induced factor (Hypoxia-inducible factor 1-alpha, HIF-1α, R&D Systems, Inc, USA), p53 protein (Human p53 ELISA Kit, Invitrogen, ThermoFisher Scientific), interleukin-6 (IL-6), cystatin C (ELISA, Vector-Best, Russia) by ELISA; CRP by immunoturbidimetric method, creatinine (Roche, Cobas c501, Roche Diagnostics, Switzerland); hematological parameters (Sysmex XN1000, Japan) at admission, on days 3 and 10 of the disease was determined.

Statistical processing of the results was carried out using the Statistica 6.0 software package; the median (Me) and percentiles ($Q_{0.25}$-$Q_{0.75}$), Spearman's correlation coefficients were determined; the prognostic significance of the indicators was assessed using ROC analysis. In the correlation analysis of indicators with the outcome of the disease, discharged

TABLE 1. CHARACTERISTICS OF NEUROLOGICAL DEFICIT IN PATIENTS WITH ISCHEMIC STROKE

Groups	Age	NIHSS	Rivermead Index	Rankin Scale	Charlson Index	n
All patients with IS	73 (67-81)	9.5 (5-13)	2.0 (1.0-7.0)	4.0 (3.0-4.0)	6.0 (4.0-7.0)	58
Discharged	71 (66-79)	7.3 (4.3-12.8)	2.5 (1.0-6.5)	4.0 (3.0-4.0)	5.0 (4.0-7.0)	44
Dead	80 (72-81)	16.0 (11.0-21.0) p = 0.022	1.0 (0.0-2.0)	4.0 (4.0-5.0)	7.5 (6.0-9.0) p = 0.004	14

Note. p, significance of differences between groups of patients discharged and dead.

patients were assigned 1 point, and those who died – 0 points. Statistical significance was taken for $p < 0.05$.

Results and discussion

Patients with IS were divided into two groups – a group of discharged patients and a group of dead patients. Patients of the selected groups significantly differed in the severity of stroke at admission and the value of the Charlson comorbidity index (Table 1). NIHSS scores and comorbidity index were associated with disease outcome ($r = -0.497$; $p < 0.001$ and $r = -0.394$; $p < 0.001$, respectively).

It is known from the literature that the level of HIF-1α in the blood of patients increases during hypoxia, which is associated with cerebral ischemia [4]. In our study, we observed a decrease in the content of HIF-1α in the blood both in discharged patients (by 2 times) and in dead patients (by 2.8 times) during all periods of observation (Table 2). It is not yet clear what mechanisms are responsible for the inhibition of HIF-1α production in the acute period of stroke.

A decrease in the content of the apoptotic protein p-53 by 3 times by the 3rd day of observation was also noted in discharged patients and 8 times in the dead. The concentration of HIF-1α was associated with the level of p-53 in the blood serum of patients on the 10th day of observation ($r = 0.999$, $p < 0.001$). We observed inhibition of apoptosis with a decrease in HIF-1α in the blood, while in the literature, inhibition of apoptosis is described with an increase in HIF-1α expression [7].

Recently, cystatin C has been considered as a biomarker of endothelial dysfunction in cerebrovascular pathology, as well as an adequate indicator of the state of renal functions, including those with normal renal function, but diagnosed with ischemic stroke [6]. The content of cystatin C in patients with IS is associated with the severity of stroke and its consequences [8]. Cystatin C is a non-glycosylated protein with a molecular weight of 13.4 kDa, an inhibitor of cysteine proteinases, synthesized by all cells containing nuclei, freely filtered through the glomerular membrane, and completely metabolized in the kidneys.

Wang Y. et al. (2019) in a meta-analysis that included nine studies involving 3773 patients with ischemic stroke, showed an association between cystatin C and the risk of ischemic stroke: it turned out that patients with ischemic stroke had significantly higher serum cystatin C concentrations compared with participants without ischemic stroke.

Our results also demonstrated a more pronounced increase in the level of cystatin C in the blood in patients with an unfavorable outcome of ischemic stroke (Table 2). On the 10th day of observation, a relationship was found between the concentrations of HIF-1α and cystatin C ($r = 0.927$; $p < 0.001$). In all periods of observation of cerebral catastrophe, a medium and strong correlation was noted between cystatin C and creatinine and CRP levels. These results may indicate dysfunction of endotheliocytes, impaired glomerular filtration, inflammation associated with hypoxia in IS.

At the same time, the feedback of HIF-1α with the content of erythrocytes, hemoglobin and hematocrit was observed ($r = -0.648$, $p < 0.05$; $r = -0.586$, $p < 0.05$; $r = -0.579$, $p < 0.05$ respectively), which can be regarded as a reflection of the hemic component of mixed hypoxia.

An experiment [12] showed that pro-inflammatory cytokines, including IL-6, are involved in the activation of HIF-1α in the rat hippocampus and contribute to cerebral damage caused by transient global ischemia. Xu S. et al., 2020 found that under hypoxic conditions, IL-6 enhances the expression of

TABLE 2. BLOOD LEVELS OF HIF-1α, p53, AND CYSTATIN C IN IS PATIENTS DISCHARGED AND DEAD

Patients with IS	Days after IS	HIF-1α, pg/mL	p53, E/mL	Cystatin C, mkg/mL
Comparison group		114 (44-116)	2.54 (1.08-4.06)	0.64 (0.62-1.17)
Discharged	1	56 (47.5-67.0) p = 0.015	1.16 (0.41-6.66)	0.87 (0.71-1.04)
	3	58.0 (48.0-66.0) p = 0.040	0.71 (0.31-3.95)	0.85 (0.76-1.02)
	10	52.0 (48.0-58.0) p = 0.033	0.5 (0.4-0.9)	1.0 (0.8-1.4)
Dead	1	42.0 (39.0-44.0) p = 0.012	2.2 (1.8- 2.5)	0.9 (0.9-1.1)
	3	40.0 (40.0-40.0) p = 0.041	0.3 (0.29-0.31)	1.22 (1.18-1.42) p = 0.027
	10	–	0.34 (0.34-0.34)	1.4 (1.3-1.6)

Note. p, significance of differences compared with data from the comparison group.

TABLE 3. BLOOD LEVELS OF IL-6 AND CRP IN IS PATIENTS DISCHARGED AND DEAD

Patients with IS	Days after IS	IL-6, pg/mL	CRP, mg/L
Comparison group		2.2 (2.0-2.7)	1.3 (1.0-4.8)
Discharged	1	10.1 (4.6-29.4)	5.0 (3.1-18.0)
	3	10.4 (2.7-30.1) $p_1 = 0.000$	10.6 (4.4-25.9) $p = 0.007$ $p_1 = 0.000$
	10	13.9 (4.6-35.7) $p = 0.058$ $p_1 = 0.000$	12.9 (4.9-40.7) $p = 0.025$ $p_1 = 0.000$
Dead	1	32.3 (24-180) $p_1 = 0.003$	19.1 (10.6-44.8) $p = 0.000$
	3	84.7 (48.9-239.7) $p = 0.011$	130 (75.8-193.0) $p = 0.000$
	10	91.9 (64.6-117) $p = 0.003$	172 (165-195) $p = 0.000$

Note. p, significance of differences compared to data from the comparison group; p_1, significance of differences between groups discharged and dead.

HIF-1α through a signal protein and transcription activator 3 (STAT3). In our study, an increase in the content of IL-6 compared with the norm by 5-6 times in discharged patients and by 14-42 times in patients with a lethal outcome was noted (Table 3). Although we did not find a direct relationship between HIF-1α and IL-6 concentrations, there was a correlation between HIF-1α and CRP levels ($r = 0.978$; $p < 0.001$). Previously, we showed [9] that in patients with IS, an increase in the concentration of IL-6 in the blood preceded an increase in the concentration of CRP; changes in indicators were interrelated and reflected the severity of ischemic stroke. We can assume an indirect effect of IL-6 on the level of HIF-1α in IS.

The content of HIF-1α in the blood of all patients with IS decreased upon admission to the hospital and remained at the same level during 10 days of observation, but only on the 10th day was the relationship of HIF-1α with the severity of neurological deficit, with the Rankin and Rivermead scale, with the index comorbidity and disease outcome ($r = 0.982$, $p < 0.001$; $r = 0.670$; $p < 0.05$; $r = -0.694$; $p < 0.05$; $r = 0.684$, $p < 0.01$; $r = -0.674$, $p < 0.001$, respectively). The prognostic significance of the level of HIF-1α in the blood on the 10th day of the disease for the outcome of IS according to the ROC analysis was AUC = 0.900, sensitivity 80%, specificity 100%.

Thus, HIF-1α plays a key role in the development of the compensatory and adaptive response of cells and tissues to ischemia and hypoxia, and is associated with endotheliocyte dysfunction, impaired glomerular filtration, and the development of inflammation in patients with ischemic stroke. The content of HIF-1α in the blood in the acute period is associated with the severity of ischemic stroke and the outcome of the disease.

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ИЛЛЮСТРАЦИИ К СТАТЬЕ «СРАВНИТЕЛЬНАЯ ОЦЕНКА ЭФФЕКТИВНОСТИ ПЕПТИДСОДЕРЖАЩЕГО ПРЕПАРАТА И ПОЛИОКСИДОНИЯ В ЛЕЧЕНИИ ХРОНИЧЕСКОГО ПАРОДОНТИТА» (АВТОРЫ: ЧУМАКОВ Н.С., ХЛЫСТОВА К.А., САРКИСЯН Н.Г., МАМЕДОВ М.М. [с. 851-854])

ILLUSTRATIONS FOR THE ARTICLE "COMPARATIVE EVALUATION OF THE EFFECTIVENESS OF A PEPTIDE-CONTAINING DRUG AND POLYOXYDONIUM IN THE TREATMENT OF CHRONIC PARODONTITIS" (AUTHORS: CHUMAKOV N.S., KHLYSTOVA K.A., SARKISYAN N.G., MAMEDOV M.M. [pp. 851-854])

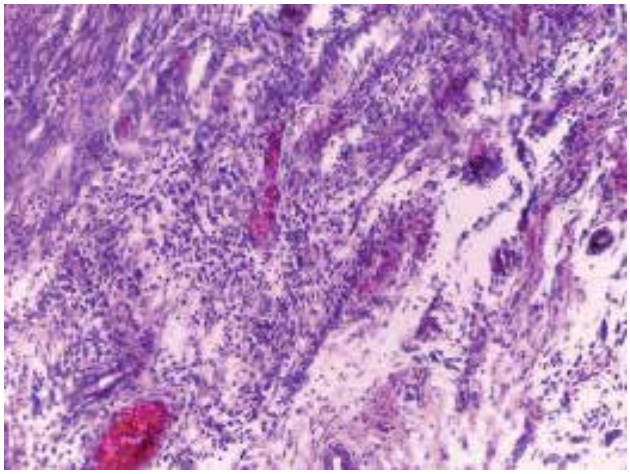

Figure 1. After treatment with the "Silativit-peptide" drug
Note. Scattered lymphocytic infiltration, vascular congestion, ×200.

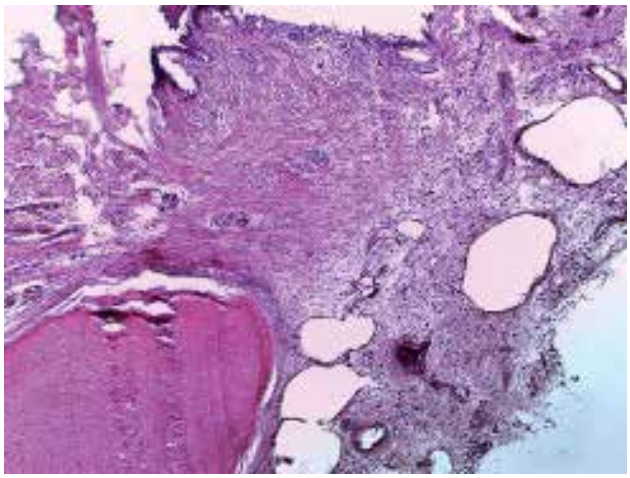

Figure 2. After treatment with the "Metrogyl Denta" drug
Note. Diffuse lymphocyte infiltration, formed connective tissue. ×100.

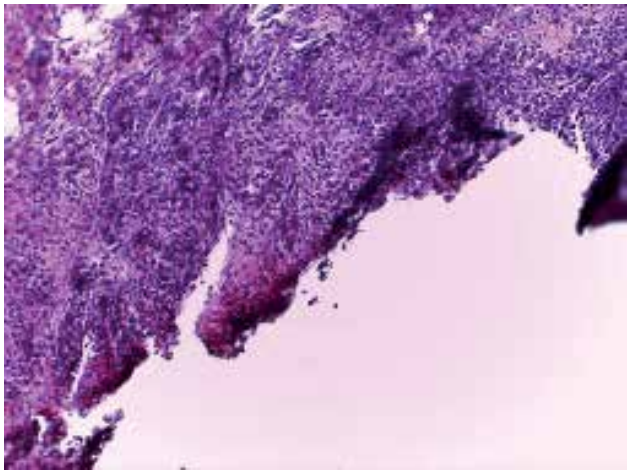

Figure 3. After treatment with the "Polyoxidonium" drug
Note. Focal mucosal necrosis, massive leucocytic infiltration, ×200.

ИЛЛЮСТРАЦИИ К СТАТЬЕ «ИММУНОЛОГИЧЕСКИЕ ИНДИКАТОРЫ ОСЛОЖНЕНИЙ ХИРУРГИЧЕСКИХ ЗАБОЛЕВАНИЙ КИШЕЧНИКА У ДЕТЕЙ» (АВТОРЫ: МУСАХОДЖАЕВА Д.А., КАРИМОВ Р.К., РАСУЛОВА С.Х. [с. 907-912])

ILLUSTRATIONS FOR THE ARTICLE "IMMUNOLOGICAL INDICATORS OF COMPLICATIONS OF SURGICAL BOWEL DISEASE IN CHILDREN" (AUTHORS: MUSAKHODZHAEVA D.A., KARIMOV R.K., RASULOVA S.H. [pp. 907-912])

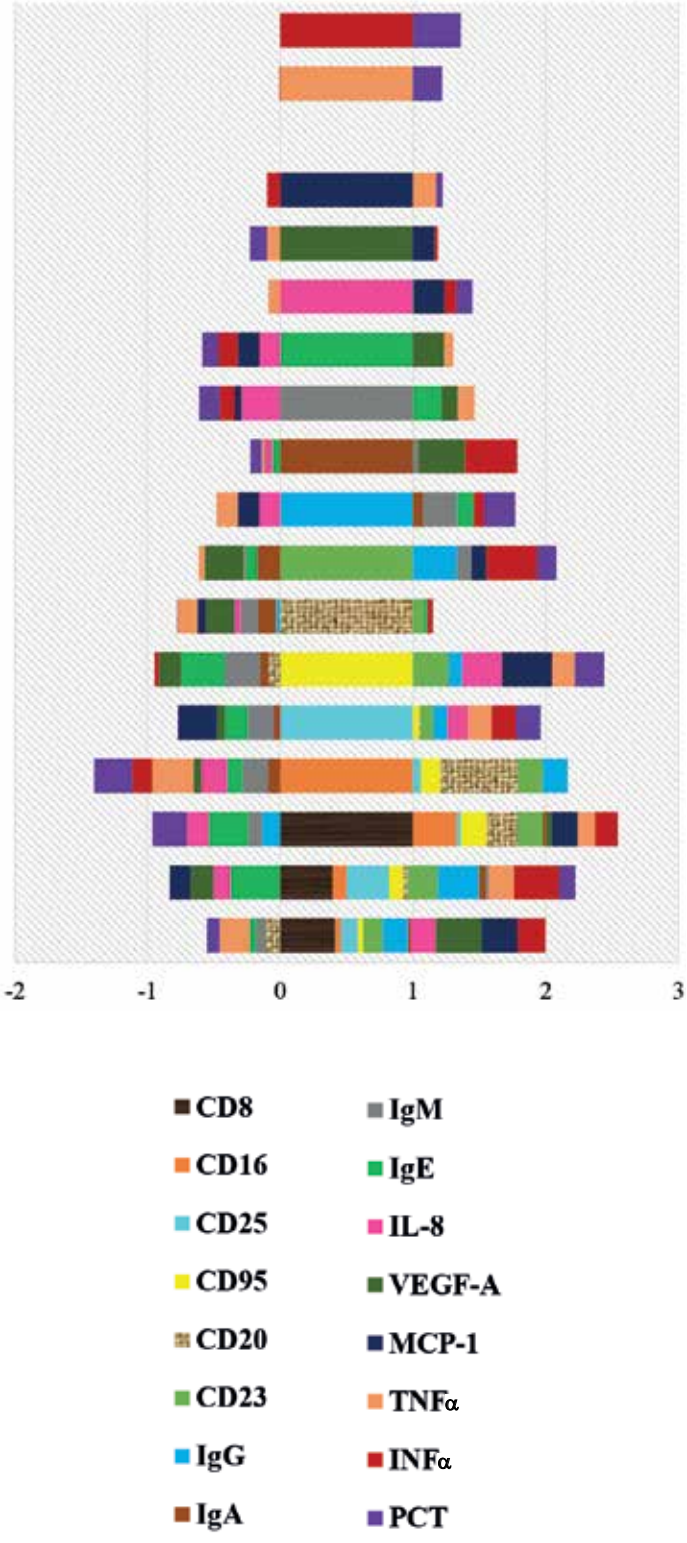

Figure 1. Correlations of immunological parameters of blood in intestinal obstruction in children

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