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Уважаемые коллеги и читатели!

Выпуск, который вы держите в руках, является уникальным, поскольку в нем опубликованы материалы XVII Всероссийского научного Форума с международным участием имени академика В.И. Иоффе «Дни иммунологии в Санкт-Петербурге», который должен был состояться 7-10 июня 2021 года, но был отменен в связи со сложной эпидемиологической обстановкой по новой коронавирусной инфекции. Конечно, мы могли бы провести форум в дистанционном формате, но, учитывая многолетние традиции данного мероприятия, которое из региональной конференции для практических врачей, проведенной в 1997 году, выросло до значимого для иммунологов всей страны научного форума с международным представительством по основным направлениям развития современной фундаментальной и клинической иммунологии, оргкомитет принял решение о проведении мероприятия исключительно в очном формате и о его переносе до периода, когда ситуация с COVID-19 в нашей стране нормализуется.

Поскольку наиболее активные участники форума подали материалы для публикации до того, как было принято решение о переносе мероприятия, мы сочли правильным опубликовать представленные краткие сообщения в ближайшем номере журнала.

За многолетнюю историю форума мы впервые опубликовали материалы в виде кратких сообщений, представленных на английском языке. Необходимо сказать, что данный опыт оказался достаточно успешным и, несмотря на ограниченный временной период приема материалов, мы увидели большую активность коллег и смогли отобрать для публикации в данном номере 54 кратких сообщения.

Редколлегия журнала «Медицинская иммунология» и оргкомитет XVII Всероссийского научного Форума с международным участием имени академика В.И. Иоффе «Дни иммунологии в Санкт-Петербурге» благодарят авторов представленных кратких сообщений и выражают надежду на скорую очную встречу в Санкт-Петербурге.

От редколлегии журнала «Медицинская иммунология»
и оргкомитета XVII Всероссийского научного Форума
«Дни иммунологии в Санкт-Петербурге»
академик РАН, д.м.н., профессор Арег А. Тотолян

Dear Colleagues and Readers,

The issue you are holding in your hands is unique as it contains materials from the XVII Russian/International Scientific Forum, "St. Petersburg Immunology Days", in honor of Academician V.I. Ioffe. The event was supposed to take place on June 7-10, 2021, but was canceled due to the difficult epidemiological situation with the novel coronavirus. Of course, we could have held the forum in an online format, but the long-term traditions of this event were also considered. What started as a regional conference for practitioners in 1997, has grown into a significant scientific forum for immunologists across Russia, along with international representation in key topics of modern fundamental and clinical immunology. Thus, the organizing committee decided to hold the event exclusively in a face-to-face format and to postpone it until the situation with COVID-19 in our country has normalized.

Since the majority of active forum participants had already submitted materials for publication before the postponement decision, we considered it appropriate to publish the presented short communications in the next issue of the journal. For the first time in the forum's long history, we have published materials in the form of short communications presented in English. It must be said that this experience turned out to be quite successful. Despite the limited time interval for receiving materials, we saw great activity among our Colleagues and were able to select 54 short communications for publication in this issue.

The editorial board of the journal "Medical Immunology" (Russia) and the organizing committee of the XVII Russian/International Scientific Forum, "St. Petersburg Immunology Days", in honor of Academician V.I. Ioffe are grateful to the authors of the presented brief reports. We express hope for a face-to-face meeting in St. Petersburg as soon as feasible.

On behalf of the "Medical Immunology" (Russia) editorial board
and the organizing committee of the XVII Russian/International Scientific Forum
"St. Petersburg Immunology Days",

Respectfully yours,

Professor Areg A. Totolian
Academician of the Russian Academy of Sciences

РЕГУЛЯТОРНЫЕ Т-КЛЕТКИ ЛИМФАТИЧЕСКИХ УЗЛОВ У *Muc2*^{-/-} МЫШЕЙ С *HELICOBACTER SPP.*

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Резюме. Иммунные процессы, связанные с формированием устойчивости к патогенам в кишечнике, зависят от микробиома. Поддержание гомеостаза в кишечнике обеспечивают регуляторные Т-клетки. При воспалительных заболеваниях кишечника (ВЗК) наблюдают как нарушение функции Т-регуляторных клеток, так и изменения микрофлоры. Развитие осложнений при этих заболеваниях сопровождается различными инфекциями. Однако некоторые представители патобионтов, например *Helicobacter spp.*, могут влиять на Т-регуляторные клетки. Одной из генетических моделей для изучения ВЗК являются мыши с нокаутом гена *Muc2*. У таких мышей, как и у людей с ВЗК, эпителиальные и иммунные клетки кишечника находятся в тесном взаимодействии с микрофлорой. Полагают, что иммунные клетки лимфатических узлов *Muc2*^{-/-} мышей чувствительны к изменению сформировавшейся у них микрофлоры, даже если в ее состав входят патобионты. В данном исследовании было показано влияние присутствия *Helicobacter spp.* на количество и процент различных типов лейкоцитов и Т-регуляторных клеток в мезентериальных лимфатических узлах *Muc2*^{-/-} мышей. Количество CD45⁺CD19⁺, CD45⁺CD3⁺, CD45⁺CD3⁺CD4⁺, CD45⁺CD3⁺CD8⁺-клеток в мезентериальных лимфатических узлах мышей *Muc2*^{-/-} был достоверно выше, чем у мышей дикого типа *Muc2*^{+/+}. Однако наличие инфекции у *Muc2*^{-/-} мышей отменяло увеличение количества CD45⁺CD19⁺, CD45⁺CD3⁺, CD45⁺CD3⁺CD4⁺, CD45⁺CD3⁺CD8⁺-клеток. У мышей дикого типа *Muc2*^{+/+} инфекция не оказывала достоверного эффекта на клетки в мезентериальных лимфатических узлах. Такое изменение в снижении иммунных клеток в мезентериальных лимфатических узлах под действием *Helicobacter spp.* может быть связано с активацией регуляторных Т-клеток. Действительно, было показано, что наличие врожденной инфекции *Helicobacter spp.* вызывало увеличение количества регуляторных Т-клеток (CD45⁺CD4⁺CD25⁺FoxP3⁺) в мезентериальных лимфатических узлах. Известно, что регуляторные Т-клетки обеспечивают противовоспалительные реакции в кишечнике. Таким образом, увеличение регуляторных Т-клеток способствует снижению всех типов иммунных клеток в мезентериальных лимфатических узлах мышей *Muc2*^{-/-} с инфекцией *Helicobacter spp.*, что способствует улучшению жизнедеятельности этих мышей и, возможно, уменьшает воспалительные реакции в кишечнике. Это может быть свидетельством того, что некоторые патобионты, приобретенные с рождения, могут быть активаторами регуляторных механизмов иммунитета и, тем самым, оказывать благоприятное воздействие на хозяина.

Ключевые слова: Т-регуляторные лимфоциты, муцин 2, микрофлора, *Helicobacter*, мыши

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LYMPH NODE REGULATORY T-CELL IN Muc2^{-/-} MICE WITH *HELICOBACTER* SPP.

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Abstract. The immune processes associated with the formation of resistance to pathogens in the intestine depend on the microbiome. The maintenance of homeostasis in the intestine is provided by regulatory T-cells. In inflammatory bowel disease (IBD), both a disturbance of the T-regulatory function and changes in microflora are observed. Aggravation of the disease is accompanied by various infections. However, pathobionts such as *Helicobacter* spp., can affect regulatory T-cells. One of the genetic models for studying IBD is Muc2 knockout mice. In these mice, as in humans with IBD, intestinal epithelial and immune cells closely interact with the microflora. It is believed that the immune cells of the lymph nodes Muc2^{-/-} mice are sensitive to changes in the microflora formed in them. In this study, the effect of *Helicobacter* spp. on the number and percentage of different types of leukocytes and T regulatory cells in the mesenteric lymph nodes of Muc2^{-/-} mice was studied. The number of CD45⁺CD19⁺, CD45⁺CD3⁺, CD45⁺CD3⁺CD4⁺, CD45⁺CD3⁺CD8⁺-cells in the mesenteric lymph nodes of Muc2^{-/-} mice was significantly higher to compare with wild-type Muc2^{+/+} mice. However, the presence of infection in Muc2^{-/-} mice canceled the increase in the number of CD45⁺CD19⁺, CD45⁺CD3⁺, CD45⁺CD3⁺CD4⁺, CD45⁺CD3⁺CD8⁺-cells. In wild-type Muc2^{+/+} mice, infection had no significant effect on cells in mesenteric lymph nodes. This change in the decrease in immune cells in the mesenteric lymph nodes under the *Helicobacter* spp. may be associated with the activation of regulatory T-cells. Indeed, it has been shown that the presence of a congenital *Helicobacter* spp. infection increased of the number of regulatory T-cells (CD45⁺CD4⁺CD25⁺FoxP3⁺) in the mesenteric lymph nodes. Well known that regulatory T-cells mediate anti-inflammatory responses in the gut. Thus, an increase in regulatory T-cells promotes a decrease in all types of immune cells in the mesenteric lymph nodes of Muc2^{-/-} mice infected with *Helicobacter* spp. It could provide an improvement in the vital functions of these mice and possibly reduces inflammatory responses in the intestine. This may indicate that some congenital pathobionts activate of the regulatory mechanisms of immunity and, thereby, have a beneficial effect on the host.

Keywords: regulatory T-cells, mucine 2, microflora, *Helicobacter*, mice

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Introduction

Microflora plays an important role in the formation of intestinal lymphoid tissue, to increase resistance to pathogens [8]. Regulatory T-cells controlled by symbiotic microflora are involved in intestinal homeostasis [4, 9]. It is known that in germ-free vs. conventional mice (GF) regulatory T-cells have less suppressive activity [13]. Certain bacterial species can influence on the function of regulatory T-cells. *Bacteroides fragilis* stimulates the production of anti-inflammatory cytokine IL-10 by CD4⁺-cells, preventing development of inflammation in mice [14]. The intestinal colonization of such bacteria in GF mice stimulates the differentiation of IL-10-producing

CD4⁺FoxP3⁺T-cells [14]. *Clostridium* spp. leads to increased number of regulatory T-cells in the gut lamina propria and stimulates IL-10 production [3]. The differentiation of regulatory T-cells can also be influenced by bacterial metabolites. Butyrate has an effect on the expression of the FoxP3 transcription factor gene by CD4⁺T-cells in mouse colon as well as *in vitro*. At the same time, dietary butyrate helps to ameliorates mouse induced colitis [7].

Muc2 knockout mice (Muc2^{-/-}) are one of the genetic models for studying intestinal inflammation [5]. At the same time, colitis in Muc2^{-/-} mice has similar features to ulcerative colitis in humans [15]. Muc2^{-/-} mice are more susceptible to various pathogens, due to impaired production of mucin2. In the presence of pathogens, these mice exhibit acute intestinal inflammation [5]. Thus, Muc2^{-/-} mice as IBD model can be used to study a relationship between changes in intestinal microflora and intestinal inflammation.

Previously in our laboratory have been shown that mice with impaired gut barrier function and mutation in the gene *Muc2* (*Muc2*^{-/-} Kaiso^{-/-}), are more sensitive to treatment with antibiotics, and the elimination of *Helicobacter* spp, compared to mice with normal gut barrier function (C57BL/6). *Helicobacter* spp. are considered pathobionts, i.e. show their pathogenic properties only in the presence of defects in immune function, or under special environmental conditions. Also *Helicobacter* spp. can induce regulatory T-cells [10]. Member of the genus *Helicobacter* (*H. hepaticus*) enable mechanisms that maintain a non-pathogenic, symbiotic relationship with the host organism [6]. Thus, the bacteria *Helicobacter* spp. found in host from birth, can act as symbionts, influencing the formation of the immune system.

Due to the lacked mucin2, the intestinal epithelial and immune cells are in closer contact with the microflora. It can be assumed that the immune cells in regional lymph nodes of *Muc2*^{-/-} mice are sensitive to changes in microbiota composition. In this study we examined an effect of inoculated *Helicobacter* spp. on number and percentage of different types of leukocytes and T-regulatory cells in the mesenteric lymph nodes (LN) of *Muc2*^{-/-} mice.

Materials and methods

The study was carried out on sexually mature female knockout *Muc2* gene knockout mice (*Muc2*^{-/-}) and their littermates with normal gene function (*Muc2*^{+/+}). Mice were free of species-specific pathogens (SPF) recommended by the Federation of European Laboratory Animal Science Associations

(FELASA), except for *Helicobacter* spp. Three to six females were housed in individually ventilated cages (Optimice, USA) under an artificial light regimen (14L: 10D), at a temperature of 20–22 °C. Animals received chop diet (Sniff, Germany) and sterile drinking water *ad libitum*.

The study was carried out on four groups of mice (two genotypes with and without *Helicobacter* spp., n = 7 per each group). Mesenteric lymph node cells were isolated and stained with PE-anti-CD3ε, FITC-anti-CD4, PE / Cy7-anti-CD8α and PE-anti-CD3ε, FITC-anti-CD19, PE / Cy7-anti-CD4 and APC-anti-CD25, AlexaFluor488-anti-FoxP3 and TrueNuclear™ Fix (BioLegend, USA) and analyzed on a Guava easyCyte 8HT Flow Cytometer (Merk, Germany).

Data statistical distribution was not normal; the analysis of the data was performed by using nonparametric criteria.

Results and discussion

The effect of the groups on the cell number in mesenteric LNs (mLNs) was found (Kruskal–Wallis test $H(3,25) = 16.08$, $p < 0.01$). The cell number in mLN of mutant mice born without infection was higher than in *Muc2*^{-/-} mice with infection, as well as in *Muc2*^{+/+} mice of the corresponding infection status (Mann–Whitney U-test $Z = 3.03$ and $Z = 2.85$, $p < 0.01$; Figure 1A). A similar effect was observed for the number of lymphocyte subsets such as CD19⁺, CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ (Figure 1A).

The study of regulatory T-cell subpopulations revealed an effect of the group on the percentage of CD25⁺FoxP3⁺-cells among CD4⁺-cells (Kruskal–

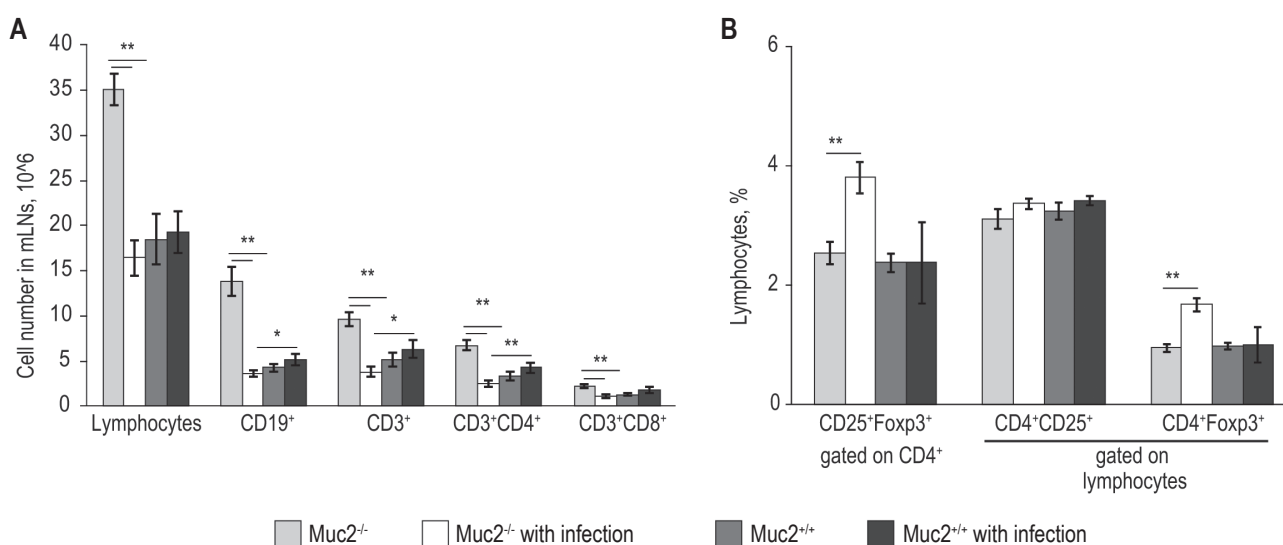


Figure 1. Lymphocytes in mLNs of *Muc2*^{-/-} and *Muc2*^{+/+} mice

Note. A, cell number of lymphocytes and different lymphocyte subsets in the mLNs. B, percentage of regulatory T-cell subpopulations. *, $p < 0.05$; **, $p < 0.01$; Mann–Whitney U-test.

Wallis test $H(3,25) = 9.15$, $p < 0.05$). The percentage of $CD25^{+}FoxP3^{+}$ -cells among $CD4^{+}$ -cells in $Muc2^{-/-}$ mice with vs. without infection was higher (Mann–Whitney U-test $Z = 2.78$, $p < 0.01$; Figure 1B). The increased number of such cells was probably due to upregulated expression of the FoxP3 protein in $CD4^{+}$ -cells (effect of the Kruskal–Wallis test $H(3,25) = 9.47$, $p < 0.05$). The percentage of $CD4^{+}FoxP3^{+}$ -cells among lymphocytes was higher in $Muc2^{-/-}$ mice with infection compared to mice without infection (Mann–Whitney U-test $Z = 2.92$, $p < 0.01$; Figure 1B). At the same time, no such effect was found for the percentage of $CD4^{+}CD25^{+}$ -cells.

Thus, the presence of *Helicobacter* spp. in $Muc2^{-/-}$ mice was associated with increased mLN percentage of regulatory T-cells ensuring anti-inflammatory responses in the intestine.

A study of lymphocytes in mLNs showed that $Muc2^{-/-}$ mice bearing *Helicobacter* spp. had increased percentage of $CD25^{+}FoxP3^{+}$ regulatory T-cells compared to mice without infections. These differences are likely due to increased expression of the

FoxP3 protein in $CD4^{+}$ -cells. Possibly, *Helicobacter* spp. activates the regulatory function of T-cells in mice, which can prevent the development of extremely strong immune responses to host microflora. It was shown that tolerogenic properties of *Helicobacter* spp. particularly were due to its potential to stimulate the production of the anti-inflammatory cytokine IL-10 [2, 11] and activate regulatory T-cells [1, 10, 12]. It is possible that the pathobiont *Helicobacter* spp. can favorably influence the state of $Muc2^{-/-}$ mice by stimulating the regulatory function. In our study, we found that *Helicobacter* spp.-free $Muc2^{-/-}$ mice had prolapse at an earlier age (2–3-months old) and significantly more frequently than in mice with infection. It was also noted that the fertility of $Muc2^{-/-}$ mice without *Helicobacter* spp. is lower than in mice with infection (unpublished data).

Thus, the presence of *Helicobacter* spp. in $Muc2^{-/-}$ mice, it was associated with an increase in regulatory T-cells, which provide anti-inflammatory responses in the intestine and improve the vital functions of mice with a pathology of thinned mucus in the intestine.

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ОЦЕНКА ВЛИЯНИЯ КУКУРБИТУРИЛОВ НА МОНОЦИТЫ И НК-КЛЕТКИ ЗДОРОВЫХ ДОНОРОВ

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Резюме. Нанотехнологии в иммунологии — перспективно развивающееся направление в фундаментальной и практической медицине. Кукурбитурилы являются макроциклическими кавитандами с определенным количеством гликолурильных фрагментов (n), определяющих размер полости данного соединения. На данный момент существует шесть синтезированных гомологов: 5, 6, 7, 8, 10 и 14. Отличаются друг от друга порталным размером и размером полостей. Характеризуются особыми физико-химическими и биологическими свойствами, такими как биосовместимость, стабильность, высокая способность к инкапсуляции химических соединений. Известно, что кукурбитурилы инкапсулируют молекулы путем формирования комплексов по типу «гость—хозяин», что позволяет высвобождать вещество из комплекса, повышать растворимость соединений и использовать кукурбитурилы как системы доставки лекарственных веществ. Иммуномодуляторная активность зависит от его специфических наноразмерных характеристик: функциональные группы, форма, размер, поверхность, растворимость в различных средах. Каждая наночастица, в зависимости от этих свойств, оказывает различные эффекты на клетки. Эффекты кукурбитурилов могут быть различными даже для одной субпопуляции клеток в зависимости от гомолога или дозировки. Взаимодействие клеток врожденного иммунитета с кукурбитурилами пока еще недостаточно изучены.

Целью этого исследования была оценка влияния кукурбит[n]урилов (n = 6, n = 7, n = 8) на клетки врожденного иммунитета — моноциты, НК-клетки, NKT-клетки.

Иммунологическое исследование включало выделение мононуклеарных клеток периферической крови условно здоровых доноров (n = 8) на градиенте плотности фиколл-урографина и проточную цитометрию с определением количества иммунокомпетентных клеток по классическим маркерам дифференциации этих клеток — CD3⁺CD16⁺CD56⁺ для НК-клеток, CD3⁺CD16⁺CD56⁺ для NKT-клеток и CD3⁺CD14⁺ для моноцитов. Активацию моноцитов определяли по экспрессии поверхностного маркера HLA-DR.

Клетки культивировали 72 часа с добавлением кукурбитурилов CB[6], CB[7] в концентрациях 0,1, 0,3, 0,5 мМ и CB[8] в концентрации 0,01 мМ, поскольку он имеет плохую растворимость.

Было отмечено достоверное снижение количества НК-клеток (p < 0,01 для исследуемых концентраций CB[7]), увеличение количества NKT-клеток (p < 0,04 и p < 0,02 для концентраций CB[6] и CB[7] соответственно). Также наблюдалась тенденция к повышению экспрессии HLA-DR на моноцитах (p = 0,06 для CB[6]).

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Учитывая такое разностороннее действие кукурбитурилов, в перспективе можно рассматривать возможность использования кукурбитурилов в качестве иммуномодуляторов, противоопухолевых агентов, при аутоиммунных заболеваниях.

Ключевые слова: кукурбитурилы, макроциклические соединения, иммунитет, врожденный иммунитет, моноциты, NK-клетки, NKT-клетки, проточная цитометрия

ASSESSING EFFECTS OF CUCURBITURILS ON MONOCYTES AND NK-CELLS IN HEALTHY VOLUNTEERS

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Abstract. Nanotechnology in immunology is a prospectively developing area in fundamental and practical medicine. Cucurbiturils are macrocyclic cavitands with a definite amount of glycoluril fragments (n) that determine the size of the cavity of these compounds. Nowadays, there are six synthesized homologues: 5, 6, 7, 8, 10 and 14. They differ from each other in the portal size and the size of the cavities. They are characterized by special physicochemical and biological properties, such as biocompatibility, stability, high ability to encapsulate chemical compounds. It is known that cucurbiturils encapsulate molecules by forming guest-host complexes, which allow the substance to be released from the complex and increase the solubility of the compounds. These advantages allow using cucurbiturils as drug delivery systems. Immunomodulatory activity of cucurbiturils depends on its specific nanoscale characteristics: functional groups, shape, size, surface, solubility in various media. Each nanoparticle depending on these properties has different effects on cells. The effects of cucurbiturils can be different even for one subpopulation of cells, depending on the homologue or dosage. The interaction of innate immune cells with cucurbiturils are not yet sufficiently characterized.

The aim of this study was to assess the effects of cucurbit[n]urils (n = 6, n = 7, n = 8) on innate immune cells – monocytes, NK-cells, NKT-cells.

The immunological research included the isolation of peripheral blood mononuclear cells from healthy donors (n = 8) on the density gradient of ficoll-urografen and flow cytometry with the determination of the amount of immunocompetent cells according to the classic markers of differentiation of these cells – CD3⁺CD16⁺CD56⁺ for NK-cells, CD3⁺CD16⁺CD56⁺ for NKT-cells and CD3⁺CD14⁺ for monocytes. Monocyte activation was determined by the expression of surface HLA-DR.

The cells were cultured for 72 hours with the addition of cucurbiturils CB[6], CB[7] at concentrations of 0.1 mM, 0.3 mM, 0.5 mM and CB[8] at concentration of 0.01 mM, due to its poor solubility.

There were a significant decrease in the quantity of NK-cells ($p < 0.01$ for the test concentrations of CB[7]), an increase in the quantity of NKT-cells ($p < 0.04$ and $p < 0.02$ respectively for the concentrations of CB[6] and CB[7]). There was a tendency to increase the expression of HLA-DR on monocytes ($p = 0.06$ for CB[6]).

Considering a variative effects of cucurbiturils, in the future it is possible to consider a possibility of using cucurbiturils as an immunomodulators, antitumor agents, in autoimmune diseases.

Keywords: cucurbiturils, macrocyclic compounds, immunity, innate immune system, monocytes, NK-cells, NKT-cells, flow cytometry

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Introduction

Cucurbiturils (CB[n]) consists of 5 to 14 glycoluril molecules, which are linked via methylene bridges.

The quantity of glycoluril monomers determines the serial number (n) of cucurbiturils. CB[n] have a cavity surrounded by highly reactive oxygen atoms. Cucurbituril through oxygen atoms interact with positively charged compounds, which are placed into CB[n]. Thermal and chemical stability, non-covalent interactions with guest-molecules, the cavi-

ties of different sizes and the most importantly is a biocompatibility of these macrocyclic-hosts allow using of cucurbiturils as delivery systems. These properties distinguish cucurbiturils from other organic nanostructures [3]. For example, the solubility of poorly soluble drug molecules can be significantly increased by complexation with CB[n] [6]. Nanotoxicity is determined by the structure of the nanomolecule and its functional groups. The toxicity of the cucurbituril should be examined for its potential pharmacological application. Cucurbiturils mainly at the concentrations up to 1 mM are not toxic to host tissues and immune cells [2, 10, 14]. However, the immunomodulatory activity of cucurbiturils has not been well studied. For example, it is known that CB[n] has an immunostimulatory effect enhancing cell proliferative activity and increasing the expression of HLA-DR on lymphocytes [11]. However, few published data on the interaction of cucurbiturils with other cells of innate immunity are available.

Natural killer cells (NK-cells) are cytotoxic cells of innate immunity with the classic phenotype $CD3^+CD16^+CD56^+$. Functional NK-cells exert activity corresponding to that of adaptive cytotoxic T-lymphocytes (T-killers), however, NK-cells recognize infected cells without involving membrane major histocompatibility complex (MHC) molecules. NK-cells destroy infected cells and also actively take part in events of anti-tumor immunity. The main function of these cells is killing target cells via one of the two ways: through the secretion of perforin and granzymes or through membrane-bound death receptors. The molecular signatures of nanomaterials can be recognized by pattern recognition receptors (PRP) expressed on the surface of innate immune cells. The function of NK-cells can be altered by interaction with nanoparticles. For example, the interaction between NK-cells and nanocarbon tubes reduces viability of NK-cells and the production of perforins and granzymes, but elevates production of ROS and cell apoptosis, which led to the suppression of NK-cell functions [1].

Natural killer T-cells (NKTs) are a subpopulation of T-lymphocytes expressing NK-cells markers and T-cells differentiation antigens ($CD3^+CD16^+CD56^+$). NKTs are the most important regulators of the immune response that protect from emergence, growth and metastasis of tumors, intracellular infections of various nature, and development of autoimmune diseases. NKT-cells maintain the induction of anti-tumor immune response by producing IFN- γ , which activates NK-cells, $CD8^+$ T-lymphocytes and macrophages. Basically, nanoparticles are used to selectively activate NKT-cells. For example,

PLGA-based nanoparticle systems designed to modulate the activity of NKT-cells to improve their antitumor function [5]. However, the effect of organic nanoparticles such as cucurbiturils on the viability of NK-cells and NKT-cells should be investigated to determine synergistic effects of cucurbiturils.

Monocytes are mononuclear cells of innate immunity displaying classic phenotype $CD14^+CD16^-$, expressing pattern recognition receptors and chemokine receptors allowing to migrate to the inflammation focus. Monocytes secrete pro-inflammatory cytokines in inflammation focus and participate in phagocytosis. The main functions of monocytes are to enable phagocytic and cytolytic activity. Monocytes express human leukocyte antigen-DR molecules (HLA-DR), which are responsible for presenting antigen to adaptive T-cells. The expression of HLA-DR indicates about cell activation and presence of foreign agents in the organism. Nanostructures can persist for some time in the systemic circulation and, apparently, affect monocytes function. Inorganic nanoparticles can be phagocytosed by monocytes and cause the production of pro-inflammatory cytokines, while the viability of monocytes is reduced [8]. However, modified inorganic nanoparticles by an organic component and organic nanoparticles can be used as darts for monocyte-targets to improve the diagnosis and treatment of diseases [7].

The purpose of the study is to assess an effect of cucurbiturils on monocytes, natural killer cells and nature killer T-cells.

Materials and methods

Heparinized peripheral blood was collected from 8 healthy donors (average 29 ± 2.4 years) after obtaining their informed consent. Peripheral blood was placed on Ficoll-urografin density gradient medium according to the protocol. Cells were centrifuged at 2.7×10^3 rpm for 20 minutes, followed by washing out peripheral blood mononuclear cells (PBMCs) twice in the appropriate phosphate-buffered saline (PBS). The amount of cells was counted in a hemocytometer added with 2% acetic acid.

After isolation, PBMCs at a quantity of 1×10^6 cells/ml were cultured with CB[6], CB[7] and CB[8] using the complete culture medium RPMI-1640, supplemented with 10% FCS, 50 mg/ml gentamicin and 25 mg/ml thienam in 24 well plate (TPP, Switzerland) for 72 h at 37°C , 5% CO_2 , 90% relative humidity. CB[6], CB[7] were added at the concentrations of 0.1 mM, 0.3 mM, 0.5 mM. CB[8] was added at a concentration of 0.01 mM due to its very low solubility in the media.

After 72h-incubation the cells were washed 2× in PBS. Fluorochrome-conjugated monoclonal antibodies were used to determined phenotypes of monocytes and NK-cells by CD3⁺ (BioLegend, USA), CD14⁺ (BioLegend, USA), CD16⁺CD56⁺ (BioLegend, USA) and HLA-DR (BioLegend, USA). The cells were labeled with 4 fluorochrome-conjugated antibodies by combining two monoclonal antibodies recognizing the human CD16⁺ and CD56⁺-cell surface antigens. NK-cells were identified by the classical phenotype CD3⁺CD16⁺CD56⁺, NKT-cells – CD3⁺CD16⁺CD56⁺ and monocytes by the CD14⁺ marker.

Samples were analyzed by flow cytometry using cytometer FACS Canto II (BD, Franklin Lakes, NJ, USA) with Diva 6.0 software (BD). Expression of cell surface receptor HLA-DR was analysed gated on CD3⁺CD14⁺ monocytes.

Statistical analysis was performed using Statistica 6.0 software (StatSoft, USA). Differences between groups were determined using non-parametric Wilcoxon matched-pairs test. P value of < 0.05 level was considered statistically significant.

Results and discussion

First, we evaluated the effect of cucurbiturils on NK-cells obtained from peripheral blood mononuclear cells from 8 healthy donors in PBMCs cultures *in vitro*. The NK-cells frequency was decreased at all three tested concentrations of CB[7] compared to the control (0 mM) (Figure 1). Interestingly, the amount of NK-cells at a concentration of 0.3 mM is higher compared to concentrations of 0.1 and

0.5 mM of CB[7]. According to literature data, some nanoparticles also lower functional activity of NK-cells and even alter their phenotype [9]. On the contrary, other nanoparticles were able to increase NK-cell proliferative and cytotoxic activity [4, 12].

Next, we evaluated the effect of cucurbiturils on a subpopulation of T-lymphocytes associated with the innate immunity – NKT-cells. Our data showed that CB[6] at a concentration of 0.3 mM and CB[7] at a concentration of 0.5 mM increased the amount of NKT-cells compared to the control group (0 mM) (Figure 2). The frequency of NKT-cells was increased compared to NK-cells, probably due to the fact that NKT-cells express T-cell antigen receptors. In previous studies we found an increase in T-lymphocytes during PBMC treated with cucurbiturils [11]. According to available literature, nanoparticles are mainly used to stimulate the NKT-cells [13].

The next step was to assess the frequency of monocytes and their activation during cultivation with CB[n]. We found no significant differences in amount of monocytes (Figure 3A), however, there was a tendency ($p = 0.06$) to enhanced expression of HLA-DR on the surface of monocytes with the CB[6] at a concentration of 0.1 mM acting probably in a dose-dependent manner (Figure 3B). This may be due to the small size of cucurbituril and its ability to penetrate immunocompetent cells.

Conclusion

To summarize the data obtained, it is clear that nanoscale compounds interact with the innate immune system, which is important for potential deve-

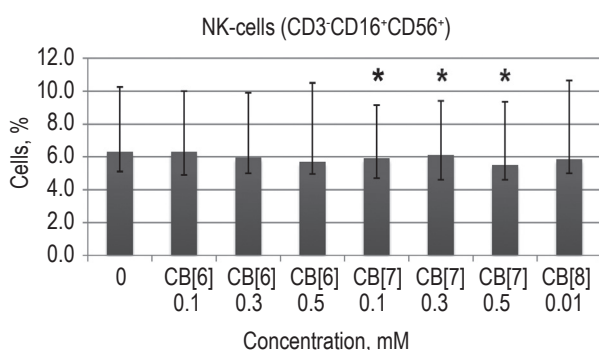


Figure 1. The frequency of NK-cells in the presence of CB[6], CB[7], CB[8]

Note. Human PBMCs were cultured with CB[6] and CB[7] at concentrations 0.1 mM, 0.3 mM, 0.5 mM and with CB[8] at a concentration of 0.01 mM for 72h. PBMCs cultured without CB[n] were used as a control (0 mM). The frequency of CD3⁺CD16⁺CD56⁺ NK-cells were evaluated by flow cytometry. Data presented as median and interquartile range ($n = 8$). * – significant differences compared to control (Wilcoxon test), $p < 0.05$.

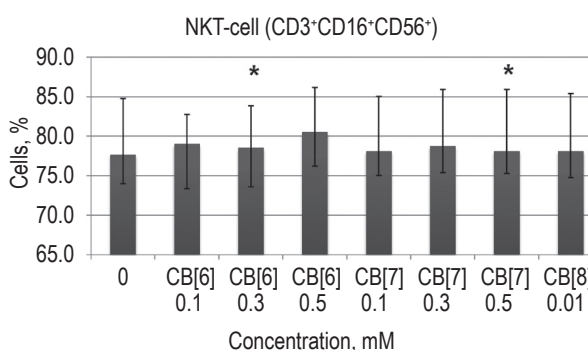


Figure 2. The frequency of NKT-cells in the presence of CB[6], CB[7], CB[8]

Note. Human PBMCs were cultured with CB[6] and CB[7] at concentrations of 0.1 mM, 0.3 mM, 0.5 mM and with CB[8] at a concentration of 0.01 mM for 72h. PBMCs cultured without CB[n] were used as a control (0 mM). The frequency of CD3⁺CD16⁺CD56⁺ NKT-cells were evaluated by flow cytometry. Data presented as median and interquartile range ($n = 8$). * – significant differences compared to control (Wilcoxon test), $p < 0.05$.

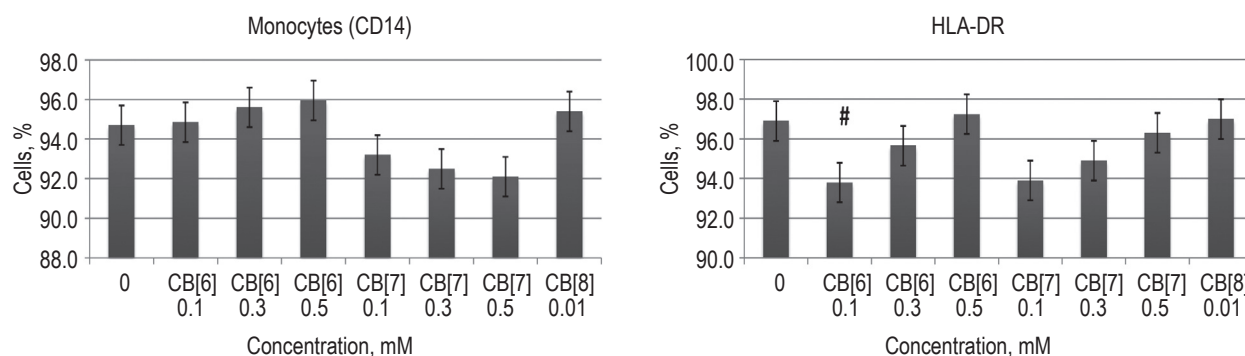


Figure 3. The frequency of monocytes and expression of HLA-DR molecules on monocytes in the presence of CB[6], CB[7], CB[8]

Note. Human PBMCs were cultured with CB[6] and CB[7] at a concentration of 0.5 mM and with CB[8] at a concentration of 0.01 mM for 72h. PBMCs cultured without CB[n] were used as a control (0 mM). The frequency of CD3⁺CD14⁺ monocytes (A) and HLA-DR expression (B) were evaluated by flow cytometry. Expression Data presented as median and interquartile range (n = 8). # – tendency compared to control (Wilcoxon test), p = 0.06

lopment and modification of immunocompatible nanomaterials. Nanoconstructions are mainly used for cancer immunotherapy, vaccines and treatment of autoimmune diseases. However, the mechanism and molecular pathways of how nanomaterials affect

the innate immune system are not clear. Therefore, more research is needed to understand the interaction of cucurbiturils with the innate immune system to develop new prospective strategies for the prevention, diagnosis or treatment of human diseases.

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РОЛЬ ГЛИКОДЕЛИНА В РЕГУЛЯЦИИ ДИФФЕРЕНЦИРОВКИ МИЕЛОИДНЫХ СУПРЕССОРНЫХ КЛЕТОК

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Резюме. Гликоделин (PP14, PAEP, альфа-2-микроглобулин, димерный гликопротеин с молекулярной массой от 42 до 56 кД) рассматривается как маркер рецептивности репродуктивной ткани. Невзирая на то, что иммунодепрессивные эффекты гликоделина хорошо известны, его роль в регуляции миелоидных супрессорных клеток (MDSC) не изучена. MDSC представляют собой гетерогенную популяцию незрелых миелоидных клеток, которые при патологических состояниях приобретают супрессорный фенотип, подавляя иммунный ответ.

Известно, что MDSC играют важную роль в поддержании иммунной толерантности в период беременности и при трансплантации. Наша гипотеза состоит в том, что гликоделин способен индуцировать формирование MDSC, поскольку уровень этих клеток во время успешной беременности повышается, в то время как спонтанное прерывание беременности и развитие преэклампсии ассоциировано с низким уровнем циркулирующего гликоделина. Таким образом, целью работы являлось изучение роли рекомбинантного гликоделина в физиологических концентрациях, в регуляции дифференцировки MDSC.

Для генерации MDSC *in vitro* мононуклеарные клетки из периферической крови добровольцев-доноров сепарировали путем центрифугирования на градиенте плотности 1,077 г/см³ (Ficoll-Нугауе, Sigma-Aldrich). Затем полученные клетки культивировали в 24-луночной планшете в концентрации 1 × 10⁶ клеток/мл в ППС с внесенными в среду цитокинами IL-6 (20 нг/мл), GM-CSF (40 нг/мл) в течение 14 суток при 5% CO₂ и 37 °C. Замена среды в культуре производилась на 7-е сутки, тогда же повторно вносили цитокины, а на 11-е сутки вносили рекомбинантный гликоделин (MyBioSource, Inc., США) в физиологических концентрациях (0,2; 2 мкг/мл), фармакологическая концентрация была 50 мкг/мл. В культурах оценивали уровень M-MDSC (Lin⁻HLA-DR⁺CD33⁺CD11b⁺CD14⁺CD66b⁻) и PMN-MDSC (Lin⁻HLA-DR⁺CD33⁺CD11b⁺CD14⁺CD66b⁺) методом проточной цитометрии (CytoFlexS (Beckman Coulter)) при помощи антител R&D Systems по стандартной методике. Статистическую обработку данных проводили в программе GraphPad Prism 6 при помощи критерия Фридмана.

Установлено, что гликоделин не оказывал существенного влияния на жизнеспособность клеток, оцениваемую методом проточной цитометрии (PI). Высокая концентрация GdA (50 мкг/мл), которая

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является фармакологической, не оказывала достоверного эффекта на дифференцировку MDSC. В то же время гликоделин в концентрациях, соответствующих нормальной беременности (0,2; 2 мкг/мл), повышал процент MDSC в индуцированных культурах мононуклеарных клеток человека.

При анализе субпопуляций установлено, что этот эффект был обусловлен повышением уровня PMN-MDSC, в то время как уровень M-MDSC достоверно не изменялся. Данный результат можно интерпретировать как фетопротективный эффект гликоделина, поскольку повышение уровня G-MDSC связывают с подавлением иммунного ответа на патернальные антигены. Известно, что уровень PMN-MDSC увеличивается в периферической крови здоровых беременных женщин на всех стадиях беременности по сравнению с небеременными, тогда как число M-MDSC остается без изменений.

В то же время у пациенток с выкидышем более чем на 30% снижено количество MDSC в крови и эндометрии, особенно в первом триместре. В период физиологической беременности PMN-MDSC накапливаются в плаценте, но при спонтанном аборте их количество уменьшается. Плацентарные PMN-MDSC эффективно подавляют Т-клеточный ответ, одновременно поляризуя CD4⁺-лимфоциты в фенотип Th2. Вероятно, PMN-MDSC играют важную роль в индуцировании и поддержании толерантности к антигенам плода, что позволяет рассматривать их как перспективную мишень терапевтического манипулирования при осложнениях беременности. В целом, мы впервые продемонстрировали влияние GdA на процесс дифференцировки MDSC.

Ключевые слова: гликоделин, миелоидные супрессорные клетки, дифференцировка, беременность, культивирование клеток, иммунная толерантность

ROLE OF GLYCODELIN IN REGULATION OF MYELOID-DERIVED SUPPRESSOR CELL DIFFERENTIATION

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Abstract. Glycodelin (PP14, PAEP, alpha-2-microglobulin, dimeric glycoprotein with molecular weight of 42 to 56 kDa) is considered as a reproductive tissue receptivity marker. Despite that glycodelin immunosuppressive effects are well-known there still remains uncovered its role in myeloid suppressor cell (MDSC) regulation. MDSC represent the heterogeneous population of immature myeloid cells that acquire suppressor phenotype while inhibiting the immune response under the pathological states. MDSC are known to play an essential role in supporting the immune tolerance in pregnancy and at transplantation. Our hypothesis suggests that glycodelin is capable of inducing the MDSC formation as the level of these cells is elevated during the successful pregnancy, whereas the spontaneous abortion and progression of eclampsia are associated with low circulating glycodelin. Therefore, the aim of the work was to analyze the role of recombinant glycodelin in physiological concentrations in regulation of MDSC differentiation. Peripheral blood mononuclear cells of donor volunteers were separated via centrifugation on density gradient of 1,077 g/cm³ (Ficoll-Hypaque, Sigma-Aldrich) to obtain MDSC generation *in vitro*. Then cells obtained were cultured in 24-well plate at a concentration of 1×10^6 cell/ml in complete medium with cytokines IL-6 (20 ng/ml), GM-CSF (40 ng/ml) therein for 14 days at 37 °C and 5% CO₂. Medium replacement was made by 7th day in culture followed by cytokine re-introduction, and on the 11th day recombinant glycodelin in physiological concentrations (0,2; 2 mkg/ml) was applied while the pharmacological concentration was 50 mkg/ml. The M-MDSC (Lin⁻HLA-DR⁺CD33⁺CD11b⁺CD14⁺CD66b⁻) and PMN-MDSC (Lin⁺HLA-DR⁺CD33⁺CD11b⁺CD14⁺CD66b⁺) level was evaluated in cultures using flow cytometry (CytoFlexS (Beckman Coulter)) and "R&D Systems" antibodies according to standard protocol. Statistical data processing was realized with GraphPad Prism software using Friedman test. It was found that glycodelin did not significantly affect cell viability being assessed with flow cytometry (PI). It was revealed that high GdA concentration (50 mkg/ml) being pharmacological did

not render significant effect on MDSC differentiation. Meanwhile, glycodelin in concentrations corresponding the healthy pregnancy (0,2; 2 mkg/ml) was stated to increase the MDSC percentage in induced cultures of human mononuclear cells. When analyzing the subsets it was disclosed that this effect was conditioned by the increase in PMN-MDSC level while the M-MDSC level remained significantly unchanged. This result could be interpreted as glycodelin fetoprotective effect as the increase of the PMN-MDSC level is associated with the suppression of the immune response to paternal antigens. The PMN-MDSC level is known to be elevated in peripheral blood of healthy pregnant women at all the stages of pregnancy as compared to non-pregnant subjects whereas the M-MDSC amount remains unaltered. Meanwhile, patients with miscarriage demonstrated more that by 30% lowering in the MDSC amount in blood and endometrium and in I trimester, in particular. During the physiological pregnancy PMN-MDSC accumulate in placenta, but at spontaneous abortion their number is found to be declined. Placental PMN-MDSC efficiently suppress the T-cell response while concurrently polarizing the CD4⁺ lymphocytes in Th2 phenotype. PMN-MDSC are suggested to play an essential role in inducing and supporting the tolerance to fetal antigens that allows considering these as promising target of therapeutical manipulation in pregnancy complications. As a whole, we have originally demonstrated the GdA effect on MDSC differentiation.

Keywords: glycodelin, myeloid-derived suppressor cells, differentiation, pregnancy, cell culture, immune tolerance

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Introduction

In 1976, D.D. Petrunin, Ju.S. Tatarinov et al. initially isolated and identified glycodelin as a novel placental antigen that was called chorionic α 2-microglobulin [8]. Subsequently, while accumulating new data on the protein location and properties, its name changed from placental α 2-microglobulin, fertility α 2-microglobulin (AMGF), and finally to specific α 2-microglobulin.

Outside Russia, several independent researchers described glycodelin as a placental protein 14 (PP14), pregnancy-associated endometrial α 2-globulin (α 2-PEG), endometrial protein 15 (EP15), progestagen-dependent endometrial protein (PEP), α -uterine protein (AUP), and progesterone-associated endometrial protein (PAEP). In 1995, A. Dell et al. suggested to define the protein as a “glycodelin” describing its unique property: a sex-dependent glycosylation to overcome confusion in terms.

Glycodelin (GdA) is considered as the receptivity marker for reproductive tissue. In 2018, glycodelin was found to prevent experimental graft rejection [2] that favors its pharmacological application. Schneider et al. [9, 10] suggested a possible usage of glycodelin in lung transplantation. Despite that, immunosuppressive effects of glycodelin are well-known [1]. Its role in the regulation of cell functions shaping immune tolerance against allogenic graft remains unclear.

In particular, the role of glycodelin in the regulation of key (with regard to evolutionary biology) cell population myeloid-derived suppressor cells (MDSC) is still uncertain. MDSC represents a heterogeneous population of immature myeloid cells acquiring

suppressor phenotype under the pathological states and repressing the immune response [3].

Recent research works demonstrated the MDSC key role in supporting immune tolerance in transplantation [12]. There is a hypothesis that glycodelin might be capable of inducing the MDSC formation because their level is elevated in successful pregnancy. In contrast, spontaneous abortion and preeclampsia progression are associated with low circulating glycodelin level.

Therefore, **this work was aimed** at examining human recombinant GdA role in MDSC formation and regulation playing an important role in allograft tolerance induction.

Materials and methods

The study was conducted according to the requirements of the Declaration of Helsinki WMA (2000) and protocol to the European Council Convention on Human Rights and Biomedicine (1999); the permission from the Ethics Committee of the IEGM UB RAS of 30.08.2019 (IRB00010009) was received.

Glycodelin was used in physiological range because it corresponds to its female peripheral blood level during pregnancy. GdA peaks at 2.0 μ g/ml by the end of the trimester I reaching as high as 0.2 μ g/ml in the trimester II, but declining to the minimal value or even not detected in the trimester III.

Recombinant glycodelin (PAEP), (*E. coli*), MBS 718444 was used; glycodelin (PAEP) was received from the <https://www.mybiosource.com/recombinant-protein/glycodelin-paep/718444>.

Fractionated peripheral blood mononuclear cells (PBMC) of apparently healthy donors (n = 6, women of 28 $\pm$ 6 years) were used. Each donor provided a written informed consent. PBMC were obtained using

density gradient (1.077 g/cm³) centrifugation (Diacoll "Dia-M", Russia).

MDSC production *in vitro*. To obtain MDSC *in vitro* PBMC were cultured in 96-well plate at 1×10^6 cell/ml concentration in complete medium (RPMI-1640, 10% FBS, 10 mM Hepes, 2 mmol L-glutamine (ICN Ph., USA) added with penicillin-streptomycin-amphotericin (100 µL per 10 ml of medium "B.I.", Israel) for 14 days (37 °C, 5% CO₂). Recombinant cytokines IL-6 (20 ng/ml), GM-CSF (40 ng/ml) (Miltenyi Biotech, Germany) were applied for MDSC induction.

A complete medium was replaced on day 7, followed by three days of exposure to recombinant glycodeclin (GdA, "MyBioSource. Inc.", *E. coli*; USA) at physiological concentrations (0.2; 2.0 µg/ml) corresponding to its level in peripheral blood of pregnant women; pharmacological concentration was equal to 50 µg/ml [7]. Thereafter, cells were cultured for another three days followed by collection using the "Accutase" according to the manufacturer's recommendations (Capricorn Scientific, Germany).

Then cells were stained to detect viability with propidium iodide (PI) (Biolegend, USA) according to the manufacturer's protocol. The percentage of live (PI⁻) cells in cultures (using a gate of living cells on FSC/SSC plot) varied within 85.5-92.1%.

Following standard washing procedures, cells were stained with antibodies to determine the MDSC percentage by flow cytometry (Lin⁻HLA-DR⁺CD33⁺CD11b⁺) as well as M-MDSC (Lin⁻HLA-DR⁺CD33⁺CD11b⁺CD14⁺CD66b⁺) and PMN-MDSC (Lin⁻HLA-DR⁺CD33⁺CD11b⁺CD14⁺CD66b⁺). Staining was made using antibodies (Alexa Fluor 750-HLA-DR, APC-Anti Siglec-3/CD33, Alexa Fluor 405-Anti-Integrin αM/CD11b, PE-CEACAM-8/CD66b and PerCP- Anti CD14, all purchased from "R&D Systems", USA) according to methodology suggested by the manufacturer. Percentage of appropriate cell subsets (MDSC, M-MDSC, PMN-MDSC) was assessed on CytoFLEX S flow cytometer (Beckman Coulter, USA). Flow cytometry data files

were analyzed using the "KALUZA Analysis 2.1 Software".

Statistical data processing was realized with GraphPad Prism 8 and the non-parametric Wilcoxon test.

Results and discussion

Experiments demonstrated that GdA resulted in elevated MDSC percentage in cytokine-induced human mononuclear cell cultures that was significant only at its low concentrations (0.2 and 2.0 µg/ml) that fits its pregnancy-related range. While analyzing the cell subsets, it was demonstrated that the rise in the MDSC level treated with GdA (0.2 and 2.0 µg/ml) resulted in significant PMN-MDSC increase, whereas the M-MDSC level was changed insignificantly. Therefore, GdA did not reliably affect the M-MDSC (Table 1). It was determined that high GdA concentration (50 µg/ml) corresponding to the pharmacological level conferred no significant effect on MDSC differentiation.

Cell viability in cultures was assessed following the propidium iodide staining. It was revealed that GdA at all concentrations did not affect cell viability in culture. Gating strategy according to light scattering parameters in FSC/SSC plot was applied to analyze solely live cells. Therefore, the effects obtained are related indeed to GdA direct influence on MDSC differentiation.

More recently, it became evident that the elevation of MDSC level also accompanied pregnancy. In 2014, it was found that the PMN-MDSC level was increased in peripheral blood of healthy pregnant women at all the stages of pregnancy as compared to non-pregnant subjects, while the M-MDSC amount remained unchanged [4]. Around the same time, it was revealed that patients with miscarriage had count of blood and endometrial MDSCs by 30% lower particularly in trimester I [6].

PMN-MDSC are known to accumulate in placenta of healthy pregnant women, while their number declines in spontaneous abortion. Placental PMN-MDSCs effectively suppress T-cell response

TABLE 1. GdA EFFECT ON MDSC PERCENTAGE IN MONONUCLEAR CELL CULTURES INDUCED BY IL-6 AND GM-CSF CYTOKINES, Me (Q_{0.25}-Q_{0.75}), n = 4

Cell type/GdA concentration	Control	GdA 0.2 µg/ml	GdA 2 µg/ml	GdA 50 µg/ml
MDSC % (CD33 ⁺ CD11b ⁺)	21.5 (15.2-26.1)	32.3 [#] (24.8-38.5)	28.2 [#] (21.5-37.4)	22.5 (16.7-30.2)
PMN-MDSC % (CD33 ⁺ CD11b ⁺ CD14 ⁺ CD66b ⁺)	12.6 (7.8-15.7)	17.4 [#] (12.6-24.3)	16.8 [#] (11.7-22.1)	15.1 (7.8-21.3)
M-MDSC % (CD33 ⁺ CD11b ⁺ CD14 ⁺ CD66b ⁺)	6.2 (3.4-7.9)	7.6 (4.6-11.3)	6.9 (5.5-10.1)	5.8 (2.8-7.6)

Note. Percentages of cells in corresponding subsets from HLA-DR⁺Lin⁻ cells are presented. #, significant differences as compared with control by Wilcoxon U test (p < 0.05).

with subsequent polarization of CD4⁺-lymphocytes to Th2-phenotype [4]. PMN-MDSC are suggested to play an essential role in inducing and supporting the tolerance to fetal antigens allowing to consider them as a promising target for therapeutic manipulation under the pregnancy complications.

Meanwhile, the question about exact nature of receptor mediating GdA effects remains open. Subsequent analysis of GdA mode of action showed that the surface L-selectin molecule on monocyte-macrophage lineage cells is a binding protein for GdA. Thus, the antibody against L-selectin reduced the GdA binding and GdA-induced IL-6 production [5]. In particular, Vijayan et al. in 2018 found potential receptors for GdA on human peripheral blood monocyte membrane.

SIGLEC-7 (sialic acid-binding immunoglobulin-like lectin 7, CD328) – receptor to GdA was identified using co-immunoprecipitation and flow cytometry. GdA was found under culture conditions to improve the expression of decidual macrophage markers IDO-1 and CD209 on GdA-polarized macrophages. SIGLEC-7 receptor blocks GdA-related biological effects on monocyte differentiation [11]. However, it is not clear through which receptor this occurred, and we can only speculate.

Therefore, we have initially demonstrated that GdA can regulate the MDSC differentiation primarily by increasing its polymorphonuclear subset (PMN-MDSC). Potentially, GdA possesses the potential to manipulate the MDSC level that, in turn, serve as the immune response suppressors.

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НАНОЧАСТИЦЫ ОКСИДА ГРАФЕНА В РЕГУЛЯЦИИ ОКИСЛИТЕЛЬНОЙ АКТИВНОСТИ МОНОЦИТОВ ЧЕЛОВЕКА

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Резюме. Благодаря своим свойствам, материалы на основе графена обладают потенциалом к использованию в биомедицине, но из-за их цитотоксического и провоспалительного воздействия на клетки практическое применение препаратов на его основе затруднено. Известно, что различные поверхностные модификации (функционализация) наночастиц оксида графена (ОГ) полиэтиленгликолем (ПЭГ) — один из способов снижения негативных эффектов графена на живые клетки. Так как применение наночастиц подразумевает их взаимодействие с иммунной системой, которая участвует в защите организма и в регуляции его функций, изучение этого вопроса крайне важно. Моноциты — клетки врожденного иммунитета и первая линия защиты человеческого организма от микроорганизмов и других чужеродных объектов. Одна из реакций моноцитов на стимул любой природы — производство активных форм кислорода (АФК). Ранее опубликованные данные демонстрируют неполную картину влияния наночастиц модифицированного оксида графена на образование АФК моноцитами человека. Таким образом, целью нашего исследования явилось оценка влияния пегилированного оксида графена (ОГ-ПЭГ и ОГ-8армПЭГ) на продукцию АФК моноцитами человека в тесте спонтанной и стимулированной люминол-зависимой хемилюминесценции (ЛЗХЛ).

В качестве объектов исследования использовались CD14⁺-клетки, выделенные из мононуклеаров периферической крови здоровых доноров. Продукция АФК стимулировалась опсонизированным зимозаном (ОЗ), в качестве контроля выступал спонтанный вариант ЛЗХЛ. В работе использовались частицы оксида графена модифицированные линейным и разветвленным (армированным) ПЭГом (ОГ-ПЭГ и ОГ-8армПЭГ) размерами 100-200 нм («малые») и 1-5 мкм («большие»), с количеством покрывающего ПЭГ около 20%. Наночастицы применяли в концентрациях 5 и 25 мкг/мл.

Установлено, что на уровне спонтанной продукции АФК наночастицы малого размера в низкой концентрации (5 мкг/мл) и наночастицы большой размерности, покрытые разветвленным ПЭГом, в обеих концентрациях, оказывают достоверные подавляющие эффекты. На уровне стимулированной продукции АФК было обнаружено, что наночастицы графена малой размерности в концентрации 25 мкг/мл также подавляли продукцию АФК, как и частицы большой размерности, покрытые линейным ПЭГом, в той же концентрации. Таким образом, нами впервые установлено, что наночасти-

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цы оксида графена, функционализированные ПЭГом, способны ингибировать продукцию активных форм кислорода моноцитами человека, в связи с чем, можно говорить об антиоксидантной активности ОГ-ПЭГ.

Ключевые слова: оксид графена, функционализированные наночастицы, полиэтиленгликоль, люминол-зависимая хемилюминесценция, моноциты, активные формы кислорода

GRAPHENE OXIDE NANOPARTICLES IN THE REGULATION OF THE OXIDATIVE ACTIVITY OF HUMAN MONOCYTES

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Abstract. Graphene-based materials have an opportunity for use in biomedicine, thanks to their properties. Nevertheless, due to its cytotoxic effects, the use of graphene-based drugs is problematic. However, the surface modification of graphene oxide (GO) nanoparticles with a polyethyleneglycol (PEG) is one way to reduce the harmful effects of graphene on cells. Applying nanoparticles implies their interaction with the immune system, which protects the body. Monocytes are innate immunity cells and the first line of defence of the human organism from microorganisms and other alien objects. One of the monocytes' reactions to a stimulus of any nature is to produce reactive oxygen species (ROS). Published data shows an incomplete picture of modified graphene oxide nanoparticles' effects on ROS formation by human monocytes. Thus, it was essential to evaluate the pegylated graphene oxide (GO-PEG and GO-8armedPEG) effect on ROS production by human monocytes, assessed by the luminol-dependent chemiluminescence (LCL). The objects of the study were CD14⁺-cells isolated from mononuclear cells of healthy donors. ROS production was stimulated by opsonized zymosan (OZ), spontaneous LCL was used as a control. PEG-modified (GO-PEG and GO-8armedPEG) GO nanoparticles with sizes of 100-200 nm ("small") and 1-5 μ m ("big") with PEG covering ~ 20% were used at concentrations of 5 and 25 μ g/ml.

The study showed that small size nanoparticles at a low concentration of 5 μ g/ml and big nanoparticles coated with 8-armed PEG at both concentrations have a significant suppressive effect on spontaneous ROS production. In the stimulated LCL reaction variant, it was found that small nanoparticles (25 μ g/ml) also have a suppressive effect on ROS production, such as big-sized particles coated with linear PEG at the same concentration. Thus, we have established for the first time that graphene oxide nanoparticles functionalized with PEG are capable of inhibiting the ROS production by human monocytes, and therefore, we can speak of the antioxidant activity of GO-PEG.

Keywords: graphene oxide, surface modification of nanoparticles, polyethyleneglycol, luminol-dependent chemiluminescence, monocytes, reactive oxygen species

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Introduction

Graphene is a lightweight two-dimensional material made from a monatomic carbon layer. Graphene and its modifications, particularly graphene oxide (GO), are currently being investigated for use in various biomedicine fields [9, 10]. However, the GO cytotoxicity and proinflammatory effects are the factors limiting the use of GO-based drugs and hinder the translation of scientific developments into prac-

tice. It is known that the surface modification (functionalization) of GO nanoparticles using the polymer-adsorbed particles is one of the ways to reduce the harmful graphene-related cell effects [2, 3]. In particular, polyethylene glycol (PEG) is the most common material used for functionalization of GO nanoparticles. In general, the use of graphene-based materials in medicine requires a thorough assessment of their biocompatibility and a detailed understanding of their interaction with immune cells.

Our work was focused on studying the modified GO preparations effect on the formation of free radicals and reactive oxygen species (ROS) by mono-

cytes, which play an important role in the body nonspecific immune defense. Scarce data on how GO nanoparticles modulate the functions of human monocytes are available. Nevertheless, it is known that GO nanoparticles could reduce the viability THP-1 monocytic line cells, whereas the GO functionalization via the amino groups (GO-NH₂) significantly reduced graphene cytotoxicity. Simultaneously, GO, and GO-NH₂ nanoparticles increased the level of proinflammatory intracellular cytokines (TNF α , MIP-1 β , IL-6) in human monocytes. [11, 14]. In 2020, it was evidenced that GO, coated with AgInS₂ crystals, increased the level of TNF α and MIP-1 β in human monocytes [5]. Thus, **our work aimed** to study PEG-modified GO nanoparticle effect on the oxidative activity of human monocytes, assessed by luminol-dependent chemiluminescence (LCL).

Materials and methods

The study was carried out in accordance with the 2000 Helsinki Declaration of the WMA and the protocol of the 1999 Council of Europe Convention on Human Rights and Biomedicine; permission from the Ethics Committee of the Institute of Ecology and Genetics of Microorganisms of the Ural Branch of the Russian Academy of Sciences (IRB00010009) dated of 30.08.2019 was obtained to use peripheral blood samples.

We used linear and armed polyethylene glycol (PEG and PEGarm, respectively)-based coated GO nanoparticles sized of 100–200 nm (“small”, GO-S) and 1–5 μ m (“big”, GO-B) (Ossila Ltd, Great Britain). For functionalization procedures, we used monochloroacetic acid (99%), 1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC, 98%), N-hydroxysuccinimide (NHS, > 98%), 8-arm-polyethylene glycol- (tripentaerythritol core) -NH₂ (8armPEG-NH₂, Mw 10,000 g·mol⁻¹), methoxy polyethylene glycol amine mPEG-NH₂ (MW 5 kDa), manufactured by Alfa Aesar, USA. All reagents were used without additional purification. GO modification with linear PEG and branched 8armPEG was carried out by using the covalent attachment of amino groups PEG-NH₂ and 8armPEG-NH₂ to the surface carboxyl groups of GO with the formation of an amide bond. For this, GO-B and GO-S solutions (2 mg/ml) were sonicated

for 30 min at 25 W and 150 W, respectively. GO carboxylation was carried out in an alkaline medium (NaOH) in the presence of Cl-CH₂-COOH with ultrasonic treatment for 1 hour. The resulting GO-COOH solution was neutralized by repeated washing with deionized water. Next, NHS (10 mmol/L), EDC (4 mmol/L), and PEG-NH₂ (or 8armPEG-NH₂) (2 mg/ml) were added to the suspension of GO-COOH (pH 5.6) under ultrasonic treatment for 5 min., to complete the reaction of PEG covalent crosslinking, the solution was left at room temperature for 24 hrs. The resulting suspensions of GO-PEG or GO-8armPEG were purified by dialysis and triple washing by centrifugation with ethyl alcohol with final drying at 65 °C under vacuum.

Fourier-transform infra-red spectra of the initial and modified GO were obtained on an IFS 66/S Bruker spectrometer in the range 400–4000 cm⁻¹. Samples for analysis were prepared by pressing KBr tablets (2 mg sample to 299 mg KBr). The absorption spectra of the initial and modified GO solutions were determined on a UV 2600 double-beam spectrophotometer in the wavelength range of 200–900 nm. The size distribution of the initial and modified GO, as well as their zeta potential in aqueous solutions, was determined by dynamic light scattering on a ZetaPALS Brookhaven instrument. Thermogravimetric analysis of the initial and modified GOs was carried out on a TGA/DSC 1 Mettler-Toledo combined TG-DSC instrument at a heating rate of 10 K min⁻¹ within the temperature range 30–900 °C in an inert atmosphere.

The studies of the pegylated GO samples showed that the chemical modification process was successful regardless of the size of the graphene particles, the aromatic structure of GO did not change, and the average size of pegylated GO nanoparticles decreased compared to the initial parameters declared by the manufacturer. The characteristics of the obtained particles are presented in Table 1.

Here we examined peripheral blood mononuclear cells (PBMC) obtained from apparently healthy donors (n = 7) isolated by density gradient centrifugation (1.077 g/cm³) (Diacoll, Dia-M, Russia). The cells were then washed from serum three times by centrifugation at 350 g for 20 minutes using RPMI-1640 medium (Sigma, USA). PBMC-derived CD14⁺ cells were isolated by the immunomagnetic separation

TABLE 1. CHARACTERISTICS OF GO-PEG NANOPARTICLES USED IN THE WORK

	OG-PEG-S	OG-PEGarm-S	OG-PEG-B	OG-PEGarm-B
Average effective diameter, nm	184 $\pm$ 73	287 $\pm$ 52	569 $\pm$ 14	1376 $\pm$ 48
Polydispersion index	0.25 $\pm$ 0.02	0.23 $\pm$ 0.02	0.21 $\pm$ 0.02	0.30 $\pm$ 0.01
Zeta-potential, mV	-31.70 $\pm$ 1.70	-34.28 $\pm$ 0.41	-39.98 $\pm$ 1.17	-53.56 $\pm$ 1.23
Coating degree, weight %	17.2 $\pm$ 1.4	20.5 $\pm$ 1.8	19.4 $\pm$ 2.2	20.5 $\pm$ 1.1

based on the MACS® technology (Miltenyi Biotec, Germany). The isolated cells were adjusted to a concentration of 10^6 cells per ml dissolved in Hanks's solution (Biolot, Russia).

The LCL reaction was carried out in the wells of a 96-well sterile plate (Nunc, Denmark) added with Hanks's solution (Biolot, Russia), graphene nanoparticles in various pharmacological concentrations (5 µg/ml; 25 µg/ml; control – spontaneous LCL), 10 µL of cell suspension and the luminol sodium salt (Sigma-Aldrich, USA) at a concentration of 20 µM [7]. In addition, pooled inactivated human serum was added to the samples to a final 10% concentration. Opsonized zymosan (OZ) acted as an inductor of oxygen burst added to a final concentration of 1.5 µg/ml.

The luminescence intensity was measured for 90 minutes with an interval of 3 minutes on a Synergy H1 hybrid reader (BioTek). The dynamic change in luminescence within 90 minutes at 37°C was assessed, and the integral indicator was calculated: the light sum (S), i.e., area under the chemiluminescence curve characterizing the total ROS synthesis in 90 min of the study and equal to the sum of all values of the luminescence intensity for each sample.

The percentage of live and dead cells was assessed by staining with propidium iodide (Thermo Fisher Scientific, USA) followed by analysis on flow cytometer (CytoFlexS, Beckman Coulter, USA). The viability of monocytes in the presence of nanoparticles averaged 96% of the total number of CD14⁺-cells.

Statistical data processing was performed by using GraphPad Prism 8 software in Friedman's test and Dunn's posthoc test for multiple comparisons.

Results and discussion

One of the key functions of monocytes is the ROS generation, which can occur when cells interact with nanoparticles of almost any nature. Thus, cell stimulation is accompanied by the superoxide anion, hydrogen peroxide, hydroxyl radical, and chlorine active form generation, which have a potent bactericidal effect; and the LCL reaction characterizes the total ROS production by isolated monocytes.

The study showed that small size nanoparticles (GO-PEG-S and GO-PEGarm-S) at a low concentration of 5 µg/ml have a significant suppressive effect on spontaneous ROS production. Interestingly, a high concentration (25 µg/ml) of small nanoparticles (GO-PEG-S and GO-PEGarm-S) did not affect this parameter. It was shown that big nanoparticles also suppressed ROS production, while the effect was exerted only by particles coated with armed PEG (GO-PEGarm-B) at both concentrations (Table 2).

In case of the stimulated ROS production, small nanoparticles (GO-PEG-S and GO-PEGarm-S) were also found to exert a suppressive effect, which, however, was evident after using a high concentration of particles (25 µg/ml) only. Only nanoparticles coated with linear PEG at a high concentration (GO-PEG-B, 25 µg/ml) displayed a significant suppressive effect among the large-sized particles.

There are various possible ways of the GO nanoparticle effect on LCL. First, GO nanoparticles can directly interact with ROS and luminol, changing the intensity of LCL, and secondly, GO can affect the ROS production by monocytes. Since GO and GO-PEG reduced the LCL in blood monocytes, we assumed that it might be accounted for by an effect of

TABLE 2. GO-PEG EFFECT ON THE OF SPONTANEOUS AND ZYMOSAN-STIMULATED CHEMILUMINESCENCE LIGHT SUM (n = 7), Me ($Q_{0.25}$ – $Q_{0.75}$)

	Control	GO-PEG-S (µg/ml)		GO-PEG-arm-S (µg/ml)		GO-PEG-B (µg/ml)		GO-PEG-arm-B (µg/ml)	
		5	25	5	25	5	25	5	25
Light sum	Spontaneous LCL (90 min incubation)								
	419 (417-521)	265 (254-307) p < 0.05*	312 (285-358)	259 (256-305) p < 0.05*	313 (300-359)	285 (263-324)	343 (333-404)	265 (244-311) p < 0.05*	271 (258-313) p < 0.05*
	OZ-stimulated (1.5µg/ml) LCL (90 min incubation)								
	4354 (3848-4812)	4026 (3757-4234)	2426 (2256-2653) p < 0.05*	4111 (3863-4498)	2538 (2249-2674) p < 0.05*	4190 (3903-4383)	2585 (2335-2871) p < 0.05*	3981 (3611-4220)	3133 (2782-3287)

Note. * p < 0.05, significant differences according to Friedman's test and Dunn's test for multiple comparisons compared to the control. Control, sample without GO-PEG (first column).

particle-driven quenched luminescence, which is not associated with its impact on cells. For this, we used a cell-free model of LCL, where hydrogen peroxide was used as a luminescence inductor. As a result, the effect of luminescence quenching by GO-PEG nanoparticles was not revealed [12].

Thus, it has been established that GO nanoparticles coated with linear and armed PEG have, first of all, suppressive effects on human monocyte ROS production. Interestingly enough, to enable such an effect, the concentration of small nanoparticles was primarily critical, and the type of pegylation was not of fundamental importance. However, for large nanoparticles the type of pegylation was of fundamental importance. Thus, at the level of spontaneous monocyte ROS production, only armed PEG-coated GO “were active”. Simultaneously, in zymosan-stimulated samples, the suppressive effect was conferred if the particles were coated only with linear PEG. In general, it is quite interesting that the overall pattern of effects was suppressive, but the nuances depended on the size, concentration, and type of nanoparticle pegylation.

Thus, we have established for the first time that graphene oxide nanoparticles functionalized with PEG are capable of inhibiting the ROS production by human monocytes, and therefore, we suggest GO-PEG-related antioxidant activity.

Specific response of monocytes, macrophages, and neutrophils to a stimulus is coupled to increased ROS production called “oxygen burst”, which is aimed at destroying foreign objects. However, the data on the antioxidant activity of GO are contradictory. In 2019 studies, it was shown that GO exhibits low antioxidant activity against H_2O_2 and OH^* , while Qui et al. considered about high antioxidant activity of GO (10 $\mu g/ml$) to OH^* , as well as its ability to neutralize the superoxide anion (at a concentration of about 80 $\mu g/treated/untreated\ ml$) [4, 8, 13]. Chemical modification of GO (doping with nitrogen, the introduction of various functional groups) significantly increases its antioxidant activity, in this regard, it can be assumed that treatment with PEG affects the neutralization of ROS [6]. However, in the study by Nilewski et al., pegylation did not significantly affect the antioxidant activity of GO to the superoxide anion [1]. At the same time, our study allowed to demonstrate that the type of pegylation is essential for conferring GO antioxidant effect.

In general, the data we obtained expand the knowledge about modified forms of GO nanoparticles in the context of their antioxidant activity, and demonstrate for the first time the data on the interaction between GO-PEG and monocytes affecting formation of reactive oxygen species therein.

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ВЛИЯНИЕ ЭЛЕКТРОПОРАЦИИ ДНК КОНСТРУКЦИЯМИ НА ДЕНДРИТНЫЕ КЛЕТКИ

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Резюме. На сегодняшний день, трансфекция клеток млекопитающих ДНК или РНК конструкциями является единственным методом доставки запрограммированной информации в ядро клетки. Одним из часто используемых методов трансфекции в работе с дендритными клетками, является электропорация. Суть метода состоит в повышении проницаемости мембраны путем проведения электрического импульса через клетку. В связи с повышенной проницаемостью мембраны повышается шанс попадания ДНК или РНК конструкций внутрь клетки, но при этом снижается выживаемость клетки после воздействия тока на плазматическую мембрану клетки.

В исследовании использовали мышей самцов линии C57Bl/6 возраста 2–4 месяца. Из бедренной кости мышей выделяли 20×10^6 клеток костного мозга, после клетки культивировали в полной RPMI-1640 среды в течение 7 суток. Для генерации дендритных клеток из клеток костного мозга, в культуральную среду добавляли 100 нг/мл gmFlt3-L на 0 день. После 7 дней культивирования, клеточную культуру электропорировали контрольными некодирующими плазмидами р5 (EP P5) или плазмидами рmaxCCR9 (EP CCR9), кодирующими мышинный рецептор хемотаксиса CCR9. В качестве контролей выступали, клеточные культуры электропорированные без плазмид (mock EP) и культуры клеток без электропорации (none EP). Электропорировали 5×10^5 клеток и оставляли на 10 минут. После 10 минут клетки собирались и рассаживались в 24-луночный планшет в 1 мл культуральной среды и кондиционной среды (1:1). Затем добавляли по 50 нг/мл Flt3-L в каждую лунку. На следующий день трансфицированные клетки оценивались с помощью метода проточной цитофлуориметрии и количественной ПЦР.

Установлено, что после электропорации в группах mock EP, EP P5, EP CCR9 относительное количество живых CD11c⁺ дендритных клеток было достоверно меньше, чем в none EP группе. Более того, в группах EP P5 и EP CCR9 было достоверно меньше живых CD11c⁺ дендритных клеток, чем в группе mock EP. Экспрессия маркера CD86 было достоверно выше в группах EP P5 и EP CCR9, чем в группах none EP и mock EP. Экспрессия I-Ab среди cDC2s было достоверно выше в группе EP CCR9 по сравнению с группой none EP. У плазмацитоидных дендритных клеток и конвенциональных дендритных клеток 2-го типа, в группах EP CCR9, экспрессия CCR9 была достоверно выше, чем в группе none EP.

Таким образом, в данном исследовании продемонстрирована эффективность электропорации, сопровождающаяся снижением выживаемости и созреванием дендритных клеток.

Ключевые слова: электропорация, плазмацитоидные дендритные клетки, конвенциональные дендритные клетки 2-го типа, ДНК конструкции, жизнеспособность, зрелость

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EFFECT OF DNA CONSTRUCTIONS ELECTROPORATION ON DENDRITIC CELLS

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Abstract. Today transfection of mammalian cell with DNA or RNA construction is the only method for delivering programmed information into the cell nucleus. Electroporation is most commonly used method of transfection in experiments with dendritic cell. The aim of electroporation is to permeabilize the membrane by passing electric impulse through the cell. Due to the increase permeability of the membrane chance DNA or RNA construction getting inside into the cell is increased, wherein survival of the cells is decreased.

In the study male mice C57Bl/6 line 2-4 months old were used. From femur bones was isolated 20×10^6 bone marrow cells, which were cultured in 20 mL of complete RPMI-1640 for 7 days. To generate dendritic cells from BM cells, 100 ng/mL of rmFlt3-L was added to culture media at day 0. After 7 days of cultivation, the cell cultures were electroporated with control noncoding plasmids p5 (EP P5) or pmaxCCR9 encoding mouse chemokine receptor CCR9 (EP CCR9). The controls were cell cultures electroporated without any plasmids (mock EP) and cell cultures without electroporation (none EP). 5×10^5 cells were electroporated and resting for 10 minutes. After 10 minutes, cells were harvested and seeded into 24-well plates in 1 mL of culture medium and conditioning medium (1:1). Then, 50 ng/mL of Flt3-L was added to each well. The next day, transfected cells were collected and used for flow cytometry, qRT-PCR analysis.

It was found that after electroporation in the groups mock EP, EP P5, EP CCR9 relative amount of live CD11c⁺ dendritic cells was significantly less than in the non EP group. Moreover, in the EP P5 and EP CCR9 groups the frequency of live CD11c⁺ dendritic cells was significantly less than in the mock EP group. Expression of the CD86 marker in the EP P5 and EP CCR9 groups was significantly higher than in the non EP and mock EP groups. Expression of the I-AB(MHCII) in the EP CCR9 group on cDC2s was significantly higher than in the non EP group. On plasmacytoid DCs (pDCs) and conventional type 2 DCs (cDC2s) in the EP CCR9 group expression of CCR9 was significantly higher than in the non EP group. Therefore, in this study, we demonstrated the effectiveness of electroporation, accompanied by the decrease in the survival rate and maturation of DCs.

Keywords: *electroporation, plasmacytoid dendritic cells, conventional dendritic cell type 2, DNA constructions, viability, maturity*

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Introduction

Electrotransfection of cells is one of the methods of transfection induced by electric field impulses used recently as an effective technique of foreign DNA introduction into cells of any origin [9, 14]. The aim of electroporation is to permeabilize cell membrane while preserving the cell viability. While applying impulses, irreversible damage occurred to the plasma membrane, from which some cells are not able to recover. The latter depends on cell ability to achieve biochemical balance after inducing intense molecules influx and efflux. If that balance is not attained, cell viability may be lost [4, 9, 13].

Comprehension of electroporation mechanism is important for improving efficiency in experiments with dendritic cells (DCs) both *in vitro* or *in vivo* [5].

Recently, methods of electroporation turned out to be more frequently applied for antigen loading of DCs compared to other methods of transfection. For example, transfection of immature DCs by using DNA or RNA constructs leads to special ability to capture and process antigens followed by their maturity [8]. Within an hour after electroporation, DCs start to express X-box binding protein 1 (XBP1), which is a transcription factor controlling biogenesis, thereby reducing stress and promoting cell survival [12]. Moreover, glycolysis in DCs depends on PI3K/Akt, mTORC1, which is one of the most common signaling pathways for proliferation in the stress response. The process is supported by the fact that glucose restriction strongly inhibits activation and lifespan of DCs after stress, which electroporation can induce [11].

In this study, we electroporated cell cultures containing murine CD11c⁺B220⁺pDCs and CD11c⁺SIRPα⁺cDC2s for assessing viability and maturation.

Materials and methods

In the study, male C57Bl/6 mice aged 2–4 months were used, obtained from the SPF vivarium of the Institute of Cytology and Genetics (Novosibirsk, Russia) and maintained at the animal facility of the Research Institute of Fundamental and Clinical Immunology on standard diet, under natural light conditions with unrestricted access to food and water.

An artificial gene encoding mouse CCR9 (UniProtKB-Q9WUT7) was designed and prepared with the optimization of codon composition for its efficient expression in mammalian cells. The artificial gene was subcloned into the pMax-plasmid vector under the control of a CMV promoter added with poly(A)tail. After transformation in *Escherichia coli* plasmid DNA was purified and sequenced.

Dendritic cells were generated from the bone marrow (BM) of UBC-GFP mice. First, 20×10^6 BM cells isolated from femur bones were cultured for 7 days in 75-cm² flasks (TPP, Switzerland) together with 20 mL of complete RPMI-1640 supplemented with 10% FCS (Biowest, France), 2 mM L-glutamine (Biolot, Russia), 10 mM HEPES (Biolot, Russia), 5×10^{-4} M 2-mercaptoethanol (Sigma-Aldrich, USA), 80 µg/mL gentamycin (KRKA, Slovenia), and 100 µg/mL benzylpenicillin (Biolot, Russia). To generate dendritic cells from BM cells, 100 ng/mL of rmFlt3-L (R&D Systems, USA) was added at day 0. Half of the growth factor-containing medium was changed every 2–3 days.

After 7 days of experiment, cell cultures were harvested and electroporated with pmaxCCR9 (EP CCR9), without plasmid (MockEP) or left untreated (non EP group). Cell cultures electroporated with non-coding plasmid p5 (EP P5) were used as control for assessing effective electroporation. For electroporation, 5×10^6 of cells were resuspended in 50 µL of OptiMEM (Thermo Fisher Scientific, USA) with or without 50 µg of pmaxCCR9 and transferred to 1-mm cuvettes (BTX, USA). Electroporation was carried out using a BTX 830 electroporator (BTX, USA) with a single pulse (strength 200 V, pulse duration 40 µs). Electroporation was followed by 10 minutes of resting, after which cells were seeded into six-well plates (TPP, Switzerland) in 5 mL of culture medium and conditioning medium (1:1). Next, 50 ng/mL of Flt3-L was added to each well. One day after transfection, cells were collected and used for flow cytometry, qRT-PCR analysis.

For flow cytometry, 5×10^5 cells were collected and incubated in the dark at room temperature with an appropriate combination of fluorescently labeled monoclonal antibodies, in staining buffer, for 20 minutes. For DC staining, anti-CD11c-PE/Cy7 (N418), anti-B220-Bv510 (RA3-6B2), anti-SIRPα-PerCP/Cy5.5(P84), anti-CCR9-PE (9B1), anti-I-Ab-APC (AF6-120.1), anti-CD83-FITC (Mitchel-19) and

anti-CD86-APC/Cy7 (GL-1) (BioLegend, USA) antibodies were used. DAPI was used to stain dead cells.

To analyze the gene expression profiles of nonEP, MockEP, and EP CCR9 DCs, total RNA was isolated using TRIZOL reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

RNA concentrations were determined by measuring the absorbance at 260 nm using a Nanodrop 2000c spectrophotometer (Wilmington, DE, USA). Reverse transcription of RNA into cDNA was performed using an MMLV cDNA Synthesis Kit (Evrogen, Russia).

Subsequently, SYBR® Green technology was used for relative mRNA quantification by qPCR in a CFX96 C1000 thermal cycler (Bio-Rad). qPCR reactions were conducted at 95°C for 4 minutes, followed by 40 cycles at 95 °C for 10 s, 65 °C for 15 s, and 72 °C for 30 s. All primer sets were obtained from Biosan (Russia).

qPCR was performed in triplicate and resulting mRNA levels were normalized to levels of the reference gene phosphoglycerate kinase 1. Melt curve analysis was performed to confirm the specificity of the amplified product. The $\Delta\Delta C_t$ method was used for data processing and analysis [7].

Statistical analysis was performed using Prism 8.0 (GraphPad Software, USA). According to the Shapiro–Wilk normality test, all data were distributed normally. Data were analyzed by parametric one-way ANOVA or two-way ANOVA with Tukey's corrected multiple comparisons. P-values ≤ 0.05 were considered statistically significant.

Results and discussion

The effect of electroporation on the viability of dendritic cells.

After electroporation, a relative amount of live CD11c⁺DCs in the mock EP, EP P5, EP CCR9 groups was significantly lowered compared to the non EP group (Figure 1A). Moreover, in the groups with plasmid-performed electroporation (EP P5 and EP CCR9), relative amount of live CD11c⁺DCs was significantly decreased compared to mock EP group (Figure 1A).

In the EP P5 and EP CCR9 groups there was a decreased level of pDCs compared to the non EP and mock EP groups. Percentage of cDC2s in the EP P5 and EP CCR9 groups was lowered compared to the non EP group (Figure 1B).

The phenotype of DCs after electroporation.

We determined the phenotype of DCs after electroporation by examining specific markers of maturity CD83, CD86 and I-Ab (MHCII).

The CD83 DC maturity marker did not significantly changed in proportion of cell expression and the mean value of fluorescence (Figure 2A, E).

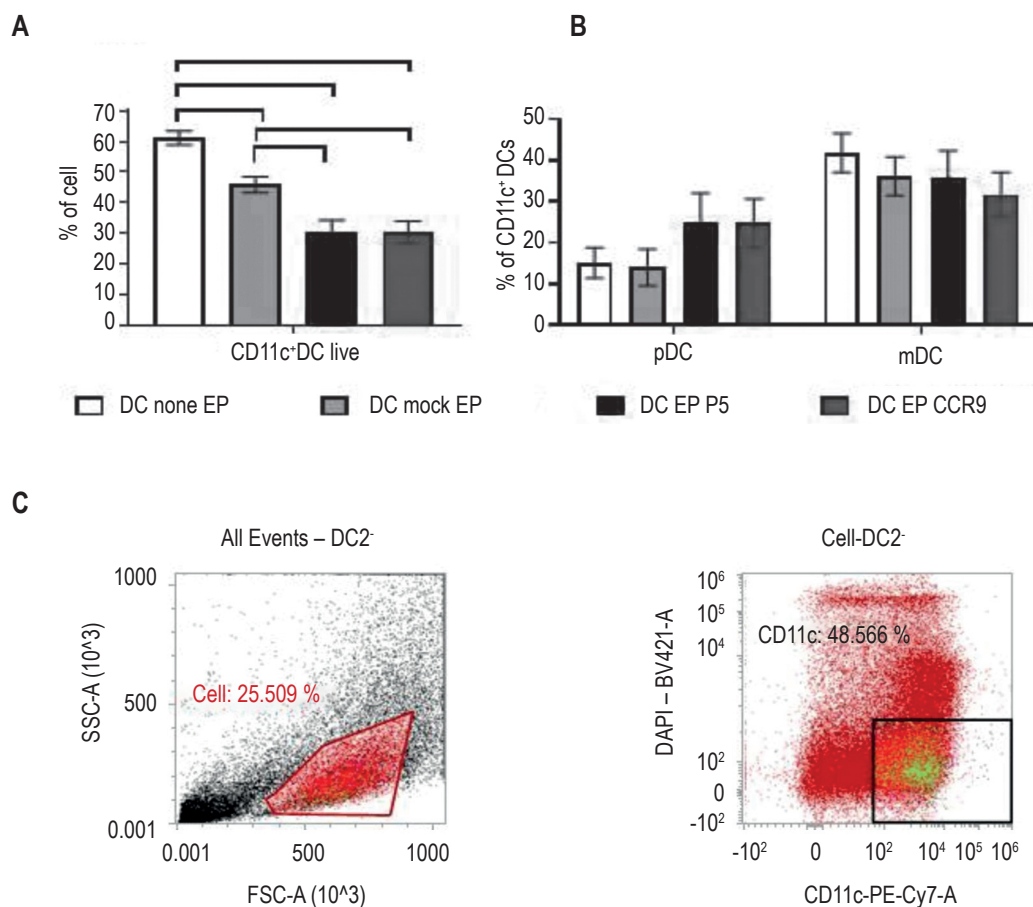


Figure 1. A, relative amount of CD11c⁺DCs live. B, relative amount of pDCs and cDC2s. C, flow cytometry gating strategy of dendritic cells, CD11c⁺live DCs

Note. Data are presented as mean $\pm$ SEM. Brackets indicate significant differences (One-way ANOVA (A), two-way ANOVA (B) with Tukey's multiple comparisons test, $p < 0.05$).

However, the frequency of CD86⁺pDCs and cDC2s in the EP P5 and EP CCR9 groups was significantly higher than in the non EP and mock EP groups (Figure 2B). Mean fluorescence intensity (MFI) of CD86 on cDC2s in the EP P5 and EP CCR9 groups was significantly higher than in the non EP and mock EP groups (2F). The frequency of I-Ab⁺ cDC2s in the EP CCR9 group was significantly higher than in the non EP group (Figure 2C). MFI of I-Ab on pDC and cDC2s in the EP CCR9 group was higher than in the non EP and mock EP groups (Figure 2G).

DCs electroporated with pmaxCCR9 DNA-plasmids express higher levels of CCR9.

Flow cytometry analysis showed that in the EP CCR9 group percentage of CCR9⁺pDCs and cDC2s was significantly higher than in the non EP group (Figure 2D). In addition, relative amount of CCR9⁺cDC2s in the EP CCR9 group was significantly higher than in the mock EP group (Figure 2D).

According to the expression levels of native CCR9 RNA and plasmid CCR9 RNA measured by qRT-PCR, the DCs electroporated with pmaxCCR9 (EP CCR9) expressed 94.4-fold higher levels of bulk CCR9 RNA compared with non-electroporated DCs (nonEP) (median $2^{-\Delta\Delta Ct}$ 119.46 and 1.27, respectively, $n = 4$). Mock electroporated DCs (MockEP) expressed bulk CCR9 RNA that was 1.4-fold lower than that of nonEP DCs (median $2^{-\Delta\Delta Ct}$ 0.85 and 1.27, respectively, $n = 4$).

The electrostatic charge of the membrane is an element responsible for maintaining the physiological identity and function of cell organelles [2]. The electrostatic charge and the balance of electrons in the environment depend on the shape of the cells, e.g., cells with oval shape have uniformly distributed electrostatic charge, whereas those with processes (dendrites) or irregularly shaped cells display different electric charge on the cell membrane, and therefore

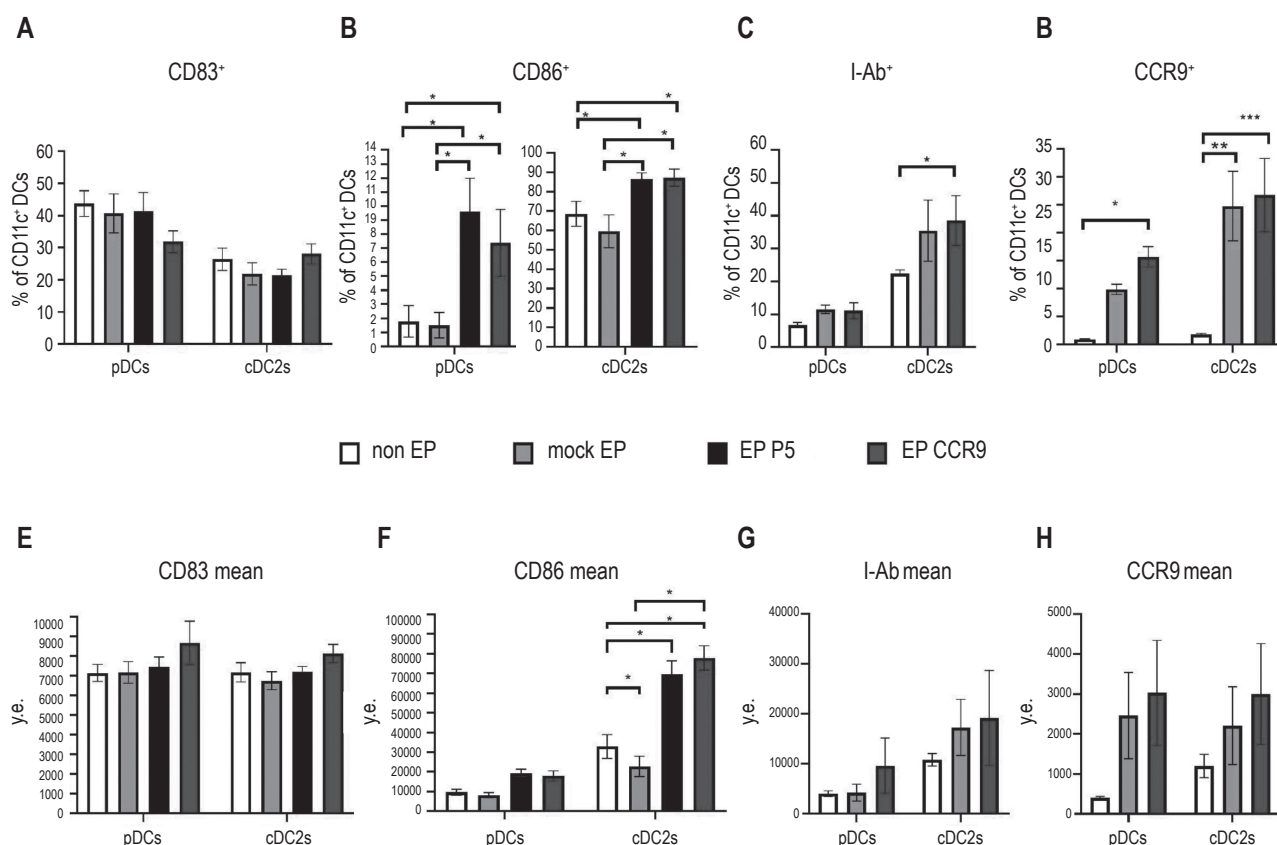


Figure 2. Relative amount of CD83 (A), CD86(B), I-Ab (C), CCR9 (D) positive DCs and mean of fluorescence intensity of CD83 (E), CD86 (F), I-Ab (G), CCR9 (H) on DCs (n = 6)

Note. Data are presented as mean $\pm$ SEM. Brackets indicate significant differences (Two-way ANOVA with Tukey's multiple comparisons test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

are less resistant to electric impulse [10]. Dendritic cells are generally of oval shape with characteristic dendrites suggesting that DCs in the groups of mock EP, EP P5 and EP CCR9 cells were less viable compared to non EP dendritic cells (Figure 1A).

Cluster of differentiation 86 (CD86) is an important marker of dendritic cell maturity. Upregulated CD86 expression leads to increased I-Ab(MHCII) surface level and potential for T-cells activation [1, 6, 8]. In this study, we observed that pDCs and cDC2s had a higher level of CD86 expression in the EP P5 and EP CCR9 groups. We confirmed an increase of I-Ab expression on cDC2s after electroporation by DNA construction encoding-CCR9 (Figure 2C).

After electroporation, qPCR data showed increased levels of bulk CCR9 RNA in the EP CCR9 group compared to the non EP group. Moreover, flow cytometry analysis showed an increase of CCR9 expression on pDCs and cDC2s in the EP CCR9 group, which confirms the effectiveness of

electroporation as a method for DNA construct transfection (Figure 2D). CCR9 is a chemokine that regulates the migration of dendritic cells from the bone marrow to the thymus for participation in negative deletion of the na ve T-cell [3].

Conclusion

The current study demonstrated a potential for dendritic cell electroporation with DNA constructions encoding plasmids CCR9. Our data confirmed the low viability and maturation of dendritic cell after electroporation, but the effectiveness of the method for delivery of DNA construction into the cell as well.

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РЕЦЕПТОР ЖИРНЫХ КИСЛОТ GPR84 И Th1/Th2-БАЛАНС В ЭКСПЕРИМЕНТАЛЬНОЙ СИСТЕМЕ *IN VIVO*

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Резюме. В ходе многочисленных исследований, в том числе в нашей лаборатории, было обнаружено, что баланс Th1/Th2 играет важную роль в регуляции реакций, определяющих исход иммунопатологических процессов как в моделях хронической РТПХ, так и в моделях острой РТПХ. Однако до сих пор открыт вопрос: от активности какого именно элемента регуляторного процесса в момент индукции РТПХ (например рецептора или фермента) зависит соотношение этого баланса. Сделано предположение, что степень активации рецептора GPR84 в момент индукции РТПХ может существенно влиять на Th1/Th2-баланс в организме и благодаря этому определяет направление развития и интенсивность патологического процесса. Целью настоящей работы являлось изучить влияние лигандов рецептора среднецепочечных жирных кислот GPR84 на баланс Th1/Th2 в экспериментальной модели *in vivo*.

В экспериментах были использованы самки мышей линий DBA/2 и гибридов (C57Bl/6 × DBA/2)F₁. В качестве исследуемых лигандов GPR84 использовали каприновую и лауриновую кислоты, а также синтетический лиганд 6-OAU. Хроническую РТПХ в полуаллогенной системе индуцировали вводя спленоциты мышей DBA/2 мышам-гибридам B6D2F₁: по 60-70 × 10⁶ клеток в/в двукратно с интервалом в 6 дней. Первое введение лигандов GPR84 производили через час после переноса донорских клеток и затем раз в сутки в течение двух недель. Эффект воздействия исследуемых препаратов на течение хронической РТПХ оценивали через три месяца после начала эксперимента.

Было показано, что введение животным лигандов к GPR84 в ходе индукции хронической РТПХ влияет на активность рецептора и на Th1/Th2-соотношение в организме. Введение синтетического лиганда 6-OAU увеличило количество животных, у которых иммунопатологический процесс развивался по Th1-зависимому варианту, почти в полтора раза по сравнению с группой без введения лиганда, что соответствовало литературным данным, полученным в системе *in vitro*. Эффект смеси каприновой и лауриновой кислот, видимо, опосредуется каким-то иным механизмом, отличным от активации GPR84. Следовательно, для осуществления перспективной возможности корректировать иммунные реакции путем включения в диету определенных жирных кислот требуются дальнейшие исследования.

Ключевые слова: системная красная волчанка, экспериментальная модель, лиганды жирных кислот, GPR84, Th1/Th2-баланс

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FATTY ACID RECEPTOR GPR84 AND Th1/Th2 BALANCE IN THE EXPERIMENTAL SYSTEM *IN VIVO*

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Abstract. While conducting numerous studies, including researchers in our laboratory, it was found that Th1/Th2 balance plays an essential role in the regulation of reactions that determine the outcomes of immunopathological processes in both chronic and acute GVHD models. However, the question about activity of which element in the regulatory process during GVHD induction (for example, a receptor or an enzyme) affects the ratio of this balance depends remains open. It has been suggested that the degree of activation of the GPR84 receptor during GVHD induction can significantly affect the host Th1/Th2 balance. And, by assessing this parameter, the direction of development and the intensity of the pathological process can be determined. The aim of this work was to investigate the effect of ligands such as medium-chain fatty acid receptor GPR84 on the Th1/Th2 balance in an experimental model in an *in vivo* system.

Female DBA/2 and hybrids (C57Bl/6 × DBA/2) F1 mouse strains were used in the experiments. The studied ligands of GPR84 were capric and lauric acids, as well as a synthetic ligand 6-OAU. Chronic GVHD in the semi-allogenic system was induced by injecting splenocytes from DBA/2 mice to B6D2F₁ hybrid mice: 60–70 × 10⁶-cells iv twice with an interval of 6 days. The first administration of the GPR84 ligands was performed one hour after the donor cell transfer and then once a day for two weeks. The effect of the study drugs on the course of chronic GVHD was assessed three months after the onset of the experiment.

It was shown that the administration of GPR84 ligands to animals during the induction of chronic GVHD affects the activity of the receptor and the host Th1/Th2 ratio. In the group with the injection of 6-OAU, the number of animals which the immunopathological process developed according to the Th1-dependent variant increased by more than 1.5-fold, compared with the control group. This fact is consistent with the literature data obtained in the *in vitro* system. Apparently, the effect of a mixture of capric and lauric acids is mediated by some other mechanism, differed from the GPR84 activation. Therefore, further research is required to realize the promising possibility of adjusting immune responses by including certain fatty acids in the diet.

Keywords: systemic lupus erythematosus, experimental model, fatty acid ligands, GPR84, Th1/Th2 balance

Introduction

In the organism of experimental animals, a balance between type 1 and type 2 T-helper cells plays a decisive role in developing the graft versus host reaction (GVHD) in the classical semi-allogenic system with adoptive transfer of lymphoid cells from one of the parental lines to hybrid mice of the first generation. Cell transfer from C57Bl/6 mice to hybrid (C57Bl/6 × DBA/2)F1 mice is accompanied by dominance of Th1-lymphocytes in immune responses and development of acute GVHD with pronounced tissue destruction and mass death of recipient animals. At the same time, if DBA/2 mice are used as donors, a different type of immune conflict develops in recipient mice: chronic GVHD with predominance of Th2-dependent reactions, intense polyclonal activation of B-lymphocytes, overproduction of antibodies and

development of immunocomplex glomerulonephritis in some animals.

Moreover, over many years of studies in our laboratory it was found that Th1/Th2 balance plays an essential role in the regulation of reactions that determine the outcomes of immunopathological processes in both chronic and acute GVHD models. In particular, it was found that recipients develop two variants of this disease after the induction of chronic GVHD. Th2-dependent response, leading to the formation of glomerulonephritis and Th1-dependent — without nephritis, but with severe immunodeficiency [3]. Development of either of such scenarios is determined by the magnitude of Th1/Th2 ratio in individual animals at early stage of chronic GVHD development. It is possible to shift the balance of T-helper cells in either direction by using various immunomodulatory agents. It makes this model a convenient and sensitive test-system for identifying

agents that affect the Th1/Th2 balance. A similar division of recipients into Th1- and Th2-dependent variants of the developing disease was found in acute GVHD [10].

It has long been established that the inability of DBA mouse lymphocytes to induce acute GVHD after cell transfer to hybrid mice is accounted for by impaired recipient CD8⁺T-cells [8]. In the critical phase of the immune conflict, such defective lymphocytes produce no sufficient IFN to sharply shift the balance towards Th1-cells and initiate massive generation of killer cells specific to the recipient antigens. Later, by backcrossing hybrids with the parental mouse strains C57Bl and DBA, it was also found that the defect inhibiting induction of acute GVHD by DBA mouse lymphocytes was genetically determined probably affecting the Th1/Th2 ratio [1]. However, the question remains as to which defective DBA mouse gene the observed differences are assigned to and how it is associated with development of opposite GVHD variants.

Based on the results of various studies carried out over the last twenty years, there is every reason to believe that the GPR84 gene is the most probable candidate for this role.

GPR84 was discovered in 2001 as an orphan receptor of the family of membrane G-protein-coupled proteins involved in cell signalling. However, several years later it was found that GPR84 may physiologically ligate medium-chain fatty acids (from C9 to C14), among which the most active were capric, undecanoic, and lauric acids as well as their hydroxylated metabolites [11]. Among the examined so far, the most important effects of GPR84 activation in cell cultures were demonstrated in activities which are directly related to the data described in this study.

Firstly, during the development of inflammatory response (after LPS injection) it was found that activation of GPR84 significantly upregulated expression of the IL-12 p40 subunit [2, 11], a cytokine that shifts the balance of helper cells towards Th1 pathway and plays a decisive role in developing acute GVHD. Secondly, the inhibitory effect of GPR84 activation on generation of Th2-lymphocytes and their production of IL-4 was established [7]. These data are in good agreement with the effect of this receptor on IL-12 expression. An increased IL-4 production by cells in mice knocked out for GPR84 has also been shown [9].

The results obtained in the study of the GPR84 gene deletion in the genome of various strains of laboratory mice are also of great importance [5]. The authors found that DBA/2 is among several strains bearing such gene deletion. Thus, the defects in CD8⁺T-cells from such mice and the corresponding

shift in Th1/Th2 balance towards Th2 can be accounted for by the decreased expression of IL-12 upon GVHD induction. These data convincingly support the assumption that the degree of GPR84 receptor activation can markedly affect the Th1/Th2 ratio *in vivo*. Therefore, it might determine modality of development and intensity of the pathological process upon induction of GVHD.

The aim of current study was to test this assumption in an *in vivo* model by administering GPR84 ligands to recipient mice upon induction of chronic GVHD.

Materials and methods

In the experiments, we used 45 female mice of the C57Bl/6, DBA/2 strains and (C57Bl/6 × DBA/2)F₁ hybrids obtained from the SPF vivarium of the Institute of Cytology and Genetics SB RAS (Novosibirsk).

Capric (C10, Acros Organics, Germany) and lauric (C12, Acros Organics, Germany) acids, as well as the synthetic ligand 6-(octylamino)-2,4(1H,3H)-pyrimidinedione (6-OAU; Abcr GmbH, Germany), were used as the GPR84 ligands.

Chronic GVHD in the semi-allogenic system was induced according to the standard regimen by inoculating splenocytes from DBA/2 mice to B6D2F₁ hybrid mice: 60–70 × 10⁶ cells iv twice with an interval of 6 days [3]. The outcome of the immunopathological process in individual animals was determined 3 months after the induction of GVHD by measuring the level of protein in the urine: with proteinuria of 3 mg/ml or more, mice were classified as a Th2-dependent variant of GVHD, leading to the formation of glomerulonephritis, in the presence of protein in urine less than 3 mg/ml – to a Th1-dependent variant of the disease.

Statistical analysis of the data obtained was carried out by using the software package Statistica 6.0.

Results and discussion

The response of C57Bl/6 and DBA/2 mice to an exogenous inflammatory stimulus was explored in a preliminary experiment. The animals were injected with LPS (sc, at a dose of 1 µg/mouse). Next, at various timepoints the intensity of the response was assessed after drug administration by measuring the neutrophil/lymphocyte ratio in peripheral blood as well as concentration of free (extracellular) DNA. It was found that magnitude of changes in such parameters after administration of LPS in DBA/2 mice was significantly lower than in C57Bl/6 mice (data not shown). This is in agreement with literature evidence regarding attenuated proinflammatory responses in DBA/2 mice under various experimental settings.

TABLE 1. INDUCTION OF CHRONIC GVHD IN AN EXPERIMENTAL MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) UNDER THE INFLUENCE OF THE SYNTHETIC LIGAND GPR84 AND A MIXTURE OF FATTY ACIDS

Groups	Th1-dependent variant of cGVHD	Th2-dependent variant of cGVHD	Th1/Th2 %	Proteinuria*
1. cGVHD (control)	4	10	29/71	5.17
2. cGVHD + 6-OAU	5	6	46/54	5.63
3. cGVHD + (C10+C12)	0	12	0/100	5.63

Note. * mean concentration of protein in the urine of mice with developed glomerulonephritis (mg/ml).

During experiments with inducing chronic GVHD, animals were divided into three groups after donor cell transfer:

Group 1 – control (n = 14) – ip injection of 0.2 ml of PBS (saline);

Group 2 – experiment (n = 11) – ip injection of 0.4 mg 6-OAU in 0.2 ml of PBS;

Group 3 – experiment (n = 12) – injection of a mixture of fatty acids (0.35 mg C10 + 0.4 mg C12 in the form of a suspension in 0.5 ml of PBS), per os (using a gastric tube).

The first injection of GPR84 ligands to all animals was performed one hour after donor cell transfer and then once a day for two weeks.

The effect of the drugs on chronic GVHD was assessed three months after the onset of experiment. By determining the number of mice with developed immunocomplex glomerulonephritis in each of the indicated groups. The results of this experiment are presented in the Table 1.

These data show that even in the in vivo test-system activation of GPR84 by a synthetic ligand also leads to marked shift in the Th1/Th2 ratio towards Th1 and increased production of proinflammatory cytokines similar to what was previously shown in experiments with cell cultures. In the group with inoculated 6-OAU, the number of animals with immunopathological process developed along Th1-dependent variant increased by more than 1.5-fold, compared with the control group. Although donor cells initiating an immune conflict could not directly respond to inoculated 6-OAU (due to the ablated GPR84 gene). Nevertheless, the reactions of such cells, being actively involved in inducing a certain Th1/Th2 balance in recipient host, were significantly modified by the injected drug. Such data may be easily explained by assuming that the effect of the GPR84 activating agent is mediated by its effects on host cells, which genome contains a normally functioning GPR84 gene inherited from the parental C57Bl/6

mice. Injection of 6-OAU into recipients activating GPR84 increases the amount of IL-12 produced by the host cells. In turn, this cytokine can also affect donor cells affecting their activity and thereby shifting the Th1/Th2 balance to the pro-inflammatory side as well as ultimately reducing the number of nephritis cases in experimental group.

A more difficult obstacle is interpretation of intriguing data obtained in mice inoculated with a mixture of fatty acids. While planning the experiments, it was assumed that a mixture of capric and lauric acids, effectively binding to the GPR84 protein and cause its activation, should trigger an effect similar to that of a synthetic ligand in our test system. However, a sharp shift in the balance of T-helper cells to the dominant Th2-cells was unexpected. At first glance, this result contradicted our initial assumption and the effect of 6-OAU. Apparently, in this case, the effect of fatty acids on the Th1/Th2 ratio is mediated in another way, differed from the activation of GPR84 by these acids. As it turned out, one of the possible explanations may be related to the activation of the hydroxycarboxylic acid receptor 3 (HCA3) by capric acid, which is included in the mixture used in our experiments. Peters A. et al. found that the hydroxylated metabolite of this acid (3-OHC10) was able to activate not only GPR84 (with the same efficiency as the native C10), but also the HCA3 receptor [6]. Similar to the medium-chain fatty acid receptor, HCA3 is expressed on immune cells. However, unlike GPR84, activation of HCA3 may also suppress the production of proinflammatory cytokines in response to LPS [4]. Thus, we can assume that capric acid, acting through cognate receptor, is capable of shifting the Th1/Th2 ratio towards the prevalence of Th2-lymphocytes. However, further research is required to find out whether a similar process underlies the discovered phenomenon or results from action of some other factors.

Conclusion

The results of this study confirm that the GPR84 ligands are able to robustly affect the Th1/Th2 ratio *in vivo*, and also indicate a promise to assess a potential for correcting immune responses by including certain fatty acids in the diet.

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ЭФФЕКТИВНОСТЬ ФОРМИРОВАНИЯ ПРОТИВООПУХОЛЕВЫХ ИММУННЫХ ОТВЕТОВ В СИСТЕМЕ ПРОФИЛАКТИЧЕСКОЙ ВАКЦИНАЦИИ МЫШЕЙ ТЕСТИКУЛЯРНЫМИ АНТИГЕНАМИ

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Резюме. Появление злокачественной опухоли ассоциируется с нарушением механизмов пролиферации, дифференцировки, способности к апоптозу. Этих изменений недостаточно для того, чтобы иммунная система распознала и уничтожила мутировавшие клетки. Причина этого явления — слабая иммуногенность опухолево-ассоциированных антигенов (ТАА). Противоопухолевая вакцинация является наиболее эффективным специфическим методом как профилактики рецидива заболевания, так и терапевтическим инструментом лечения в онкологии. Одним из главных условий эффективности противоопухолевой иммунотерапии является повышение иммуногенности опухолевых клеток. Иммунизация моно- или олиго-ТАА-производными пептидами не обеспечивает общее подавление развития опухоли и даже создает благоприятные условия для селективного роста отдельных клонов опухолевых клеток, не имеющих общих АГ с вакцинальными клетками. Ксеногенные АГ обладают высокой иммуногенностью и эффективны в разрушении иммунной толерантности к человеческим аналогам, представленным на опухолевых клетках. В нашей работе мы использовали тестикулярные АГ барана в качестве источника ксеногенных ТАА. Яички овец содержат большой набор ТАА. Экспериментальные мыши были иммунизированы липосомальной тестикулярной вакциной, полученной из яичек барана. Через месяц после вакцинации мышам подкожно имплантировали опухолевые клетки карциномы LLC. Обнаружено, что продолжительность жизни мышей опытной группы была в 2 раза выше по сравнению с сингенным контролем, при этом у 20% из них опухоль не развивалась вообще. В спленоцитах мышей, у которых не было опухолей, определяли Т-регуляторные клетки и Т-клетки памяти. Мы обнаружили достоверное снижение как наивных Т-регуляторных ($CD4^+CD25^+$), так и активированных ($CD4^+CD25^+FoxP3^+$), а также Т-памяти ($CD4^+CD44^+$), в том числе популяцию центральных Т-памяти ($CD4^+CD44^+CD62L^+$) в селезенке предварительно иммунизированных мышей по сравнению с лимфоцитами мышей, полученных из интактной селезенки. Исследование содержания $IFN\gamma$ и IL-10 в супернатантах мышинных спленоцитов, полученных от вакцинированных мышей без

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опухолей, показало достоверное снижение количества IL-10, но не IFN γ . На основании полученных результатов мы полагаем, что иммунизация ксеногенными опухолевыми АГ может привести к формированию эффективного противоопухолевого ответа, направленного на опухолеассоциированные АГ, имеющиеся на собственной опухоли.

Ключевые слова: противоопухолевые вакцины, карцинома легких Льюиса, опухолевые антигены, тестикулярные антигены, иммунотерапия, иммунный ответ

EFFICIENCY OF THE FORMATION OF ANTITUMOR IMMUNE RESPONSES IN THE SYSTEM OF PREVENTIVE VACCINATION OF MICE WITH TESTICULAR ANTIGENS

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Abstract. Appearance of a malignant tumor is associated with impaired mechanisms of proliferation, differentiation, apoptosis ability. However, these changes are not enough for immune system to recognize and destroy mutated cells. Weak immunogenicity of tumor-associated antigens (TAA) and the insufficiency of co-stimulating molecules on the surface of tumor cells is a reason for this phenomenon. Since biochemical processes of tumor cells and healthy tissue cells are identical, therefore creation of effective chemotherapeutic drugs is limited not by selectivity of their action. So antitumor vaccination is the most effective specific method for both preventing recurrence of a disease and a therapeutic treatment tool in oncology. Xenogeneic proteins are highly immunogenic and effective in breaking immune tolerance to human analogs. In our work, we used sheep testicular AG as a source xenogenic TAAs. Sheep testicles contain a large set of TAAs. Experimental mice were immunized with type liposomal testicular vaccine from sheep, one month after vaccination, to induce tumor growth, cells of carcinoma LLC were implanted in mouse. The life expectancy of the experimental group of mice was 2 times higher compared to the syngenetic control and 20% of them did not develop the tumor at all. In the spleen of mice who did not have tumors after pre-vaccination sheep liposomal testicular AG, T-regulatory cells and T-memory cells were measured. We found a credible decrease in both naive Treg (CD4⁺CD25⁺), activated (CD4⁺CD25⁺FoxP3⁺) and both T-memory (CD4⁺CD44⁺) and central memory (CD4⁺CD44⁺CD62L⁺) in spleen pre-vaccination mice with compared to the contral intact spleen. Content of IFN γ and IL-10 in supernatants of mouse splenocytes derived from vaccinated mice with no tumors was investigated and showed a reliable decrease in the amount of IL-10, but not IFN γ . We believe that immunization with xenogenic tumor AGs can lead to the formation of a protective antitumor response.

Keywords: cancer vaccines, Lewis lung carcinoma, tumor-specific antigens, testis antigens, immunotherapy, immune response

Introduction

Conventional anti-cancer treatment is mainly based on surgical operation, radiological therapy and chemotherapy. During treatment, the chemotherapy-resistant cells survive and obviously acquire selective growth advantages over drug-susceptible cells. Accumulation of drug-resistant cancer cells over time usually converts into less efficient chemotherapy as compared to the previous treatment cycles. However, tumor cells can be differentiated from normal cells and carry surface immunogenic markers. The latter (tumor-associated antigens (TAA)) are targeted by the immune system [7]. TAAs include: oncogene products, mutated mucins, viral antigens, fetal antigens, and cell differentiated antigens. Activation of naive T-cells occurs due to TAA recognition coupled to

MHC molecules, lymphocyte T-cell receptor and non-antigen-specific costimulatory signal. Co-stimulatory molecules such as B7-1/CD80, B7-2/CD86, CD40 are expressed on professional APCs, but are usually downmodulated on tumor cells. There are mechanisms for evading immune surveillance of malignant cells, which plays a key role in tumorigenesis. Thus, transformed cells survive and produce clones.

The immune system consists of both innate and adaptive arms to prevent oncogenesis.

Over the last few years, different types of cancer vaccines have been developed, including cell-based vaccines (i.e. DC-based Sipuleucel), protein/peptide vaccines, tumor cell-based vaccines, viral/bacterial-based vaccines and gene-based vaccines, including RNA and DNA vaccines [10]. However, clinical ap-

plication of cancer vaccines were not very effective due to the fact that most TAAs are presented by non-mutated weakly immunogenic proteins. Furthermore, immunizations with mono- or oligo-TAA-derived peptides often fail to control the overall tumor development, and even creates favorable conditions for selective growth of the particular tumor cell clones that lack vaccine Ags [4]. Therefore, compared to individual TAA-derived peptides, multi-antigen-based vaccine can elicit strong polyclonal immune responses to different TAAs. Autologous tumor cell vaccines are typically combined with an immunostimulatory adjuvant (e.g., *Bacillus Calmette-Guérin*, BCG). Allo-geneic tumor cell vaccines are typically prepared from two or three established human tumour cell lines [3]. Finally, to our knowledge, xenogeneic cell-based vaccines could constitute the most effective approach to stimulate antitumor immune responses in clinical cancer settings [11, 12]. This opinion is substantiated by observations that xenogeneic proteins are highly immunogenic and effective in breaking immune tolerance to human analogs. In addition, a wide variety of animal tumor cell lines are available, which could be exploited to design vaccines with maximal antigenic overlap with target tumors in order to elicit strong polyclonal tumor-specific immune responses.

Vaccination of mice with a TAA xenogeneic tumors induces a specific humoral and CD8⁺T-cell-response against TAA tumors in mice [8]. Vaccination of tumor-bearing rats with antigens (CSH-275) from human colonadenocarcinoma showed prolonged survival time compared to control group. An effective antitumor immunity was also revealed in an experimental model of B-cell lymphoma using the xenogeneic cell vaccine "trioma". Immunotherapy of mouse solid tumors with xenogeneic cell vaccine consisting of endothelial cells has revealed a significant protection against tumor growth, regression of prevalent tumors and extended survival rate of tumor-bearing mice. Similar results were obtained while using a xenogeneic cell vaccine for the treatment of B16 melanoma in mice. The use of xenogeneic homologous proteins, in some mouse solid and hematopoietic tumors, mounts an effective protective and therapeutic antitumor immunity [14].

In our study, we used sheep testicular AG as a source of xenogenic TAAs. Sheep testicles contain a large set of TAAs [1, 2] and can be used to induce a prominent antitumor immune response.

Materials and methods

Experimental animals (mice)

The experiments were carried out on adult (4-5 months) specific pathogenic-free C57BL/6 (B6, H-2b) mice obtained from the nursery of laboratory animals "Rassvet", Siberian Branch of the Russian Academy of Medical Sciences (Tomsk). Animals (males weighing 15-20 grams) were housed per 10 mice/cage,

under normal conditions, with a 12-hour light regime, drinking water ad libitum, on granulated diet.

Tumor cell line

Tumor cell line of murine Lewis lung carcinoma LLC was obtained from the Cancer Research Center of the Russian Academy of Medical Sciences (Moscow).

Vaccine preparation

The sheep testicles were homogenized to further isolate cells followed by two washouts with RPMI-1640 medium by centrifugation at 1500 rpm for 30 min. After the final washout, the cells were resuspended in physiological saline and to count cell number in hemocytometer. The viability of tumor cells usually ranged within 90-100% of the total cell number. The cell concentration was adjusted to 50 million/ml. To obtain a first and the second type liposome vaccine, the antigenic material was freeze-dried and placed in 50 ml vials at the required weight equivalents. The method for obtaining liposomal vaccine contains know-how approach.

Experiment model

Experimental mice were immunized with type liposomal testicular vaccine obtained from sheep testicles 3 times at weekly intervals. 3 groups of mice were randomized for different routes of administration as well as vaccine types: control (without vaccination), liposomal testicular vaccine (LTV) and syngeneic testicular vaccine (STV). Vaccines were dried, and diluted in 0.9% NaCl, in a total volume of 400 µl/mouse for inoculation.

One month after vaccination, of Lewis lung carcinoma LLC (200 thousand cells per mouse) cells were implanted under the skin of the anterior abdominal wall of mice to induce tumor growth. On days 7-9 after inoculation, tumor growth was noticeable in all animals.

Survival assessment

Mice (n = 10) inoculated with LLC Lewis lung carcinoma tumor cells were placed in cage after conducting a course of vaccination to assess host survival. The survival rate was 50% compared to the control group. The data were depicted in plots.

Measurement of serum cytokines

Concentration of IFN γ and IL-10 were assessed by ELISA in the mouse sera, which were stored at -180C until use. Enzyme-linked immunosorbent assay was performed on an ELISA processor "Berthold Technologies Tristar LB 941" at a wavelength of 450 nm. Assays were performed by using commercial ELISA kits (manufactured by Tonbo Biosciences, International, Inc.).

Assessment of cell subpopulations by flow cytometry

Percentage of splenic Treg and T-memory cell subsets was measured by staining with anti-CD4-FITC, CD8-FITC, CD44-PE, CD62-APC, CD4-APC, CD25-FITC, FOXP3-PE (BioLegend, BD Bioscience) anti-

bodies on BD FACSCanto II flow cytometer according to the manufacturer's instructions.

Statistical data analysis

Data (mean values added with appropriate standard deviations) were reproduced in at least 3 independent experiments. Statistical data processing was carried out by using the nonparametric Mann–Whitney test as well as Kaplan–Mayer criterion.

Results and discussion

The experiments were carried out as preventive vaccination. The mice were initially immunized by xenogeneic (relative to mice) liposomal testicular AG (the main experimental group) and mouse syngeneic testicular AG (syngeneic control) (see Materials and Methods). One month after vaccination, the mice were vaccinated with the LLC tumor, the control group of mice was not previously vaccinated, and only tumor cells were injected. Next, we assessed the duration of mouse lifespan that was found in 50% of control and syngeneic control mice was 20 and 20–25 days, respectively. The mice in the experimental group lived by 2-fold longer, about 41–43 days, and 20% developed no tumors (Figure 1). Splenocytes from tumorfree mice after LTV pre-vaccination were assessed for quantity of T-regulatory cells and T-memory cells (Table 1). We found markedly decreased percentage both of naive Treg ($CD4^+CD25^+$) and activated ($CD4^+CD25^+FoxP3^+$) in spleen of pre-vaccinated mice compared to intact animals: from 12.84 ± 0.77 to 5.85 ± 0.47 in the $CD4^+CD25^+$ population and from

0.95 ± 0.17 to 0.33 ± 0.07 among $CD4^+CD25^+FoxP3^+$ subset. Similar dynamics of such parameters were found in memory T-cells. We found a significant decrease in both splenic T-memory ($CD4^+CD44^+$) and central memory ($CD4^+CD44^+CD62L^+$) cells after pre-vaccination compared to intact mice: from 68.81 ± 3.71 to 31.89 ± 1.46 in the $CD4^+CD44^+$ population and from 4.35 ± 0.59 to 0.7 ± 0.06 in the $CD4^+CD25^+CD62L^+$ population.

In order to determine the importance of contribution by regulatory and memory T-cells in formation of anticancer immunity, we immunized intact mice with LTV and xenogeneic splenocytes. In the spleen of vaccinated mice T-regulatory cells and T-memory cells were measured.

We did not observe any prominent changes in the number of $CD4^+CD25^+$, $CD4^+CD25^+FoxP3^+$, $CD4^+CD44^+$, $CD4^+CD44^+CD62L^+$ while comparing control mice after vaccination with LTV and xenogeneic splenocytes.

Thus, we found no direct effect of vaccination on reducing number of regulatory and memory T-cells in mice. Level of $IFN\gamma$ and IL-10 in supernatants of mouse splenocytes isolated from vaccinated tumor-free mice was investigated and showed a marked decrease in IL-10, but not $IFN\gamma$ level (Table 2).

In our study, we showed that the formation of an effective anticancer response is associated with a significant reduction in the Treg-cell number in the spleen that was confirmed by numerous research data. Studies performed in several experimental mo-

TABLE 1. CONTENTS OF TREG AND TEM IN SPLENOCYTES OF MICE IMMUNIZED WITH SHEEP LIPOSOMAL TESTICULAR VACCINES

Contents of Treg and Tem in splenocytes of mice immunized with Sheep Liposomal Testicular vaccines, which did not have tumors after implantation of LLC carcinoma (n = 10, p ≤ 0.01)				
	CD4 ⁺ CD25 ⁺	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	CD4 ⁺ CD44 ⁺	CD4 ⁺ CD44 ⁺ CD62L ⁺
1. Liposomal testicular AG	5.85±0.47	0.33±0.07	31.89±1.46	0.700±0.068
2. Intact group	12.84±0.77	0.95±0.17	68.81±3.71	4.35±0.59
Contents of Treg and Tmem in splenocytes of mice immunized with Liposomal Testicular vaccines				
	CD4 ⁺ CD25 ⁺	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	CD4 ⁺ CD44 ⁺	CD4 ⁺ CD44 ⁺ CD62L ⁺
1. Liposomal testicular AG	10.17±1.03	1.13±0.19	65.84±2.05	4.57±0.52
2. Sheep xenogeneic splenocytes	16.22±0.24	0.73±0.11	73.46±2.06	5.35±0.31
3. Intact group	12.84±0.77	0.95±0.17	68.81±3.71	4.35±0.59

TABLE 2. CONCENTRATION OF $IFN\gamma$ AND IL-10 IN MOUSE SPLENOCYTE SUPERNATANTS

Concentration of $IFN\gamma$ and IL-10 in mouse splenocyte supernatants in the prophylactic variant of immunization of mice with testicular AG of a ram with no tumor growth (n = 5)		
Cytokines	Testicular vaccine	Control group
IL-10	18.610±0.514**	25.90±2.41
$IFN\gamma$	141.70±39.34	205.70±28.08

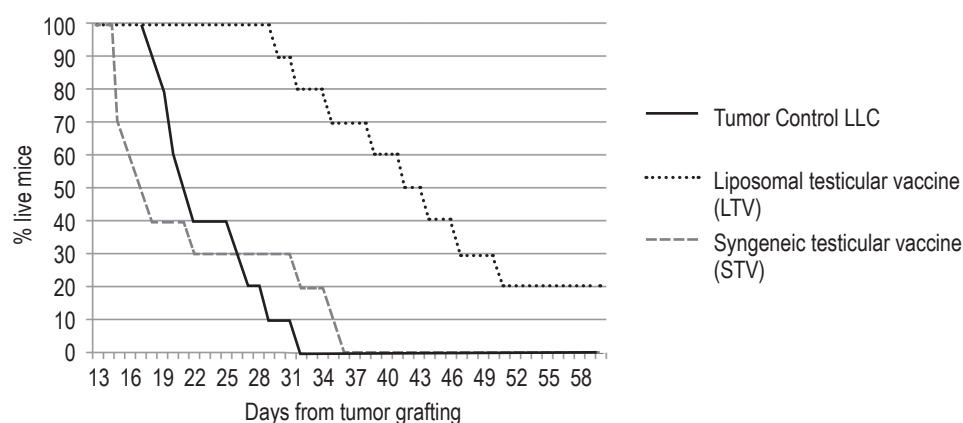


Figure 1. Survival of LLC tumor-bearing mice with prophylactic vaccination

dels have demonstrated that Tregs depletion with anti-CD25 mAbs enhanced development of anti-tumour immunity followed by tumour rejection [6, 9]. Previous preclinical studies were shown to reduce Treg number and enhance Ag-specific immune responses by IL-2/diphtheria toxin fusion protein Denleukin diftotox [15]. Various studies reported that activated Tregs express high levels of immune checkpoint molecules, including CTLA-4 and PD-1 [5], thereby allowing to consider them as a promising target for Ab-based immune-therapeutics treatment. Indeed, anti-PD-1 mAb is currently used against different types of cancer (lung, colon, melanoma, renal cell carcinoma) due to its ability to reduce immunosuppressive Tregs activity [13].

Hence, our study showed that owing to xenogenic vaccination it is possible to achieve 100% efficiency in establishing anticancer reactions, manifested by massive inoculation of tumor cells to vaccinated mice resulting in no overt tumor disease.

Based on the data obtained, we believe that immunization with xenogenic tumor AGs can lead to formation of protective antitumor response targeting own tumor-associated AGs. In addition, significant changes were recorded in some immune parameters in tumor-free mice compared to immunized control animals. Further studies on immunity parameters are required, which will allow us to confidently determine significant immune links underlying formation of prominent antitumor protection.

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ВЛИЯНИЕ ГОМЕОСТАТИЧЕСКИХ ЦИТОКИНОВ – IL-7 И IL-15 НА Т-РЕГУЛЯТОРНЫЕ КЛЕТКИ *IN VITRO*

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Резюме. Цитокины IL-7 и IL-15 являются наиболее важными гуморальными факторами, обеспечивающими восстановление пула Т-лимфоцитов после лимфопении в процессе гомеостатической пролиферации (ГП). Однако неизвестно, могут ли эти цитокины обеспечивать гомеостатическое поддержание и пролиферацию Т-регуляторных клеток (Treg). Также, учитывая связь между гомеостатической пролиферацией и развитием аутоиммунных заболеваний, интересно, могут ли цитокины ГП вызывать переход Treg-клеток в Th17-лимфоциты. Поэтому целью данного исследования было изучить влияние гуморальных факторов ГП (IL-7 и IL-15) на Treg-клетки *in vitro*. В исследовании использовалась периферическая кровь 22 здоровых доноров. Фракцию МНК получали центрифугированием в градиенте плотности фикола. Для стимуляции пролиферации использовались цитокины ГП – IL-7 и IL-15, а также комбинация IL-2 с анти-CD3-антителами. Интенсивность пролиферации Treg оценивалась с помощью проточной цитометрии с использованием CFSE в общих культурах МНК по фенотипу CD3⁺CD4⁺CD25⁺FoxP3⁺, а также в чистой популяции Treg-клеток CD3⁺CD4⁺CD25⁺CD127^{lo}, полученной с помощью иммуномагнитной сепарации с использованием набора MACS Treg Isolation Kit. Также во время культивирования оценивалась экспрессия основного транскрипционного фактора Th17-лимфоцитов – RORγt в CD3⁺CD4⁺CD25⁺FoxP3⁺-лимфоцитах. Было обнаружено, что IL-7 и IL-15 могут поддерживать Treg-клетки численно и по фенотипу, поддерживая экспрессию FoxP3 в Treg-клетках. При этом, в отличие от цитокинов ГП, стимуляция IL-2 + анти-CD3 индуцировала экспрессию FoxP3 *de novo* в CD4⁺ лимфоцитах. Также было обнаружено, что IL-7 и IL-15 могут вызывать пролиферацию Treg-клеток, однако она значительно менее интенсивна по сравнению со стимуляцией IL-2 + анти-CD3. При этом цитокины ГП не обладали способностью индуцировать экспрессию RORγt как в Treg клетках, так и в CD4⁺ лимфоцитах. Таким образом, было показано, что IL-7 и IL-15 потенциально могут участвовать в поддержании общего пула клеток Treg во время лимфопении, когда возникает дефицит IL-2, и не индуцируют экспрессию RORγt. Однако, как эти цитокины влияют на функциональную активность Treg-клеток, остается неясным и требует дальнейших исследований.

Ключевые слова: Т-регуляторные клетки, IL-7, IL-15, гомеостатическая пролиферация, RORγt, FoxP3

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INFLUENCE OF HOMEOSTATIC CYTOKINES – IL-7 AND IL-15 ON T-REGULATORY CELLS *IN VITRO*

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Abstract. Cytokines IL-7 and IL-15 are the most important humoral factors providing T-conventional cell pool reconstitution during homeostatic proliferation caused by lymphopenia. However, whether these cytokines can provide homeostatic maintenance and proliferation of T-regulatory (Treg) cells is largely unknown. Considering the association between homeostatic proliferation and the development of autoimmunity, we decided to investigate the ability of these factors to cause differentiation of Treg-cells into Th17-lymphocytes. Therefore, the purpose of this study was to investigate the influence of humoral factors of homeostatic proliferation (IL-7 and IL-15) on Treg-cells *in vitro*. The study used peripheral blood sampled from 22 healthy donors. PBMC fraction was isolated by Ficoll density gradient centrifugation. Proliferation was induced by IL-7, IL-15, and by a combination of IL-2 with anti-CD3-antibodies. The proliferation intensity of Tregs was evaluated by flow cytometry using CFSE in PBMC cultures by phenotype CD3⁺CD4⁺CD25⁺FoxP3⁺ and in the previously purified population of CD3⁺CD4⁺CD25⁺CD127^{lo}-cells. In this case Treg-cells were obtained by immunomagnetic separation from PBMCs using a MACS Treg Isolation Kit. Also, the ROR γ t expression in CD3⁺CD4⁺CD25⁺FoxP3⁺-cells was evaluated during cultivation. Here, we have shown that IL-7 and IL-15 could support Treg-cells by number and phenotype. Also, we revealed that these factors provide FoxP3 expression in Treg-cells; meanwhile, stimulation with IL-2 + anti-CD3 can also cause induction of FoxP3 expression *de novo* in conventional CD4⁺ cells. Also, we have shown that IL-7 and IL-15 can cause lower-intensity proliferation of Treg-cells in comparison with IL-2 + anti-CD3. Herewith homeostatic cytokines didn't have the ability to induce ROR γ t expression in both T-regulatory cells and CD4⁺ conventional T-lymphocytes. Thus, it has been shown that IL-7 and IL-15 can potentially participate in maintaining the total pool of Treg-cells during lymphopenia, when IL-2 deficiency occurs, without causing the induction of ROR γ t expression. However, how homeostatic cytokines affect the functional activity of Treg-cells remains unclear and requires further investigation.

Keywords: T-regulatory cells, IL-7, IL-15, homeostatic proliferation, ROR γ t, FoxP3

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Introduction

T-regulatory (Treg) cells are well known to play an important role maintaining autotolerance to self-antigens. Treg-cells have high inhibition potential due to the wide spectrum of suppression such as contact and distant mechanisms. The main factors providing Treg homeostasis are IL-2, “tonic” TCR signaling (TCR – T-cell receptor), and costimulation signals [10]. The importance of these factors for maintenance of the Treg population was shown in different animal models and in humans [7, 12, 15]. In the last 20 years, the disturbance in IL-2/IL-2R signaling or lack of costimulation cues was demonstrated to decrease Treg-cell functional activity and cause diverse autoimmune disorders [11]. It is worth noting that the main transcriptional factor of Treg-cells is FoxP3, which accounts for Treg-cell suppression activity and expression of multiple functional molecules driven by relevant genes [6]. Thus IL-2, TCR signaling, or

costimulation signals take a part in providing the expression of FoxP3 in Treg-cells. Taking into account that CD4⁺- and CD8⁺-lymphocytes are the main producers of IL-2 for Treg-cells, it can be assumed that deficiency of this cytokine during lymphopenia due to the lack of cell producers can negatively impact on the homeostasis of the Treg pool [11]. Indeed, several studies demonstrate the negative influence of lymphopenia on the Treg population associated with the lack of FoxP3 expression and decreased suppression activity of ex-FoxP3-cells [3, 9]. The lack of FoxP3 expression in Treg is known to be associated with differentiation to Th17-lymphocytes due to the induced ROR γ t expression [2]. The reconstitution of the T-cell pool by adoptive transfer prevents the negative influence of lymphopenia by increasing IL-2 production. The important role of IL-7 and IL-15 in T-cell repopulation during lymphopenia is well known [1, 5]; however, how these cytokines affect the homeostasis of the Treg-cell population and whether they have a potential to support FoxP3 expression and result in Treg proliferation remains understudied.

Thus, the goal of this study was to evaluate the influence of IL-7 and IL-15 on FoxP3 and ROR γ t expression and assess their potential to cause Treg-cell proliferation.

Materials and methods

This study was performed by using peripheral blood samples collected from 22 apparently healthy donors aged from 27 to 70 years (49 ± 13 years). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. Phenotyping of Treg was performed by flow cytometry. All monoclonal antibodies were purchased from BioLegend (San Diego, CA, USA): CD3 by PE/Cy7, CD4 by APC/Cy7, CD25 by APC, and FoxP3 by PE were used for CD3⁺CD4⁺CD25⁺FoxP3⁺ phenotype; CD3⁺CD4⁺CD25⁺CD127^{lo} phenotype: CD3 by PE/Cy7, CD4 by APC/Cy7, CD25 by PE, and CD127 by PerCP/Cy5.5. Intracellular staining was performed by using True-Nuclear Transcription Factor Buffer Set according to the manufacturer's instructions (BioLegend). For evaluation of ROR γ t expression, monoclonal antibodies were used (ROR γ t by PerCP; R&D, Minneapolis, MN, USA). Isotype and FMO controls were used for the estimation of ROR γ t expression. Treg-cells were pre-stained by CFSE for the proliferation assay. The CFSE-stained PBMCs were cultured in 48-well flat-bottom plates (TPP, Trasadingen, Switzerland) for 4 days by placing 1.5 million cells per ml, and then CD3⁺CD4⁺CD25⁺FoxP3⁺-cell proliferation was evaluated by flow cytometry. The CFSE-stained Treg-cells were also cultured with PBMC in 200 μ l of total volume in a 1-to-1 ratio (30,000 Treg per 30,000 PBMC) for 4 days in round-bottomed plates. In this case, Treg-cells were obtained by immunomagnetic separation from PBMCs using a MACS Treg Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). The population was isolated to contain CD3⁺CD4⁺CD25⁺CD127^{lo}-phenotype (purity was $93 \pm 4\%$). The cell culture was performed in RPMI-1640 media supplemented with 10% FCS and antibiotics. Three types of stimulation protocols were used: IL-2 (100 U/ml; NPK BIOTECH, coupled with anti-CD3 antibodies (1 μ g/ml; Sorbent, Pasadena, TX, USA), IL-7 (50 U/ml; MyBiosource, San Diego, CA, USA), and IL-15 (50 U/ml; MyBiosource). The cells were cultured without stimulation as a negative control.

Statistical analysis was performed by GraphPad Prism software (v. 7.03; GraphPad, La Jolla, CA, USA). Nonparametric methods were applied for descriptive statistics (median with interquartile range). For multiple independent groups, the comparison was conducted with the Kruskal–Wallis test, while for multiple dependent groups comparison was performed by using the Friedman test. Post hoc analysis was

performed by using Dunn's test. The differences were considered significant at $p < 0.05$.

Results and discussion

After cell culture, the percentage of CD25⁺FoxP3⁺-lymphocytes was higher at all stimulation conditions than in the control stimulation-free setting ($3.5 \pm 1.7\%$), whereas the percentage of Treg-cells was higher by adding IL-2 + anti-CD3 ($9.7 \pm 3.4\%$) vs IL-7 ($7.9 \pm 2.8\%$) or IL-15 ($7.7 \pm 3.1\%$). At the same time, the amount of Treg-cells was lower without stimulation than in the samples from peripheral blood before cultivation ($6.2 \pm 1.5\%$). This result agrees with our previous data [8]. To directly evaluate the influence of homeostatic cytokines – IL-7 and IL-15 on the expression of FoxP3, we assessed the FoxP3 MFI in Treg-cells at different stimulation conditions (Figure 1). We revealed a significantly decreased FoxP3 expression in cells without stimulation. Meanwhile, stimulation with homeostatic cytokines – IL-7 and IL-15 supported the expression of FoxP3 in Treg-cells. It should note that the maximal FoxP3 expression was upon IL-2 + anti-CD3 stimulation, exceeding the values for peripheral blood.

The significant decrease in the percentage of CD25⁺FoxP3⁺-cells and FoxP3 MFI expression in the control cells without stimulation was due to decreased CD25 (IL-2R) expression in the absence of the key stimulation factors [4]. Here, stimulation with IL-7 or IL-15 could similarly support the Treg amount and FoxP3 expression albeit to a lesser extent than IL-2 + anti-CD3, which suggests that the cytokines IL-7 and

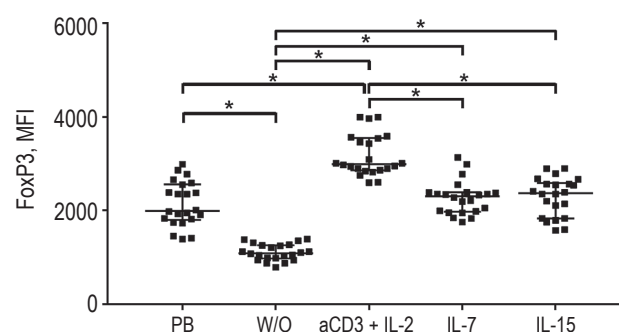


Figure 1. MFI of FoxP3 in Treg-cells in various cell culture conditions. Expression of FoxP3 in Treg-cells significantly decrease during culture without stimulation. Meanwhile, stimulation with IL-7 or IL-15 supports FoxP3 expression in Treg cells even to a lesser extent than IL-2 + anti-CD3

Note. $n = 22$, data are presented as the median and interquartile range of $Q_{0.25}$ – $Q_{0.75}$; the comparison was performed by using the Friedman test, post hoc analysis – Dunn's test. MFI, Mean Fluorescent Intensity; PB, peripheral blood; W/O, without stimulation; aCD3, anti-CD3 antibodies. *, $p < 0.001$

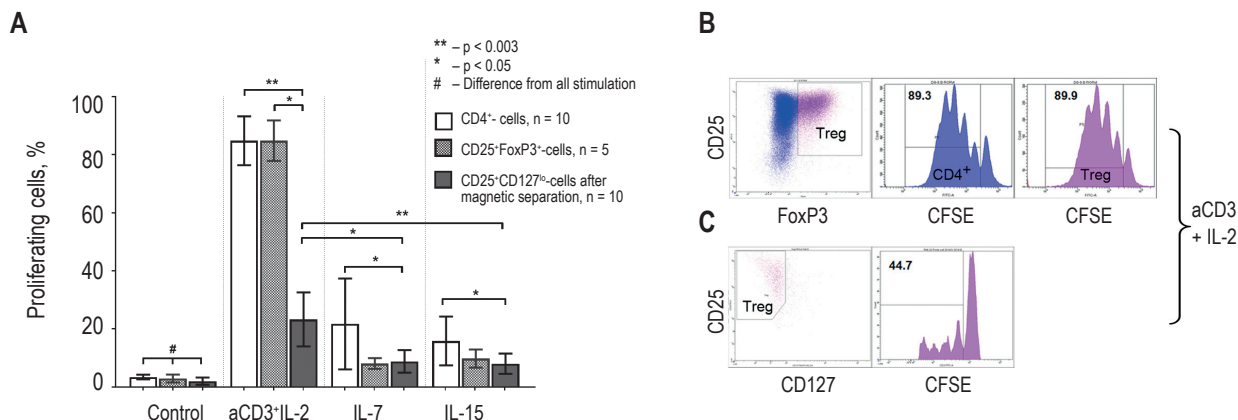


Figure 2. A, the level of proliferation of CD4⁺ and Treg-cells under various culture conditions. IL-2 + anti-CD3 stimulation, in contrast to effects of IL-7 or IL-15, can induce FoxP3 expression in conventional CD4⁺ T-cells. B, a gating strategy for CD25⁺FoxP3⁺ cells in total PBMC cultures and proliferation of CD4⁺ and CD25⁺FoxP3⁺ cells in PBMC cultures. C, a representative proliferation of Treg-cells obtained by magnetic separation

Note. The data are presented as a median and interquartile range of $Q_{0.25}$ - $Q_{0.75}$. The comparison was performed by using the Kruskal-Wallis test for unrelated groups and the Friedman test for related groups. The post hoc analysis in both cases used Dunn's test.

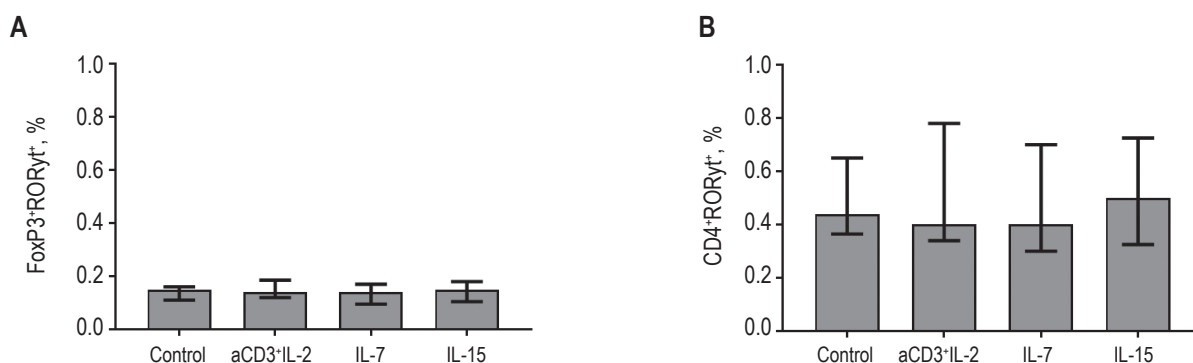


Figure 3. Percentage of FoxP3⁺RORγt⁺ lymphocytes gated on CD25⁺FoxP3⁺ cells (A) and the percentage of CD4⁺RORγt⁺ lymphocytes gated on CD4⁺ cells (B) in PBMC cultures; n = 5. Homeostatic cytokines – IL-7 and IL-15 caused no induction of RORγt in Treg and conventional CD4⁺ lymphocytes *in vitro*

Note. The data are presented as a median and interquartile range of $Q_{0.25}$ - $Q_{0.75}$. The comparison used the Kruskal-Wallis test.

IL-15 can potentially support the expression of FoxP3 in Treg-cells *in vivo* during lymphopenia.

The induction of Treg-lymphocytes from effector CD4⁺ cells is well known to occur under certain conditions. Such induced Treg-lymphocytes are not mainly “true” regulatory T-lymphocytes because they are prone to lose FoxP3 expression and may differentiate into pathogenic Th-17-lymphocytes in some situations [14]. Thus, it seems important to determine an underlying mechanism resulting in increased percentage of CD25⁺FoxP3⁺ lymphocytes in PBMC cultures occurred exposed to these stimulation conditions: i.e., either due to proliferation or induction. Hence, we compared the proliferation of CD25⁺FoxP3⁺ lymphocytes in PBMC cultures with

the proliferation of previously purified Treg-cells. Figure 2 shows the data obtained for the proliferation of CD3⁺CD4⁺CD25⁺FoxP3⁺ effector cells, CD3⁺CD4⁺CD25⁺FoxP3⁺ Treg-cells in PBMC cultures and for “pure” CD3⁺CD4⁺CD25⁺CD127^{lo} Treg-lymphocytes.

The differences revealed in the proliferation of Treg-lymphocytes in PBMC cultures from the proliferation of Treg-cells isolated by magnetic separation are associated with the induction of *de novo* CD25⁺FoxP3⁺ lymphocytes from conventional CD4⁺ T-cells under stimulation by IL-2 combined with anti-CD3. However, this effect was not observed during the stimulation with IL-7 and IL-15. Figure 2 shows that the proliferation of Treg-cells was significantly

lower than that of CD4⁺ lymphocytes under all stimulation conditions. Nonetheless, cytokines IL-7 and IL-15 could induce the proliferation of Treg-cells, although to a lesser extent, than the stimulation with IL-2 + anti-CD3, which may be important under lymphopenia.

In the next step of our study, we tested whether homeostatic cytokines can induce Treg differentiation into Th-17-lymphocytes. The analysis of ROR γ t expression revealed no increased percentage of transient FoxP3⁺ROR γ t⁺ and CD4⁺ROR γ t⁺-lymphocytes after any culture conditions (Figure 3), which suggests that the cytokines IL-7 and IL-15 cannot induce *in vitro* expression of the transcription factor ROR γ t, at least in healthy donors.

A significantly higher level of Treg-cell proliferation under the influence of the IL-2 + anti-CD3 combination vs IL-7 and IL-15 was detected, which was expected due to being associated with the nature of such stimuli. According to some assumptions, stimulation with anti-CD3 antibodies is an analog of strong TCR stimulation, while IL-2 is the pivotal cytokine involved in activating and maintaining Treg pool by accounting for STAT5 expression [14]. Thus, the data obtained allow us to conclude that the homeostatic cytokines – IL-7 and IL-15 can effectively support Treg-lymphocyte quantity and FoxP3 expression at least *in vitro*. These results are generally

consistent with the most published data, which indicate about potential of common γ -chain-driven cytokines to positively influence Treg-cell homeostasis particularly due to activation of the STAT5 signaling pathway [13]. No increase in ROR γ t induction was detected under IL-7 and IL-15 stimulation, which confirms the ability of IL-7 and IL-15 to maintain the homeostasis of the Treg population resulting in no induction of Th-17-cells.

Conclusion

In general, these results indicate the ability of cytokines IL-7 and IL-15 to provide homeostatic maintenance of the Treg population under IL-2 deficiency. However, the lower proliferation of Treg-lymphocytes compared to CD4⁺-lymphocytes may lead to a delayed recovery of Treg pool and may impair immune balance under lymphopenia. Herewith, it is worth noting that humoral factors IL-7 and IL-15 do not increase ROR γ t expression or contribute to Treg differentiation towards exFoxP3⁺ROR γ t⁺-cells.

Authors' contributions

Daniil Shevyrev performed all *in vitro* experiments, analysed the data, and wrote the manuscript. Vladimir Kozlov conceived and designed experiments, gained financial support, and finally approved the manuscript.

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ВЛИЯНИЕ РАСТВОРИМЫХ ФАКТОРОВ МАКРОФАГОВ M2-ФЕНОТИПА НА ДИФФЕРЕНЦИРОВКУ КЛЕТОК ЛИНИИ SH-SY5Y

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Резюме. Известно, что макрофаги играют важную роль в запуске и регуляции процессов нейрорегенерации. Макрофаги характеризуются выраженной пластичностью, что проявляется в их способности изменять свой фенотип в зависимости от сигналов микроокружения. В условиях патологии одним из индукторов формирования противовоспалительного M2-фенотипа макрофагов является взаимодействие с апоптотическими клетками (эффероцитоз). Ранее нами был разработан оригинальный протокол генерации M2-подобных макрофагов, поляризованных в результате эффероцитоза в условиях депривации сыворотки. Целью настоящего исследования явилась оценка нейрорегенеративного потенциала таких макрофагов (M2 (LS), Low Serum). Исследовали влияние кондиционных сред M2 (LS) на процесс дифференцировки клеток линии SH-SY5Y в сравнении с ретиноевой кислотой (RA). В качестве морфологических критериев дифференцировки оценивали относительное содержание дифференцированных клеток, т.е. клеток с длиной нейритов, превышающей длину тела клетки, а также среднюю длину нейритов на 3-и, 7-е и 13-е сутки. Наряду с этим оценивали соотношение нейроноподобных (N-тип) и эпителиально-подобных (S-тип) клеток в культурах. Клетки SH-SY5Y характеризовались низким уровнем спонтанной дифференцировки как в стандартных условиях (10% FBS), так и при депривации сыворотки (1% FBS). При обработке ретиноевой кислотой клетки SH-SY5Y прекращали делиться и подвергались нейрональной дифференцировке. Культивирование клеток SH-SY5Y в присутствии кондиционной среды (КС)-M2 (LS) (30% v/v) также приводило к значимому возрастанию относительного содержания дифференцированных клеток, средней длины нейритоподобных отростков, а также изменению баланса клеток S- и N-типа в сторону выраженного преобладания последних. При этом выраженность морфологических признаков дифференцировки на ранних этапах дифференцировки (3-и сут.) была достоверно снижена по сравнению с уровнем RA-индуцированных изменений и достигала уровня позитивного контроля только на более поздних стадиях (к 13-м сут.) ($p < 0,05$). В отличие от ретиноевой кислоты, КС-M2 (LS) индуцировали нейрональную дифференцировку клеток SH-SY5Y без подавления их пролиферативной активности. Полученные данные могут свидетельствовать о нейрорегенеративном потенциале M2 (LS) макрофагов

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in vitro, который опосредуется через растворимые факторы и проявляется в усилении дифференцировки клеток SH-SY5Y.

Ключевые слова: макрофаги, M2 фенотип, клетки SH-SY5Y, дифференцировка, ретиноевая кислота, нейрорегенерация

EFFECT OF M2 MACROPHAGE-DERIVED SOLUBLE FACTORS ON DIFFERENTIATION OF SH-SY5Y CELLS

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Abstract. Macrophages play a key role in triggering and regulation of neuroregeneration. The characteristic feature of macrophages is pronounced plasticity, which manifests itself in the ability of macrophages to change their functional phenotype depending on the micromilieu. Apoptotic cell clearance (efferocytosis) is an important inducer of a macrophage polarization to M2 phenotype under pathological settings. Previously, we have developed an original protocol for the generation of M2-like macrophages, polarized by efferocytosis under serum-deprived conditions (M2 (LS), Low Serum). The present study was aimed to assess a neuroregenerative potential of M2 (LS) macrophages. We studied their effect on the differentiation of SH-SY5Y cells in comparison with retinoic acid (RA). As the morphological criteria of differentiation we have assessed the relative content of differentiated cells, i.e., cells with a neurite length exceeding the cell body length, and the average neurite length on days 3, 7, and 13. The ratio of neuron-like (N-type) and epithelial-like (S-type) cells in cultures was also assessed. SH-SY5Y cells were characterized by a low level of spontaneous differentiation, both under standard conditions (10% FBS) and serum deprivation (1% FBS). Upon RA treatment, SH-SY5Y cells stopped proliferating and underwent neuronal differentiation. Cultivation of SH-SY5Y cells in the presence of M2 (LS) conditioned medium also led to a significant increase in the relative content of differentiated cells, the average length of neurite-like processes, as well as a change in the balance of S- and N-type cells towards a pronounced predominance of the latter. The morphological features of differentiation were significantly less pronounced at early stage (day 3) of differentiation as compared with the RA-induced changes and reached the level of positive control only at later stages (day 13) ($p < 0.05$). In contrast to retinoic acid, M2 (LS) conditioned medium induced neuronal differentiation of SH-SY5Y cells without suppressing their proliferative activity. The data obtained may indicate a high neuroregenerative potential of M2 macrophages *in vitro*, which is realized through soluble factors and manifests itself in promoting SH-SY5Y differentiation.

Keywords: macrophages, M2 phenotype, SH-SY5Y cells, differentiation, retinoic acid, neuroregeneration

The work was carried out at the expense of the federal budget for fundamental research on the topic “Justification and development of new technologies for immunomodulation, stimulation of reparative processes and correction of behavioral and addictive disorders based on the use of myeloid, lymphoid and stem cells and/or their secretome products”.

Introduction

Macrophages (Ms), being an essential component of innate immunity, play a key role in inflammation, regeneration, and homeostasis maintenance. The main

features of Ms are pronounced plasticity and heterogeneity manifested as a potential to change their functional phenotype in response to various microenvironmental signals with formation of many activation states between two opposite “extremities” — classically activated M1 and alternatively activated M2 Ms. A defining M1 characteristic is proinflammatory activity, whereas M2 exhibit anti-inflammatory properties and play a crucial role in stimulating reparative processes and resolution of inflammation [25]. M2 phenotype Ms are induced by a variety of stimuli, such as IL-4/IL-13, immunosuppressive cytokines (IL-10,

TGF- β), immune complexes, hormones and vitamins (dexamethasone and vitamin D3). Apoptotic cell clearance (efferocytosis) is an important inducer of M2 macrophage polarization under pathological settings. Previously, we have developed an original protocol for the M2-like Ms generation based on the culture of human blood monocytes under serum-deprived conditions, which contributes to the development of deprivation apoptosis with the subsequent engulfment of apoptotic cells by macrophages.

The Ms obtained in this way are characterized by low antigen-presenting and proinflammatory activities as well as a high level of growth and neurotrophic factors secretion (VEGF, IGF-1, EPO, BDNF, EGF, FGF-basic) [3, 22]. The combination of these properties allows to consider M2 (LS) as a Ms functional phenotype with a high neuroregenerative potential which was confirmed by pilot clinical trials in patients with CNS pathology [4, 23]. *In vitro* studies have revealed one of potential mechanisms for M2 (LS) neuroregenerative effects exerted as the capacity to enhance proliferation and reduce apoptosis of SH-SY5Y neural cells [19]. At the same time, along with increased neural stem cells proliferation, for a successful neuroregeneration and their subsequent differentiation and integration into existing neuronal network were required [24]. However, an influence of M2 (LS) on the neural cell differentiation *in vitro* has not been previously studied.

The present study was aimed to assess the influence of M2-derived soluble factors on the SH-SY5Y neuroblastoma cells differentiation. To this end, we assessed the effects of M2 (LS) conditioned medium on: 1) the number of cells with neurite (s) exceeding cell body lengths; 2) average neurite length in SH-SY5Y cell culture.

Materials and methods

Ms were generated from an adhesive fraction of human peripheral blood mononuclear cells (PBMCs), as described earlier [22]. Briefly, $3-5 \times 10^6$ /ml PBMCs were incubated in RPMI-1640 (Biolot, St. Petersburg, Russia) medium supplemented with 0.05 mM 2-mercaptoethanol, 2 mM sodium pyruvate 0.3 mg/ml L-glutamine, 1% nonessential amino acids, 100 μ g/ml gentamycin, 2% autoplasm and 50 ng/ml recombinant human GM-CSF (R&D Systems, USA). After 18-24 hours, the non-adherent cells were removed, and the adhesive cells were cultured for 7 days. The conditioned Ms medium was collected, centrifuged, and stored at -20 °C.

SH-SY5Y cells were cultured in DMEM/F12 (Biolot, St. Petersburg, Russia) medium containing

10% FBS (BioWest, France) (standard conditions) or 1% FBS (conditions of deprivation) at 37 °C in the CO₂ incubator. Cells were passaged after reaching 70-80% monolayer. Cells of passages 3-5 were used for the experiments.

To assess cell differentiation, SH-SY5Y cells were cultured in 6-well plates (4×10^5 cells per well) in DMEM/F12 medium with 1% FBS, supplemented with retinoic acid (RA, 10 μ M; positive control) or M2 (LS) conditioned medium (30% (v/v), experiment) for 3, 7 and 13 days.

Morphometric analysis was performed using the ImageJ software and the NeuronGrowth plugin [6]. At least 100 cells were analyzed in different fields of view, counting the number of neurite-bearing cells, the length of which exceeded the length of the cell body, and the average neurite length. Three independent experiments were performed. Morphometric analysis was performed at indicated time points (3, 7, and 13 days). For each cell, the length of the primary neurite, defined as a single neurite, or the longest neurite, for cells with more than one neurite, was measured.

Statistical analysis was performed using the STATISTICA software version 8.0 (StatSoft, Inc., USA). The data are presented as median (Me) and interquartile range (IQR, $Q_{0.25}-Q_{0.75}$). To reveal a significant difference of the values compared, the Mann-Whitney nonparametric U test was used. A significance level was set at $p < 0.05$.

Results and discussion

Effect of M2 (LS) conditioned medium on the number of differentiated SH-SY5Y cells

Undifferentiated SH-SY5Y cells tend to grow in clumps which are clusters of rounded cells in the center and cells with short processes / neurites along the edges. During the differentiation, the morphology of the culture changes: cells do not form clusters; cell bodies stretch out, acquiring a pyramidal shape with growing neurites; and the processes vs. cell body elongation significantly exceeds. The predominance of the length of the processes over the cell body is one of the main morphological markers of differentiation.

As shown in Table 1, when SH-SY5Y cells were cultured in standard conditions (10% FBS) the relative number of differentiated cells (i.e., cells with neurite length exceeding the length of the cell body) at different stages of differentiation (3, 7, and 13 days) did not outreach 10%, which indicates a low level of spontaneous differentiation. Under deprivation conditions, the number of differentiated cells was decreased almost by 2 times (4.5-5%). In the presence of retinoic acid (positive control), as

TABLE 1. EFFECT OF M2 (LS) CONDITIONED MEDIUM ON MORPHOLOGICAL MARKERS OF DIFFERENTIATION IN THE SH-SY5Y CELL CULTURES

Day	Control (10% FBS)	Deprivation (1% FBS)	RA (10 μ M)	M2 (LS) CM (30% (v/v))
	1	2	3	4
Relative amount of differentiated cells, %				
3	9.4	4.5	22.7	15.1
7	5.9	5.0	28.4	13.3
13	5.0	1.0	16.0	36.0
Average length of neurites, μ m				
3	24.7 18.6-34.8	16.8 13.5-23.0 2-1: $p_u < 0.005$	37.5 27.8-54.5 3-2: $p_u < 0.005$ 3-1: $p_u < 0.005$	32.1 24.9-43.3 4-3: $p_u < 0.005$ 4-2: $p_u < 0.005$ 4-1: $p_u < 0.005$
7	22.6 15.4-30.9	18.4 13.1-26.1 2-1: $p_u < 0.005$	47.1* 33.5-63.3 3-2: $p_u < 0.005$ 3-1: $p_u < 0.005$	32.4 25.0-44.2 4-3: $p_u < 0.005$ 4-2: $p_u < 0.005$ 4-1: $p_u < 0.005$
13	22.5 17.5-26.4	19.0 11.1-29.7	49.3* 40.5-74.3 3-2: $p_u < 0.005$ 3-1: $p_u < 0.005$	46.209* # 35.6-60.0 4-2: $p_u < 0.005$ 4-1: $p_u < 0.005$

Note. The data are presented as median (Me) and interquartile range (IQR, 25-75% quartiles). *, $p_u < 0.005$ vs day 3; #, $p_u < 0.005$ vs day 7. CM, conditioned medium.

TABLE 2. EFFECT OF M2 (LS) CONDITIONED MEDIUM ON THE RATIO OF DIFFERENT CELL TYPES IN SH-SY5Y CELLS CULTURES

Type of cells	Day	Spontaneous differentiation		Induced differentiation	
		10% FBS	1% FBS	RA (10 μ M)	M2 (LS) CM (30% (v/v))
N-type	3	79	78	89	93
	7	80	81	75	95
	13	63	37	86	98
among them differentiated	3	12.7	6.4	27	17
	7	5	5	28	17
	13	8	3	19	37
S-type	3	21	22	11	7
	7	21	19	25	5
	13	37	63	14	2
N/S-type ratio	3	4:1	4:1	8:1	13:1
	7	4:1	4:1	3:1	19:1
	13	2:1	0.6:1	6:1	49:1

Note. The data of a representative experiment. The relative content of cells was determined by analyzing at least 100 cells. RA, retinoic acid; CM, conditioned medium.

expected, the number of differentiated cells increased significantly, several times higher than a spontaneous differentiation level ($p < 0.05$ as compared with standard and “deprivation” controls). In the presence of M2 (LS) conditioned medium, the percentage of differentiated SH-SY5Y cells also increased. At the early stages of differentiation (3 and 7 days), the number of differentiated cells was lower compared to the positive control. However, by the day 13, the percentage of these cells increased sharply that was more than 2 times higher than in RA-differentiated cultures.

Effect of M2 (LS) conditioned medium on the average length of neurites in SH-SY5Y cultures

The data in Table 1 demonstrated that the average length of neurites in cells, cultivated under standard conditions (10% FBS), was 23–25 μm at all stages of differentiation. Under serum deprivation, the average neurite length significantly decreased down to 17–19 μm . In RA-induced cultures, the average neurite length was increased by 1.5–2 times and 2.2–2.6 times compared with the standard control and the deprivation control, respectively. The increase in neurite length registered on the day 3 continued by the day 7 and further remained at the level reached. Cell cultivation in the presence of M2 (LS) conditioned medium resulted in significantly increased average neurite length vs. standard and deprivation controls. This effect manifested itself on day 3 of culture and persisted up to day 7. At the same time, at the early stages of differentiation, the neurite length in cultures with M2 (LS) conditioned medium was significantly lower than that in the positive control (RA). However, the significantly increased neurite length as high as the level of RA-induced values was observed by the day 13.

Effect of M2 (LS) conditioned medium on the SH-SY5Y cell N/S-type ratio

It is known that the cultures of undifferentiated SH-SY5Y cells contain two morphologically distinct cell types: S-type (adhering to the substrate, epithelial-like cells without processes) and N-type (cells with neurite-like processes). The relative number of each cell type under differentiation conditions is shown in Table 2. The relative number of N- and S-type cells during differentiation of the SH-SY5Y cell line depending on the inducing stimulus is well visualized as the N/S ratio. At the early stages of differentiation, regardless of the serum percentage, the number of N-type cells was 4 times higher than the number of S-type cells. At later stages, a decrease in the number of N-type cells and an increase in S-type cells were observed, which led to a final difference of 2 or more times (2:1 and 0.6:1). RA-induced differentiation

was accompanied by an increase in the proportion of N-type cells and a decrease of S-type cells by the days 3 and 13. At the intermediate stage (7 days), the number of S-type cells, on the contrary, increased, which led to a reduction in the N/S ratio from 8:1 to 3:1. The most considerable change in the balance between these cell types was observed in cultures with M2 (LS) conditioned medium: the overwhelming majority of cells was of N-type cells (93, 95, 98%), while the S-type was represented by rare single cells (7, 5, 2%). Interestingly, the majority of N-type cells in these cultures were presented by cells with short processes, not exceeding the length of the cell body (i.e. undifferentiated cells).

The aim of the study was to assess an effect of M2 (LS) Ms on the SH-SY5Y cell differentiation. To address it, we have compared the influence of M2 (LS) conditioned medium and retinoic acid (RA), a well-known and widely used inducer of neuronal differentiation.

The data obtained showed that M2 (LS) conditioned medium, similar to RA, stimulates the differentiation of SH-SY5Y cells exhibited as increased relative content of differentiated cells, the average length of neurite-like processes, as well as a change in the ratio between S- and N-type cells shifted to a pronounced predominance of the latter. At the same time, it is important to note some differences between differentiation processes, induced by retinoic acid and macrophages. Thus, the effect of RA is manifested already at early stage of differentiation (day 3), leading to a significant increase in number of differentiated cells and average neurite length as compared to control cultures. Effect of M2 (LS) conditioned medium is also clearly detected at early stages of differentiation. However, morphological features of differentiation during this period are significantly less pronounced compared to those in RA-induced cultures and reaches the level of positive control only at later stages (by the 13th day). At the same timepoint (day 13) in RA-stimulated cultures a decrease in the relative number of differentiated cells is recorded that may be associated with the long-term presence of retinoic acid as a monostimulus in the culture. Indeed, in many protocols long-term cultivation implies the use of an additional differentiating stimulus (for example, BDNF, IGF-1, laminin coating of plastic, etc) [1].

Some authors have described the presence of two morphologically distinct types of undifferentiated SH-SY5Y cells: S-type (adhering to the substrate, epithelial-like cells without processes) and N-type (cells with neurite-like processes) [21]. Short-term RA treatment (up to 5 days) induces differentiation of N-type cells, while longer-term treatment promotes

proliferation of S-type cells [5]. The authors have also shown that S-type cell proliferation depends on the serum level. These data make possible to explain our results on a twofold increase in S-type cells in RA-induced cultures and a higher proportion of S-type cells in cultures added with 10% FBS.

A distinctive feature of cultures with the addition of M2 (LS) conditioned medium is the preservation of the proliferative activity of SH-SY5Y cells, which is confirmed by the previously obtained data and is consistent with the data obtained by other authors. For instance, Popova et al showed that SH-SY5Y cells continue to proliferate during differentiation [17]. High N-type/S-type cell ratio (N:S, 13:1–49:1), observed in the presence of M2 (LS) conditioned medium and exceeding this index in RA-treated cultures, indicates the predominant proliferation of N-type cells. This provides a considerable number of undifferentiated N-type cells capable of further differentiation. It is reasonable to assume that the differentiation of these certain cells provide a significant enhancement of SH-SY5Y neural differentiation at later stages of differentiation. Thus, soluble factors of M2 (LS) macrophages induce both proliferation and neural differentiation of SH-SY5Y cells. M2 macrophages produce cytokines and growth factors, which activate neurogenesis, oligodendrogenesis and angiogenesis, contributing to the appearance of new cellular elements [2, 26]. By producing trophic and growth factors, M2 Ms provide axonal remodeling, i.e., formation of new axonal collaterals by stimulating the growth of axons, the formation of new synapses and remyelination of axons [8, 14, 20]. We have previously shown that M2 (LS) actively secrete a complex of various neurotrophic, neuroprotective and angiogenic factors, including IGF-1, VEGF, erythropoietin, etc. [3].

It is known that IGF-1, whose production by M2 (LS) macrophages exceeds the level of its production by M1 and M2 macrophages by double-digit rates [3], is able to induce neural differentiation of dental pulp stem cells and stimulate differentiation of SH-SY5Y

cells *in vitro* [7, 11]. Moreover, IGF-1 increases the proliferative activity of SH-SY5Y cells [13]. Thus, IGF-1 can significantly contribute to the stimulatory effects of M2 (LS) conditioned medium on proliferation and differentiation of SH-SY5Y cells.

Erythropoietin (EPO) is currently considered as a pleiotropic cytokine which has neurotrophic, neuroprotective, antiapoptotic and mitogenic effects, and plays an important role in the regeneration of various tissues, including nervous tissue [16]. However, EPO influence on SH-SY5Y cell differentiation remains unclear. Prego et al. showed that EPO inhibits not only apoptosis, but also the differentiation of SH-SY5Y cells induced by staurosporin [18]. In contrast, Yuste et al demonstrated the preservation of the differentiation potential in a similar model [27]. Since M2 (LS) produce a high EPO level, and SH-SY5Y cells express the EPO receptor, it is reasonable to assume that it stimulates SH-SY5Y cells proliferation. At the same time, taking into account the data of Prego et al, it is impossible to rule out a negative EPO effect on the differentiation of SH-SY5Y manifested as the “delayed” stimulation of the process.

VEGF plays an important role not only in vasculo/angiogenesis, but also in the regulation of neurogenesis, stimulating the proliferation of neuronal precursors either directly [9, 28], or through brain derived neurotrophic factor (BDNF) produced by endothelial cells [12]. Neuroprotective properties of VEGF have been demonstrated both *in vitro* and *in vivo* [10, 15]. The level of VEGF production by M2 (LS) macrophages is significantly higher than that by M1 and M2a (IL-4) [3]. In addition, M2 (LS) also produce BDNF, which promotes differentiation of SH-SY5Y cells and being included into some standard differentiation protocols for these cells [1].

Taken together, the data obtained indicate a high neuroregenerative potential of M2 macrophages *in vitro* revealed by the induced differentiation of SH-SY5Y cells and enabled via soluble factors.

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ВЛИЯНИЕ НА ИММУНОКОМПЕТЕНТНЫЕ КЛЕТКИ ПРИ ТЕРАПИИ МЕЗЕНХИМАЛЬНЫМИ СТВОЛОВЫМИ КЛЕТКАМИ В СРАВНЕНИИ С ТЕРАПИЕЙ НА ОСНОВЕ МИКРОВЕЗИКУЛ У МЫШЕЙ С ХРОНИЧЕСКИМ ЗАБОЛЕВАНИЕМ ПОЧЕК

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Резюме. Мезенхимальные стволовые клетки, которые обладают как плюрипотентными, так и иммунорегуляторными функциями, активно изучаются как один из способов лечения во многих областях медицины, включая реабилитацию в нефрологии. Однако в некоторых статьях, посвященных лечению различных органических патологий с помощью МСК, было доказано, что эффекты от применения МСК в регенеративной медицине заключаются в том, что после трансплантации в поврежденные органы они могут ингибировать апоптоз клеток, способствуют ангиогенезу для лучшего кровоснабжения и в некоторых случаях стимулируют местную пролиферацию клеток в поврежденных тканях. Другими словами, про-регенерационные функции были обусловлены их паракринными эффектами без влияния дифференциации.

При введении мышам МСК было показано иммуноподавляющее действие, что в конечном счете приводило к уменьшению воспаления и улучшенной регенерации почек. Одним из механизмов восстановления могут быть МВ — внеклеточные тельца, продуцируемые МСК. Как стало известно из последних сообщений, МВ сохраняют некоторые функции клеток, такие как перенос микроРНК и т.д. Считается, что терапевтический эффект МСК может быть во многом связан с их иммуносупрессорными свойствами.

Целью данного исследования было изучение иммунорегуляторных свойств микровезикул (МСК-МВ, МВ) в сравнении с мезенхимальными стволовыми клетками (МСК). Эксперимент проводился на мышах линии СВА возрастом 3-4 месяца, использовалась модель глицерол-индуцированной хронической почечной недостаточности (ХПН). МСК были получены из костного мозга сингенных мышей большеберцовой и бедренной костей. Клетки культивировались в культуральном матрасе до достижения монослоя. МВ были получены из МСК путем апоптоза при помощи культивирования в бессывороточной среде в условиях депривации кислорода в течении суток. МСК и МСК-МВ вводились в хвостовую вену мышей, забор органов производился на 11-е сутки после введения МСК и МСК-МВ.

Методом проточной цитофлуориметрии оценили состояние клеточного иммунитета у мышей с хронической почечной недостаточностью (ХПН) после введения мезенхимальных стволовых клеток

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(МСК) и полученных из них микровезикул (МСК-МВ) на 11-е сутки эксперимента. В результате наблюдалось снижение количества Т-регуляторных клеток $CD4^+CD25^+Foxp3^+$, повышение содержания $CD4^+CD44^+CD62^+$ и $CD8^+CD44^+CD62^+$ Т-клеток памяти.

Влияние МВ не существенно отличалось от МСК. Это может свидетельствовать о том, что терапевтические свойства МСК обусловлены в большей степени, если не полностью МСК-МВ.

Ключевые слова: мезенхимальные стволовые клетки, внеклеточные пузырьки, микровезикулы, хроническая почечная недостаточность, почечное повреждение, бесклеточная терапия

MESENCHYMAL STEM CELLS COMPARED WITH MICROVESICLES-BASED THERAPY IMPACTS IMMUNOCOMPETENT CELLS IN MICE WITH CHRONIC RENAL DISEASE

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Abstract. Mesenchymal stem cells which have both pluripotent and immunoregulatory functions are being actively studied as one of the treatment ways in many fields of medicine, including rehabilitation in nephrology. However, in some articles were dedicated to the treatment of different organic pathology with MSC's was proved that the only reported effects from the application of MSC's in regenerative medicine are that, after transplantation to damaged organs, in a paracrine manner they may inhibit apoptosis of cells, promote angiogenesis for a better blood supply, and in some cases stimulate cells that have survived in damaged tissues to proliferate in order to replenish dying tissue fragments. In other words, the pro-reparational functions were due to their paracrine effects without the impact of differentiation. Microvesicles are one of the components of this pro-regenerative effect. And although immunoregulatory action has been shown for MSCs, it remains poorly understood for microvesicles.

The aim of this study was to study the immunoregulatory properties of microvesicles (MSC-MB, MB) in comparison with mesenchymal stem cells (MSC). The experiment was carried out on CBA mice 3-4 months old, a model of glycerol-induced chronic renal failure (CRF) was used. MSCs were obtained from the bone marrow of syngeneic mice of the tibia and femur. MVs were obtained by apoptosis by culturing in a serum-free medium under oxygen deprivation conditions. MSC and MSC-MB were injected into the tail vein of mice; organs were harvested on the 11th day after administration of MSC and MSC-MB.

The state of cellular immunity in mice with chronic renal failure (CRF) after the introduction of mesenchymal stem cells (MSC) and microvesicles derived from them (MSC-MB) on the 11th day of the experiment was assessed by flow cytometry. As a result, there was a decrease in the number of T-regulatory cells $CD4^+CD25^+Foxp3^+$, an increase in the content of $CD4^+CD44^+CD62^+$ and $CD8^+CD44^+CD62^+$ memory T-cells.

The effect of MVs was not significantly different from that of MSC. This may indicate that the therapeutic properties of MSCs are determined to a greater extent, if not completely, by MSC-MB.

Keywords: mesenchymal stem cells, microvesicles, microvesicles, chronic renal failure, chronic kidney disease, acellular therapy

Introduction

Mesenchymal stem cells exerting both pluripotent and immunoregulatory functions are actively studied as one of the treatment approaches in many fields of medicine, including rehabilitation in nephrology. However, some articles [3, 10] dedicated to the treatment of different organic pathology with MSCs confirmed that the only reported effects from MSC

application in regenerative medicine revealed that, after transplantation to damaged organs, they may in a paracrine manner inhibit apoptosis of cells, promote angiogenesis for a better blood supply, and in some cases stimulate cells survived in damaged tissues to proliferate in order to replenish dying tissue fragments. In other words, the pro-reparational functions were due to their paracrine effects without the impact on differentiation.

In experimental models *in vivo* the immunosuppressive functions of MSCs were proved, which resulted in decreased inflammation and improved kidney regeneration [3].

Extracellular vesicles are subdivided into exosomes, microvesicles, and apoptotic bodies. Exosomes are the smallest vesicles (30-100 nm) released via fusion of multivesicular bodies containing intraluminal vesicles with the plasma membrane. Microvesicles are vesicular structures (0.1-1.0 μ m) shed by outward blebbing of the plasma membrane. The largest EVs (1-5 μ m) are apoptotic bodies that are formed during the late stages of apoptosis [2]. ExMVVs are small circular membrane fragments secreted from the endosomal compartment or shed from the cell surface by blebbing of cell surface membrane. Large ExMVVs are released during cell-surface budding, and their sizes range from 100 to 1000 nm in diameter, their functions are presented with removing cell unwanted substances, protecting specific types of cells from complement-mediated cytolysis and transferring multiple receptors, proteins, genetic material (including mRNA and microRNA (miRNA)) and lipids [2, 5, 6, 14]. According to the recent publications, ExMVVs play a crucial role in cell-cell communication, tissue homeostasis, cell differentiation, and organ development and remodeling [5]. Microvesicle shedding is also increased after cell activation due to cell injury, proinflammatory stimuli, hypoxia, oxidative stress, or shear stress [1, 6].

Some studies showed that EVs from healthy individuals contain active caspase-3 that was not found in the parental cells, suggesting that caspase-3 might have been removed from the cells to ensure survival. Intriguingly, dying cells release microvesicles bearing the adaptor protein Crkl during the early stages of apoptosis induced by the caspase 3 cascade. These microvesicles were isolated from glomeruli after injury and were shown to induce compensatory proliferation signaling in recipient cells. Taken together, release of microvesicles may remove cell toxic substances but also induce repair in neighbor cells [8].

It was proved, that those MSCs from mouse models have an immunosuppressive effect, which ultimately led to decreased inflammation and improved renal regeneration. One of the recovery mechanisms can be executed by MVs – extracellular bodies produced by MSCs. It is believed that the therapeutic effect of MSCs may be largely mediated by their immunosuppressive properties. Thus, the aim of this study is to assess the contribution of MVs immunoregulatory activity.

Several studies have shown a significant contribution of the immune system to the processes of damage and restoration of renal tissue [15]. In acute kidney injury, regardless of origin, local increased activity of many components of the immune system

is observed [4, 8, 9, 15, 4]. Experiments with adoptive transfer of activated macrophages, T- and B-lymphocytes have shown their negative role in pathogenesis of renal failure. At the same time, MSCs have immunoregulatory properties [10], which can also be possessed by the MSCs-produced MVs, but it remains unstudied.

Thus, **the aim of this work** is to study the immunoregulatory properties of MVs produced by MSCs and their contribution to the pro-regenerative effect of MVs on renal tissue.

Materials and methods

Chronic renal failure model

The experiment was carried out on 3-4 month old CBF mice. Chronic renal failure was induced by applying three doses of 8.6 mg /kg of 50% glycerol intramuscularly every 7 days. The resulting rhabdomyolysis has a complex (ischemic, toxic, retention) effect on the kidneys, with a predominant lesion of the proximal tubule epithelium. In this model, necrosis of epithelial cells of the proximal renal tubules is observed, accompanied by a rather significant increase in the level of serum urea. All experiments on animals were carried out in accordance with the “Rules for carrying out work with the use of experimental animals” (Appendix to the order of the Ministry of Health of the USSR No. 755 of 1977).

Isolation of bone marrow mesenchymal stromal cells

Mesenchymal stromal cells were obtained from the bone marrow of syngeneic mice by adhesion to culture plastic template. Bone marrow derived from femur and tibia was suspended in RPMI 1640 culture medium. The bone marrow stroma was mechanically disrupted with a glass homogenizer, cells were washed out twice, and placed in Tissue Cultural Flask 75 cm², TPP, in complete growth medium based on RPMI 1640 supplemented with 10% FBS (Fetal Bovine Serum) Qualified, LOT 42Q3684K, Gibco. The non-adherent fraction of bone marrow cells was removed by replacing culture medium, starting from day 3. MSCs showed a typical fibroblast-like phenotype and formed a continuous monolayer by 4 weeks of culture. MSCs were removed with a 0.25% Versen-trypsin solution, washed out twice from the culture medium, and resuspended in physiological saline.

Isolation of microvesicular particles

After reaching a monolayer, some MSCs were subjected to apoptosis by cultivation under oxygen deprivation and serum-free medium for 1 day. Under these conditions, the cells entered apoptosis, their production of microvesicular particles, especially the 100-1000 nm fraction, was markedly elevated. After 24 hours, the supernatant was centrifuged at 2000 g for 15 minutes to remove cell debris followed by cen-

trifugation at 16000 g for 60 minutes at 4 °C. The resulting pellets were resuspended in 100 µl saline. To standardize the MV suspension, protein content was determined by the Bradford method.

Material intake

2 weeks after the last injection, MSC and MSC-MV were inoculated into the tail vein, and on the day 4 and 11 afterwards, splenocytes were collected by using flow cytometry. The cells were frozen according to the protocol. The studies were carried out with the approval of the Ethics Committee of the Republican Scientific and Practical Center for Mental Health (meeting of 04/11/2011).

Thus, 4 groups were randomized: intact mice, CRF, CRF + MSC, CRF + MV, with 10 mice per each group. Negative control – intact mice injected with 0.9% NaCl, positive control – chronic renal failure. MSCs were injected at a dose of 1 million/mouse, by applying similar amount of MVs.

Histological examination

Mouse kidneys were excised and fixed in 4% formalin solution, and dehydrated according to a standard technique and paraffin-embedded. Paraffin sections 4-5 µm thick were obtained by using an HM 340E rotary microtome (Carl Zeiss, Germany), stained with hematoxylin and eosin, Sirius red, and by Mallory's method. Light-optical examination

TABLE 1. CONCENTRATION OF ALBUMIN (ng/ml), CREATININE (ng/ml), FABP1 (ng/ml)

a) Albumin ng/ml				
	Intact	CRF	CRF + MSC	CRF + MSC-MV
4 th day	66.11±13.64	492.50±68.32*	153.1±15.5*	179.30±31.10*
11 th day		187.00±37.97	187.00±37.97	97.220±1.912*
b) Creatinine ng/ml				
	Intact	CRF	CRF + MSC	CRF + MSC-MV
4 th day	2.244±0.173	4.156±0.524*	1.986±0.192*	1.648±0.080**
11 th day		4.818±1.749	2.064±0.197	1.5360±0.03523
c) FABP1 ng/ml				
	Intact	CRF	CRF + MSC	CRF + MSC-MV
4 th day	0.802±0.216	0.849±0.894	0.844±0.185	0.987±0.188
11 th day		1.085±0.367	0.639±0.163	0.780±0.087*

Note. *, p < 0.05; **, p < 0.01.

TABLE 2. CONTENT OF CD4⁺CD25⁺FoxP3⁺ REGULATORY CELLS, CD4⁺CD44⁺CD62⁺ AND CD8⁺CD44⁺CD62⁺T-CELLS OF MEMORY. DAY 11

a) CD4 ⁺ CD25 ⁺ FoxP3 ⁺			
Intact	CRF	CRF + MSC	CRF + MSC-MV
0.068±0.034	9.788±3.494*	0.293±0.067*	0.5530±0.1714*
b) CD4 ⁺ CD44 ⁺ CD62 ⁺			
Intact	CRF	CRF + MSC	CRF + MSC-MV
6.940±4.326	1.4325±0.6311	27.230±5.321*	3.7325±0.9294
c) CD8 ⁺ CD44 ⁺ CD62 ⁺			
Intact	CRF	CRF + MSC	CRF + MSC-MV
4.370±2.896	1.1750±0.6969	17.0930±0.6043*	3.270±1.529

Note. *, p < 0.05.

and microphotography were performed by using an Axioskop 40 microscope (Carl Zeiss, Germany).

Morphometric analysis of kidney structure was performed on paraffin sections. The following morphometric parameters were determined: the diameters of the superficial renal glomeruli (renal corpuscles); diameter of the collecting ducts and the height of relevant cells, measured in the middle third portion of the renal medulla. Morphometric calculation was performed in the field of view with a 10/25 eyepiece and a 63 objective (Figure 1).

Confocal microscopy

Some cells were stained with Annexin 5 to confirm the onset of apoptosis 24 hours after keeping cells under conditions of oxygen deprivation in serum-free medium (Figure 2). Staining was performed by using TACS® Annexin V-FITC Apoptosis Detection Kit Cat Number 4830-01-K in *in situ* detection protocol for adherent cells.

To do this in per ~ 5 cm² sample we used: 10X Binding Buffer 10 µL; Propidium Iodide 10 µL; TACS Annexin V-FITC* 1.0 µL; Distilled water 79 µL, in total volume 100 µL. To prepare 400 µL of 1X Binding Buffer / sample for washing cells after incubation we diluted 10X Binding Buffer in distilled water. Next, we discarded PBS wash from the culture, washed cells twice for 2 minutes in excess volume of 1X Binding Buffer at room temperature. Cells were analyzed immediately under fluorescence microscope using a fluorescent mounting medium.

Statistical processing of results

The data obtained during the study were processed by the one-way ANOVA method, t-test. The values were calculated by using the GraphPad Prism 8 software.

Flow cytometry

During the study BD FACSCanto II and CytoFLEX, Beckman Coulter Flow cytometers were used. Frozen mouse splenocytes were used as well. Standard staining protocol with monoclonal antibody cocktail anti-mouse CD4-FITC, CD8-FITC, CD44-APC, CD62L-PE, CD4-APC, CD25-FITC, FoxP3-PE (all BD Biosciences) was used for detecting extracellular and intracellular markers for further detection of memory T-cells CD4⁺CD44⁺CD62L⁺, CD8⁺CD44⁺CD62L⁺ and regulatory T-cells CD4⁺CD25⁺FoxP3⁺.

Biochemical analysis of the samples

Changes in blood and urinal parameters (creatinine, albumin and FABP1) were examined. The following kits were used:

- BioVision, Creatinine (Mouse) ELISA Kit rev 03/18, Catalog Number E4369-100;
- Abcam, Mouse Albumin ELISA Kit, Catalog Number ab108792;

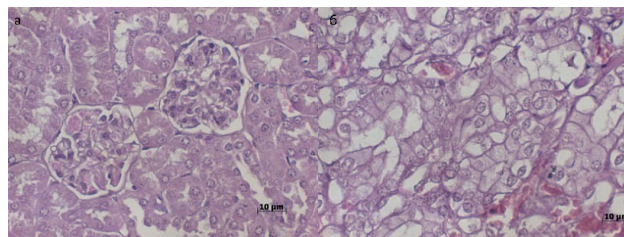


Figure 1. CRF (A) Kidney glomeruli, proximal and distal tubules. (B) The collecting ducts and loops of Henle in the middle part of the medulla of the kidney

Note. Staining with hematoxylin and eosin.

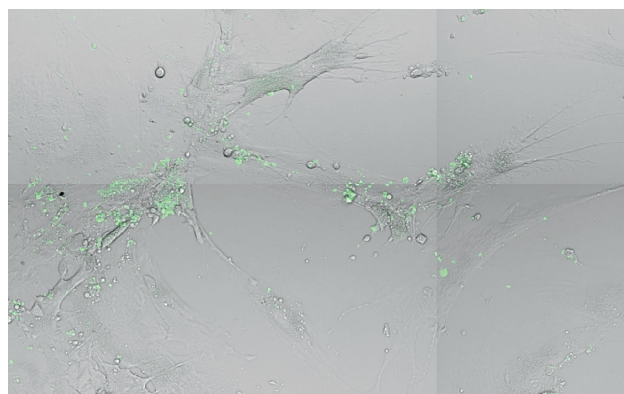


Figure 2. Image shows a typical fibroblast-like structure of MSCs, Annexin V glows green

– RnDSystems, Mouse/Rat FABP1/L-FABP Immunoassay Quantikine® ELISA, Catalog Number RFBP10.

Results and discussion

Mice with induced chronic renal failure (CRF) showed morphological changes in the renal structure and functional biochemical parameters of the excretory system, which were studied after exposure to MSC or MSC-MB on day 4 and 11 after intravenous inoculation of MSCs or MSC-MB. Concentration of urine albumin tended to decrease in mice after exposure to MSC and MSC-MB, but creatine level did not significantly changed (Table 1).

As a marker of kidney damage, the serum creatinine is usually used for calculating the glomerular filtration rate [4]. However, the concentration of creatinine can depend on various factors such as the patient age, medication administration, etc. The creatinine level may not increase immediately, but 72 hours after kidney damage coupled to more than 50% parenchyma dysfunction and significantly changed filtration rate [12]. Proteinuria and albuminuria also not always reflect extent of kidney damage. These parameters point at changes in the glomerular

apparatus, but provide no information about damage to the interstitial tissue and kidney tubules [9].

Biological markers of kidney damage include fatty acids binding proteins (FABPs) also produced in the renal tissue; two types of marker have been revealed: FABP1 (or L-FABP) is synthesized in the epithelial cells of the proximal renal tubules, and H-FABP in the distal renal tubules [12]. L-FABP is not virtually detected in urine of healthy people, but its excretion is increased significantly when interstitial tissue is damaged. Increased expression of L-FABP in tubular cells and its excretion in the urine have been described in animals with ARI (acute renal injury). In 2011, the role of L-FABP was established as an early predictor of kidney damage – in response to massive proteinuria, hyperglycemia, increased blood pressure, upon damage in the proximal kidney tubules coupled to elevated expression of the renal L-FABP gene, which increases excretion of L-FABP in urine. Protein level increased with established ARI of various etiologies, including acute tubular necrosis, sepsis, and nephrotoxin administration. Also, this protein was approved as a new early predictor of kidney damage, published by the Ministry of Health, Labor and Welfare in Japan [12] as well as in case of proximal tubule protein overload.

Kidney morphological examination clearly shows changed characteristic of chronic renal failure, both in the cortex and in the medulla. Dystrophy of cells of the distal nephron of the connecting tubule, hypertrophy of cells of collecting ducts, hypertrophy of cells of Henle's loops are noted, rheological properties of blood are impaired including leakage of blood plasma proteins as well as recorded erythrocyte sludge. On day 4, mice inoculated with MSCs had diameter of the collecting ducts significantly reduced compared to the "CRF" and "CRF + MSC-MV" groups. The height of cells of the collecting ducts was significantly lower compared to "CPI" group. Dystrophy of the cells of the collecting ducts and loop of Henle in the renal cortex and medulla was recorded visually. In addition, blood rheological properties were impaired. The therapeutic effect of MSC microvesicle vs MSCs was markedly lower on morphological characteristics: the diameter of the collecting tubules was significantly larger, the height of the collecting tubule cells was bigger. Dystrophy of the cells of the collecting ducts in the middle and lower parts of the medulla, as well as the cells of Bellini's ducts, were also noted visually.

CD4⁺CD44⁺CD62L⁺ and CD8⁺CD44⁺CD62L⁺ T-memory cells and CD4⁺CD25⁺FoxP3⁺ T-regulatory cells were detected and analyzed by flow cytometry (Table 2). This immunological examination showed that memory CD4⁺CD44⁺CD62L⁺ and CD8⁺CD44⁺CD62L⁺ T-cells were increased after transplantation of MSC and MSC-MV. However, regardless

of the expected results (due to anti-inflammatory effects of regulatory T-cells their quantity had to increase) level of CD4⁺CD25⁺FoxP3⁺ regulatory cells was dramatically decreased in mouse splenocytes. It might be due to either cell migration to the focus of damage (e.g. a kidney) or other anti-inflammatory mechanisms.

Most of the studies aimed to study CRF are dedicated to autoimmunological and mechanical mechanisms of a renal failure, but toxic immune-related origin of this disease is poorly explored. However, regardless of it, toxic renal failure remains widely spread in human population due to chemotherapy of multiple illnesses such as tuberculosis, cancer, sugar diabetes etc. Moreover, treatment of renal failure also remains less efficient compared to other renal diseases and a long-term perspective always results in a substitution therapy. Advancement in this area would contribute drastically into the improved patients' quality of life and life expectancy.

According to the literature, extracellular vesicles may exert both a positive role in the regeneration process and a negative effect, up to contributing to the development of diseases, causing inflammation, vascular damage and thrombosis. Examining both positive and negative effects on the progression of diseases, including kidney disease, may be a key for organ-preserving and regenerative medicine. Microvesicles derived from mesenchymal stem cells have a pro-regenerative effect in acute renal failure [5].

At this stage of the study, an increased level of regulatory T-cells was recorded in chronic renal failure, that declined during transplantation of both MSCs and microvesicles. At the same time, there is a decrease in the levels of all biochemical blood parameters, as well as albumin in the urine, especially on day 11, which may highlight the onset of the regenerative process. This can also be indicated by increased number of memory T-cells, of both CD4⁺CD44⁺CD62L⁺ and CD8⁺CD44⁺CD62L⁺ subsets. Regarding T-regulatory cells in the majority of published studies on chronic and acute diseases, Treg depletion exacerbated kidney disease, while their enrichment, on the contrary, increases, which contributes to a high anti-inflammatory effect [11, 13]. There is evidence that certain factors can contribute to both regenerative and toxic effects. In our study, the number of T-regulatory cells not only failed to increase, but significantly decreased on day 11. In published studies on the effect of MSCs on acute renal failure, it was found that a large Treg quantity was accumulated in mouse kidneys and spleens [13]. Thus, in chronic renal failure, it is especially important to study all aspects of the immune response. Based on literature data [7] we also carried out several enzyme-linked immunosorbent assays to study serum level of IFN γ , IL-4, IL-2, IL-10, but no significant changes were observed. Thus, we may

suggest that even chronic renal failure was associated with pro-regenerative effect after inoculation of microvesicles obtained from mesenchymal stem cells that was comparable to that one after MSC transfer. In addition, further examination on both pro-regenerative effect of microvesicles obtained from MSCs, and their effect on T-regulatory and T-memory cells remain to be conducted.

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КОРРЕКЦИЯ АГРЕССИВНОГО ПОВЕДЕНИЯ ТРАНСПЛАНТАЦИЕЙ МОДУЛИРОВАННЫХ *IN VITRO* ИММУНОКОМПЕТЕНТНЫХ КЛЕТОК

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Резюме. Агрессия — серьезная биомедицинская проблема, связанная с большим процентом пациентов и отсутствием селективных корректирующих средств. Наиболее часто повышенная агрессивность наблюдается у пациентов с депрессивными расстройствами, шизофренией, реактивными психозами и расстройствами адаптации, которые, как известно, характеризуются иммунологической дисфункцией. Нейролептики достаточно широко используются в клинической практике для коррекции психомоторного возбуждения: антипсихотическое действие указанных препаратов проявляется в достижении седативного эффекта. Однако, как и другие психоактивные вещества, они обладают рядом побочных эффектов, ограничивающих их длительное использование, что ограничивает их длительное применение и определяет необходимость поиска новых подходов к коррекции аффективных расстройств. Экспериментальное моделирование агрессии — один из основных подходов к изучению ее патогенетических механизмов и поиску новых эффективных средств для терапии. Изучение патогенетических механизмов агрессии и поиск подходов к ее терапии в рамках нейроиммунного взаимодействия в настоящее время является чрезвычайно перспективными. Имеется большое количество клинических и экспериментальных данных, указывающих на взаимосвязанные изменения функциональной активности нервной и иммунной систем при агрессии. Ведущим звеном патогенетического механизма агрессии является нарушение выработки и взаимной регуляции цитокинов, нейротрансмиттеров, нейропептидов, факторов роста, гормонов, действие которых опосредуется клеточными элементами иммунной системы. Существенная роль иммунокомпетентных клеток в патогенезе агрессии, равно как и однонаправленное действие большинства психоактивных препаратов на клеточные элементы иммунной и нервной систем, позволяет рассматривать иммунокомпетентные клетки в качестве модельного объекта для воздействия на межсистемные функциональные связи для редактирования агрессивного фенотипа. Целью настоящего исследования было изучение влияния трансплантации модулированных *in vitro* нейролептиком иммунокомпетентных клеток на поведенческий фенотип и содержание цитокинов в головном мозге агрессивных сингенных реципиентов. Агрессивное поведение было сформировано у активных мышей-самцов (СВА × С57Bl/6) F1 в результате опыта 20-кратных побед в межцовых конфронтациях (метод парного дистантного сенсорного контакта). Спленциты агрессивных мышей обрабатывали *in vitro* хлорпромазином и внутривенно вводили сингенным агрессивным реципиентам. Было продемонстрировано, что модулируемые *in vitro* хлорпрома-

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зином спленоциты агрессивных мышей после трансплантации редактируют поведение агрессивных сингенных реципиентов на фоне снижения в патогенетически значимых для агрессии структурах головного мозга цитокинов IL-1 β , IL-2, IL-6, IFN γ и повышения уровня IL-4. Механизмы коррекции агрессивной стратегии поведения модулированными *in vitro* нейрорепрессором иммунокомпетентными клетками обсуждаются.

Ключевые слова: агрессивное поведение, иммунокомпетентные клетки, хлорпромазин, структуры головного мозга, цитокины

AGGRESSIVE BEHAVIOR CORRECTION BY THE TRANSPLANTATION OF *IN VITRO* MODULATED IMMUNE CELLS

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Abstract. Aggression is a serious biomedical problem associated with a high percentage of patients and a lack of selective corrective agents. The most frequent increase in aggressiveness occurs in patients with depressive disorders, schizophrenia, reactive psychoses and adjustment disorders, which are known to be characterized by immunological dysfunction. Antipsychotics are widely used in the correction of psychomotor agitation; the antipsychotic effect of these drugs is manifested in the achievement of a sedative effect. However, like other psychoactive substances, they have a number of side effects that limit their long-term use and determines the need to search for new approaches to the correction of affective disorders. Experimental modeling of aggression is one of the main approaches for studying its pathogenetic mechanisms and searching for new effective therapeutic agents for the treatment. The study of the aggression pathogenetic mechanisms and the search for approaches to therapy within the framework of neuroimmune interaction is currently extremely promising. Currently, there is a large number of clinical and experimental data indicating interrelated changes in the functional activity of the nervous and immune systems during aggression. The leading links in the pathogenetic mechanism of aggression is the violation of the production and mutual regulation of cytokines, neurotransmitters, neuropeptides, growth factors, hormones, the effects of which are mediated by the cellular elements of the immune system. Given the immune cells essential role in the pathogenesis of aggression and the psychoactive substances unidirectional effect on the immune and nervous cells, make it possible to consider immune cells as model objects for influencing the intersystem functional relationship in order to edit the aggressive phenotype. The aim of the study was to investigate the effect of *in vitro* neuroleptic-modulated immune cells transplantation on behavioral phenotype and brain cytokines in aggressive syngeneic recipients. Aggressive behavior was formed in active male mice (CBA $\times$ C57Bl/6) F1 as a result of the experience of 20-fold victories in inter-male confrontations (distant sensory contact model). Aggressive mice splenocytes were treated *in vitro* with chlorpromazine and intravenously injected to syngeneic aggressive recipients. It has been demonstrated that modulated *in vitro* by chlorpromazine splenocytes of aggressive mice after transplantation edit the syngeneic aggressive recipient's behavior against the background of a decrease in cytokines IL-1 β , IL-2, IL-6, IFN γ and an increase in IL-4 in pathogenetically significant for aggression brain structures. The mechanisms of the aggressive behavior correcting effect of modulated immune cells are discussed.

Keywords: aggressive behavior, immune cells, chlorpromazine, brain structures, cytokines

Introduction

In human society, increased aggressiveness is one of the main social and health problems. More than a million people around the world die each year as a result of violent clashes. Aggression is one of the lea-

ding causes of death among people aged 15 to 44 years. Moreover, according to the World Health Organization experts, 20 hospitalizations associated with physical aggression per every such death [15].

Psychoactive drugs used for its correction are quite efficient, the activity of these drugs is manifested

particularly in achieving a sedative effect. At the same time, the positive sedative action of neuroleptics is accompanied by a number of side effects, such as the occurrence of addiction, dependence formation, endocrine disorders, as well as the possibility of inducing late psychoses (psychoses of dopamine hypersensitivity) that occur due to their prolonged use and aggravated course of the underlying disorder, which limits a potential to apply antipsychotics and determines the search for new approaches to correct affective disorders. The most frequent increase in aggressiveness occurs in patients with depressive disorders, schizophrenia, reactive psychoses and adjustment disorders, which are known to be characterized by immunological dysfunction [13, 14, 15]. While using experimental models of various forms of aggression, it was also revealed that an aggressive state is characterized by altered functional activity of immune system. When mice and rats were selected to model high and low aggressiveness, it was found that highly vs. low aggressive animals exhibit higher immunological reactivity [5]. Aggression formed under conditions of prolonged social stress in rats and mice of different strains is accompanied by increased primary immune response to T-dependent antigens, T-cell proliferation, redistribution of T-lymphocyte subpopulations in the bone marrow, blood and spleen. Moreover, it was found that immune dysfunction associated with impaired cytokine production by the immune and brain cells can be involved in the mechanisms of aggressive behavior development [1, 3, 5, 6, 13, 14]. It is known that the immune system cells exert regulatory effect on functions of the central nervous system. It has been established that immune cells are capable of regulating behavioral reactions both in normal conditions and in neuroimmune pathology, and their products display psycho- and neurotropic effects [2, 3, 7, 8, 9, 13, 14]. The unidirectional effect of most psychoactive substances on the nervous and immune system cells suggest the possibility of using immune cells in psychiatry to correct disorders with a pronounced neuroimmune pathogenesis component.

Considering the issues noted above, **the aim of this work** was to study effect of *in vitro* neuroleptic drug-modulated immune cell transplantation on behavioral phenotype and brain cytokines level in aggressive syngeneic recipients.

Materials and methods

Four-month-old male (CBA × C57Bl/6) F1 mice were used in the study; the average weight of the animals was 20-25 grams. The animals were housed in laboratory vivarium, 10 animals per cage, for at least 2 weeks before experiments, reared on a standard diet, with drinking water ad libitum, normal

light regime. The experiment was carried out in accordance with the rules adopted by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), the rules of laboratory practice (Order of the Ministry of Health of the Russian Federation of June 19, 2003, No. 267).

Considering the presence of individuals with active and passive types of behavior in the male population (CBA×C57Bl/6) F1 characterized by certain structural and functional characteristics of the nervous and immune systems and different psychophysiological responses to stressful influences [7, 10 12], in order to form homogeneous experimental groups of animals, all mice were pre-tested in the “open field” and only those showing an active type of behavior were included in the study. Aggressive behavior in active mice was formed under conditions of prolonged social stress: experience of victories in inter-male confrontations for 20 days (distant sensory contact model) as described earlier [6].

Splenocytes from aggressive mice were isolated under sterile conditions and treated *in vitro* with chlorpromazine (15×10^6 cells/150 µg drug) for 25 minutes. The concentration of the drug used for cell culture was determined by recalculating the therapeutic dose, taking into account the body weight and metabolic characteristics of animals, as well as preliminary testing [8, 11]. Next, following 3 washouts, splenocytes pre-cultured with chlorpromazine were intravenously inoculated to syngeneic aggressive recipients at a concentration of 15×10^6 cells dissolved in 0.3 ml of saline per animal. In the control group, the preparation and transplantation of splenocytes were carried out under similar experimental conditions, without adding chlorpromazine.

Recipients behavioral phenotyping consisted of assessing exploratory behavior (EB) in the Open Field test, as described earlier [7] and behavior in Porsolt test using a modern hardware and software complex EthoVision XT (Noldus Information Technology, The Netherlands).

Cell proliferative activity after the *in vitro* chlorpromazine treatment was assessed by a standard radioactive label (H3-thymidine) incorporation in the nucleoprotein fractions of cells.

The cytokine profile was assessed by ELISA for mouse cytokines manufactured by R&D Systems (Great Britain), according to the manufacturer's instructions.

Statistical data analysis was performed using an analytics software portfolio Statistica 10.0 for Windows (StatSoft, Tulsa, OK, USA). Normally distributed data with low variance were analyzed by using Student's t-test; in case the data were not normally

distributed, Mann–Whitney U test was applied. Results are presented as mean (M) and standard error (SE) ($M \pm SE$). $P \leq 0.05$ was considered statistically significant.

Results and discussion

Previously, the features of the aggressive mice immune cells functional activity were described [6]. Splenocyte pre-exposure to chlorpromazine modulated their functional activity, revealed as significantly decreased spontaneous proliferative activity (628.9 ± 204 cmp/min and 144.0 ± 47.6 cmp/min in the control and experimental groups respectively; $p < 0.05$).

It has been shown that repeated experience of aggression, accompanied by victories, leads to changed behavior in male mice with increased motor activity, irritability, severe anxiety, and the appearance of stereotypies [4, 11, 12, 14]. Transplantation of chlorpromazine-modulated splenocytes in aggressive mice-recipient was accompanied by changed EB parameters, manifested as decreased horizontal motor activity parameters, reflecting the behavioral motor component (peripheral: 144.0 ± 13.9 and 70.7 ± 9.9 in the control and experimental groups, respectively; central: 8.0 ± 4.0 and 0.4 ± 0.1 in the control and experimental groups, respectively; total: 152.0 ± 17.9

and 71.1 ± 10.1 in the control and experimental groups, respectively; $p < 0.01$), and vertical motor activity, reflecting the exploratory component of the behavior (free postures: 0.8 ± 0.2 and 0.2 ± 0.5 in the control and experimental groups, respectively; sideways: 2.2 ± 1.1 and 0.7 ± 0.1 in the control and experimental groups, respectively; total vertical activity: 2.9 ± 1.3 and 1.0 ± 0.6 in the control and experimental groups, respectively. $p < 0.01$). At the same time, aggressive recipients also showed decreased latency period for entering the central squares of the field with a decreased emotional reactivity, recorded by the number of fecal boluses (3.7 ± 1.2 and 1.4 ± 1.1 in the control and experimental groups, respectively; $p < 0.05$), which indirectly indicates the anxiolytic effect of the immune cell transplantation in this group of recipients.

Assessment of the aggressive recipient's behavior in the forced swimming test showed a pronouncedly reduced periods of mobility and increased periods of passive water swimming (drift + complete immobility) [11]. The data obtained indicate aggressive behavior editing in mice-recipients.

As mentioned above, cytokines are involved in the central mechanisms of various behavioral reactions regulation and significantly contribute to the development of mental disorders. The aggressive behavior strategy formation correlates with changes in cytokine profile in some brain structures, such as

TABLE 1. CYTOKINES CONTENTS IN VARIOUS BRAIN STRUCTURES IN AGGRESSIVE MICE-RECIPIENTS (CBA $\times$ C57Bl/6) F1 AFTER THE CHLORPROMAZINE-MODULATED IMMUNE CELLS TRANSPLANTATION, $M \pm SE$

Brain structures	Cytokines (pg/ml)				
	IL-1 β	IL-4	IL-6	IFN γ	IL-2
Hypothalamus					
Control	220.05 $\pm$ 28.40	15.65 $\pm$ 5.90	1350.4 $\pm$ 203.8	217.4 $\pm$ 14.7	24.01 $\pm$ 7.41
Experimental	189.79 $\pm$ 25.40	22.41 $\pm$ 5.60*	1900.1 $\pm$ 259.3	172.94 $\pm$ 18.03*	27.71 $\pm$ 7.77
Hippocampus					
Control	208.83 $\pm$ 27.30	21.07 $\pm$ 6.40	1760.5 $\pm$ 245.4	234.16 $\pm$ 26.40	59.17 $\pm$ 10.90
Experimental	102.9 $\pm$ 16.7*	20.08 $\pm$ 6.30	1190.4 $\pm$ 117.2*	158.27 $\pm$ 18.80*	28.93 $\pm$ 7.89*
Frontal cortex					
Control	212.36 $\pm$ 27.60	25.78 $\pm$ 6.90	1830.1 $\pm$ 251.5	231.74 $\pm$ 26.2	27.22 $\pm$ 7.72
Experimental	132.66 $\pm$ 29.70*	25.55 $\pm$ 6.60	1940.8 $\pm$ 262.5	226.40 $\pm$ 25.6	19.84 $\pm$ 6.98
Striatum					
Control	163.35 $\pm$ 22.70	23.18 $\pm$ 6.60	1740.0 $\pm$ 242.7	226.13 $\pm$ 25.60	49.34 $\pm$ 9.93
Experimental	175.03 $\pm$ 23.90	24.11 $\pm$ 6.70	2004.0 $\pm$ 272.8	205.67 $\pm$ 23.60	41.47 $\pm$ 9.14

Note. Control, group of mice-recipients after the transplantation of splenocytes pre-cultured without chlorpromazine. Experimental, group of mice-recipients after the transplantation of pre-cultured with chlorpromazine splenocytes; testing period – 5 min; * $p < 0.01$, as compared to control.

the hypothalamus, hippocampus, striatum and frontal cortex; particularly increased levels of IL-1 β , IL-2, and IL-6 has been shown in mice dominating in inter-male collisions [1, 4].

We have shown that behavioral changes in aggressive recipients mentioned above were accompanied by changes in the cytokines content in some brain structures: IL-1 β , IL-2, IL-6, IFN γ levels in the hippocampus were decreased; IL-4 level in the hypothalamus was increased and IFN γ – decreased; decreased IL-1 β level in the frontal cortex was recorded (Table 1).

Opposite changes in the brain cytokines during formation of behavioral aggressive strategy and its arrest by chlorpromazine-modulated splenocytes testify in favor of the cytokine-mediated psycho-

neuromodulating effect bound to transplanted immune cells. Changes in the activity of the 5-HT and DA-systems playing an important role in the psychoneuroimmunomodulation are observed in the brain of animals with long-term experience of aggression [4, 12, 13]. Taking into account the well-known cytokine effect on the 5-HT and DA-systems activities [3, 4, 13], it can be assumed that the key cytokine modulation after the immune cell transplantation changing the brain neurochemical system reduces aggressive manifestations.

So, chlorpromazine-modulated immune cells have a positive aggressive behavior editing effect being involved in the mechanisms underlying the development of aggressive reactions.

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ИММУНОКОМПЕТЕНТНЫЕ КЛЕТКИ В КАЧЕСТВЕ ПОТЕНЦИАЛЬНОГО ТЕРАПЕВТИЧЕСКОГО АГЕНТА В ЛЕЧЕНИИ ДЕПРЕССИИ

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Резюме. Иммунная и нейроэндокринная системы играют решающую роль в поддержании динамического гомеостаза в нормальных условиях и при психической дезадаптации. Психо- и иммунопатология тесно взаимосвязаны: патологические изменения в функционировании обеих систем происходят одновременно и взаимозависимы. Депрессия, как психическое расстройство, — серьезная проблема общественного здравоохранения. Экспертные оценки показывают рост заболеваемости депрессией в будущем. Однако применяемая в настоящее время терапия депрессии не обеспечивает полного излечения. Известно, что нарушение нейроиммунного взаимодействия является существенным звеном в патогенезе заболевания, негативно влияя на его течение, ухудшая клиническую картину, снижая эффективность терапии, поэтому актуален поиск нового подхода к лечению. Имеется достаточное количество данных о ведущей роли иммунных клеток и их биологически активных продуктов в патогенезе депрессии. Однонаправленное действие большинства психоактивных препаратов на центральную нервную систему и иммунную систему подтверждает межсистемную взаимную регуляцию и позволяет рассматривать иммунные клетки в качестве модельных объектов для влияния на межсистемную функциональную взаимосвязь; в силу чего, клеточная иммунотерапия может быть методом выбора при лечении депрессивных расстройств. Мы впервые продемонстрировали возможность направленной регуляции поведения экспериментальных животных путем трансплантации иммунных клеток с определенными функциональными характеристиками, в том числе с функциональной активностью, модулированной экстракорпорально психоактивным веществом. Основываясь на предыдущих результатах, мы исследовали влияние иммунных клеток, модулированных *in vitro* кофеином, на иммунный и поведенческий фенотипы у депрессивно-подобных сингенных реципиентов. Было показано, что трансплантация прекультивированных с кофеином спленоцитов депрессивно-подобных доноров вызвала редактирование депрессивно-подобного поведения у сингенных реципиентов, что проявилось в снижении ангедонии, стимуляции ориентировочно-исследовательского поведения в тесте «открытое поле» и двигательной активности в тесте вынужденного плавания по Порсолту. Поведенческие изменения у депрессивно-подобных реципиентов после клеточной трансплантации регистрировались на фоне снижения уровня провоспалительных цитокинов (TNF α , IL-1 β , IL-6, IFN γ) и увеличения уровня IL-10 в патогенетически значимых для депрессивно-подобного состояния структурах головного мозга (гиппокампе, гипоталамусе, фронтальной коре, стриатуме), что свидетельствует о снижении нейровоспаления. Выявлена также модуляция функциональной активности

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иммунной системы реципиентов. Цитокин-опосредованные механизмы редактирования депрессивно-подобного поведения модулированными *in vitro* кофеином иммунными клетками обсуждаются.

Ключевые слова: нейроиммунное взаимодействие, депрессивно-подобное поведение, иммунокомпетентные клетки, кофеин, мозг, цитокины

IMMUNE CELLS AS A POTENTIAL THERAPEUTIC AGENT IN THE TREATMENT OF DEPRESSION

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Abstract. The immune and neuroendocrine systems play a critical role in maintaining a dynamic homeostasis in normal conditions and at mental maladaptation. Psycho- and immunopathology closely interrelated: pathological changes in the functioning of both systems occur simultaneously and are interdependent. Depression, as a mental disorder, is a major public health concern. The estimations are showing rise of the depression's incidence in the future. However, currently used therapy of depression doesn't provide a complete cure. It is known that a violation of neuroimmune interaction is an essential link in the pathogenesis of the disease, having a negative impact on its course, making the clinical picture worse, reducing effectiveness of the therapy, therefore, it's urgent to search for a new treatment approaches. There are a sufficient amount data on the immune cells and their biologically active products leading role in the pathogenesis of depression. The unidirectional effect of most psychoactive substances on the central nervous system and the immune system confirms intersystem mutual regulation and allows considering the immune cells as model objects for influencing the intersystem functional relationship; so, cells immunotherapy can be the method of choice in the treatment of depressive disorders. We first demonstrated the possibility of animal's behavior directed regulation by the transplantation of immune cells with definite functional characteristics, including those with functional activity modulated extracorporeally by a psychoactive substance. Based on the previous results we investigated the effect of the *in vitro* caffeine- treated immune cells on the behavior and immune phenotypes in depressive-like syngeneic recipients. Transplantation of caffeine-treated splenocytes from depressive-like donors has been shown to induce depressive-like behavior editing in syngeneic recipients, which was manifested in anhedonia decrease, stimulation of exploratory behavior in the Open Field test and motor activity in the Porsolt forced swimming test. Recipient's behavioral changes were registered on the background of decreased brain pro- inflammatory cytokines (TNF α , IL-1 β , IL-6, IFN γ) and IL-10 increased in some pathogenetically significant for depressive-like state brain structures (hippocampus, hypothalamus, frontal cortex, striatum), which indicates a decrease in neuroinflammation. It was also detected recipient's immune system functional activity modulation. The cytokines-mediated mechanisms of depressive-like behavior editing by the *in vitro* caffeine- modulated immune cells are discussed.

Keywords: neuroimmune interaction, depressive-like behavior, immune cells, caffeine, brain, cytokines

Introduction

The immune and neuroendocrine systems play a critical role in maintaining a dynamic homeostasis in normal conditions and at mental maladaptation. Psycho- and immunopathology are closely interrelated: pathological changes in the functioning of both systems occur simultaneously and are interdependent. For decades, the consequences of acute and chronic stress caused by information overloads, environmental problems, natural disasters, man-made disasters, military clashes, interethnic conflicts, acts of terrorism, which can lead to painful

“breaking” of socio-biological mechanisms of adaptation, decrease in adequacy while assessing the environment and how consequences, the formation of disorders in the psychoemotional sphere, manifested in the development of behavioral disorders, including depression. All this leads to their steady growth in modern world and an increase in the number of related somatic diseases. Depression, as a mental disorder, is a major public health concern. The estimations demonstrate a rise of the depression incidence in the future. However, currently used therapy of depression does not provide a complete cure. It is known

that a disturbed neuroimmune interaction is an essential link in the disease pathogenesis, having a negative impact on its course, aggravating clinical picture, reducing effectiveness of the therapy [1, 3, 4, 5, 7, 14]; therefore, it is urgent to search for new treatment approaches. Obviously, this is due to formation of a “vicious circle”, which can be broken only by normalizing the neuroimmune regulatory relationship. Cell immunotherapy can be a method of choice in treatment of depressive disorders. There is a whole body of data on the immune cells and their biologically active products exerting a leading role in the pathogenesis of depression [1, 3, 4, 5, 6, 7, 14]. The unidirectional effect of most psychoactive substances on the central nervous system and the immune system confirms intersystem mutual regulation and allows to consider the immune cells as model objects for influencing the intersystem functional relationship. Cell technologies related to critical technologies, based on manipulation of cells outside the body, as a result whereby the cells acquire a higher therapeutic potential. It is assumed that cellular immunotherapy will gain an advantage over pharmacological treatment, since the therapeutic effect will be manifested unnecessarily long-term treatment of patients and the body will be “deprived” of drug side effects. Introduced cells due to the presence of chemotactic signals have a great opportunity to enter the focus of damage. It is also important that injected cells are capable of secreting a wide spectrum factors produced at physiological concentrations and having a regulatory effect on the functional activity of both homeostatic systems, which is absolutely impossible to accomplish by using a complex of individual regulatory molecules. In addition, cellular immunotherapy will allow to overcome the resistance of patients currently manifested in psychiatry to the generally accepted psychopharmacological agents. We first demonstrated the possibility of animal behavior directed regulation by the transplantation of immune cells with defined functional characteristics, including those with functional activity modulated by a psychoactive substance, which was later confirmed by other researchers [2, 8, 9, 10, 12, 13]. It was shown also the ability of splenocytes with *in vitro* caffeine modulated functional activity to stimulate passive behavior in animals in a dose-dependent manner [12, 13].

Based on the previous results we investigated an effect of *in vitro* caffeine-modulated immune cells on the immune and behavior phenotypes in depressive-like syngeneic recipients.

Materials and methods

The 3.5–4 months old (CBA × C57Bl/6) F1 depressive-like male mice, weighing 25–30 g were used as donors and recipients. Mice were obtained from

the husbandry of the Institute of Pharmacology and Regenerative Medicine named after E.D. Goldberg, Tomsk National Research Medical Centre, Siberian Branch of the Russian Academy of Sciences). The animals were housed on a standard diet, with drinking water *ad libitum*, as well as under normal light conditions in cages of 10 mice/cage for at least 2 weeks prior to experiments. The experiment was carried out in accordance with the rules adopted by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), the rules of laboratory practice (Order of the Ministry of Health of the Russian Federation of June 19, 2003, No. 267).

The presence in the population of (CBA × C57Bl/6) F1 mice individuals with active and passive types of behavior, representatives of which have certain structural and functional characteristics of the nervous and immune systems and various psychophysiological forms of reaction to stress factors has been shown [8, 15]. Based on the above noted, homogeneous experimental groups were formed after preliminarily testing of all mice in the “open field” followed by including only individuals with passive behavior as being least resistant to stress influences [15]. Depressive-like phenotype in these mice ($n = 129$) was developed by modeling repeated social defeat-induced depression using the sensory contact model, as was described earlier [6, 12]. Depression-like behavior was characterized by forced swimming and “open field” tests, and via automatic measurement of sucrose preference in IntelliCage (fully automated system for behavioral and cognitive phenotyping, TSE-systems).

Immune cells for transplantation were obtained under sterile conditions from splenocyte suspension, modulated *in vitro* with caffeine and injected intravenously to syngeneic recipients, as was described earlier [12]. In the control group of animals, immune cell preparation and transplantation were carried out under the similar experimental conditions; the only exception was that the last were cultured without caffeine. Behavioural pattern of depression-like recipients 48 hours after the immune cell transplantation was evaluated in the “open field” test (locomotion, exploration and anxiety) and Porsolt forced swimming test (depression-like behaviour) using a modern hardware and software complex EthoVision XT (Noldus Information Technology, The Netherlands). Anhedonia was assessed in the sucrose preference test based on individual consumption of a 1% sucrose solution and water under *ad libitum* conditions for 10 days in IntelliCage.

Lysates of various brain regions (hippocampus, hypothalamus, striatum, frontal cortex) were obtained from recipient mice by homogenisation in RPMI-1640 medium supplemented with 0.1% Triton X-100,

followed by centrifugation (10,000 rpm, 3 min). Cytokine (IL-1 β , IL-2, IL-4, IL-6, IL-10, TNF α , IFN γ) concentrations were measured in supernatants by ELISA using relevant kits (R&D Systems, UK), according to the manufacturer's instructions.

Statistical data analysis was performed by using an analytics software portfolio Statistica 10.0 for Windows (StatSoft, Tulsa, OK, USA). Normally distributed data with low variance were analysed using Student's t-test; in case of no normal distribution, Mann–Whitney U test was applied. Results are presented as mean (M) and standard error of the mean (SE) (M $\pm$ SE). $P < 0.05$ was considered statistically significant.

Results and discussion

Anhedonia is considered as one of the main symptoms of major depression in humans, also being the main sign of depression in experimental models

and estimated by decreased animal consumption of sucrose solution. Assessing mouse behavior in Intelli Cage allowed to find that depressive-like recipients after the transplantation of caffeine-modulated immune cells in a free choice demonstrated increased consumption and preference of 1% sucrose solution, as compared with the control group [11, 12].

Behavioral despair in mice is a primary screening test for antidepressants. Forced swimming test revealed in depressive-like recipients a significantly increased mobility and high mobility time, decreased periods of passive swimming (drift + complete immobility) with the disappearance of complete immobility periods in water. Differences in these indicators between the control and experimental groups of depressive-like recipients were recorded within 10 days after cell transplantation and were associated with the stimulation of neurogenesis in the hippocampus [11, 12].

TABLE 1. DEPRESSIVE-LIKE RECIPIENTS (CBA $\times$ C57Bl/6) F1 BEHAVIORAL PARAMETERS IN THE OPEN FIELD TEST (M $\pm$ SD)

	Horizontal activity			Vertical activity
	Number of crossed peripheral square	Number of crossed central square	total	Rearing postures (total)
Control (n = 61)	3.2 $\pm$ 2.8	0	3.2 $\pm$ 2.8	0.2 $\pm$ 0.9
Experimental (n = 68)	103.0 $\pm$ 49.9*	0.7 $\pm$ 2.4*	103.7 $\pm$ 46.4*	2.9 $\pm$ 3.2*

Note. Note. Control, group of mice-recipients after the transplantation of splenocytes pre-cultured without caffeine. Experimental, group of mice-recipients after the transplantation of caffeine-treated splenocytes; testing period – 5 min; * $p < 0.01$, as compared to control.

TABLE 2. CYTOKINES CONTENTS IN VARIOUS BRAIN STRUCTURES IN DEPRESSIVE-LIKE RECIPIENTS (CBA $\times$ C57Bl/6) F1 AFTER THE CAFFEINE-TREATED IMMUNE CELLS TRANSPLANTATION, M $\pm$ SE

Brain structures	IL-1 β	IL-4	IL-6	IFN γ	IL-10	IL-2	TNF α
Hypothalamus							
Control	233.5 $\pm$ 29.8	15.5 $\pm$ 5.8	1261.0 $\pm$ 195.1	225.3 $\pm$ 25.5	19.9 $\pm$ 6.9	16.9 $\pm$ 6.6	0
Experimental	124.7 $\pm$ 18.9*	23.2 $\pm$ 6.6	732.1 $\pm$ 142.2*	106.1 $\pm$ 13.6*	26.7 $\pm$ 7.6	19.8 $\pm$ 6.9	
Hippocampus							
Control	377.2 $\pm$ 38.1	19.1 $\pm$ 6.2	1863.0 $\pm$ 255.3	352.9 $\pm$ 38.3	5.21 $\pm$ 1.50	39.5 $\pm$ 8.9	10.6 $\pm$ 4.1
Experimental	245.8 $\pm$ 31.0*	33.9 $\pm$ 7.7	815.7 $\pm$ 150.6*	132.6 $\pm$ 16.3*	26.1 $\pm$ 7.6*	48.8 $\pm$ 9.8	1.0 $\pm$ 1.0*
Frontal cortex							
Control	274.5 $\pm$ 33.8	25.7 $\pm$ 6.9	2012.0 $\pm$ 270.2	273.8 $\pm$ 30.4	29.9 $\pm$ 7.9	24.2 $\pm$ 2.4	0
Experimental	206.6 $\pm$ 27.1	24.5 $\pm$ 6.7	1992.0 $\pm$ 268.2	98.3 $\pm$ 12.8*	33.3 $\pm$ 8.3	46.8 $\pm$ 3.6	
Striatum							
Control	70.3 $\pm$ 13.4	14.7 $\pm$ 5.8	836.7 $\pm$ 152.7	92.9 $\pm$ 12.3	7.7 $\pm$ 5.8*	22.7 $\pm$ 7.2	4.3 $\pm$ 5.4
Experimental	57.8 $\pm$ 12.2	13.7 $\pm$ 5.7	848.9 $\pm$ 153.9	83.3 $\pm$ 11.3	26.0 $\pm$ 7.2	31.6 $\pm$ 8.1	0.0 $\pm$ 5.0

Note. As for Table 1. n = 12 in each group; * $p < 0.05$, as compared to control (Mann–Whitney U Test).

We have shown also that *in vitro* caffeine-modulated immune cell transplantation in depressive-like recipients caused stimulation of motor and exploratory activities in the “open field” test (Table 1). Hence, caffeine-treated immune cell transplantation caused the behavioral phenotype editing, which was manifested in the anhedonia decrease, stimulation of exploratory behavior and motor activity in the forced swimming test.

Scientific community widely debates about phenomenon of “cytokine-induced depression”. A number of cytokines, in addition to immunological effects, may cause symptoms of depression. Such effects are typical for signaling molecules, providing conjugation cascade mechanisms of inflammation and immune response. These cytokines affect the nervous system functional activity, being one of depression-related pathogenic factors [1, 4, 5, 6, 7, 14]. Behavioral changes mentioned above after the caffeine-treated immune cell transplantation were accompanied by decreased levels of several pro-inflammatory cytokines (TNF α , IL-1 β , IL-6, IFN γ) and increased IL-10 in some brain structures pathogenetically significant for depressive-like state, which indicates a decreased neuroinflammation (Table 2).

Caffeine-treated immune cell transplantation also led to recipient immune system functional activity modulation expressed in stimulating the immune

response, the proliferative activity of splenocytes and reducing tryptophan catabolism therein [11]. A decreased production of pro-inflammatory cytokines (IL-1 β , IL-6 spontaneous production, IL-6, IFN γ and TNF α mitogen-induced production), increased production of IL-2 and anti-inflammatory cytokines IL-4, IL-10 by depressive-like recipient spleen cells (data not shown) were observed. Multiple studies have reported that baseline levels of certain cytokines or cytokine changes during treatment with specific antidepressants were associated with antidepressant treatment response [4, 7]. Hence, the modulation in the central and peripheral cytokine production may be one of the mechanisms for immune and behavior phenotypes editing after the *in vitro* caffeine-treated immune cells.

Conclusion

The current data demonstrated that *in vitro* caffeine-modulated immune cells in depressive-like recipients, affecting the main pathogenetic mechanisms of depression, have a positive psychoneuro-immunomodulating effect, which determines the possibility and prospects of immunotherapy of depression by autologous immune cells with modulated *in vitro* functional activity.

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ЦИТОКИНОВЫЙ ПРОФИЛЬ В КРОВИ И РЕПАРАЦИЯ В ОЧАГЕ ПОВРЕЖДЕНИЯ В ДИНАМИКЕ ЭКСПЕРИМЕНТАЛЬНОЙ ТЕРМИЧЕСКОЙ ТРАВМЫ В УСЛОВИЯХ ЛОКАЛЬНОГО ИЛИ СИСТЕМНОГО ПРИМЕНЕНИЯ МЕЛАТОНИНА

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Резюме. Ожоговый травматизм представляет одну из ключевых медико-социальных проблем. Несмотря на значительные достижения в комбустиологии, медленное заживление и присоединение инфекции составляют ключевые проблемы у ожоговых больных, приводящие к удлинению сроков госпитализации, снижению качества жизни и эмоциональным расстройствам. До 70% всех осложнений после термической травмы (ТТ) связаны с инфекцией — это, прежде всего, пневмония, инфекции мочевыводящих путей. Формирование инфекционных осложнений, включая сепсис, после ТТ связывают с избыточными иммуносупрессивными реакциями, в том числе как компенсацию на длительный, устойчивый провоспалительный ответ, в частности обусловленными гиперпродукцией и эффектами IL-10, IL-4, TGF- β . Цель работы: изучить влияние системного и локального в составе оригинальной дермальной пленки (ДП) применения МТ на показатели репарации и концентрацию в сыворотке некоторых цитокинов в динамике экспериментальной ТТ. Работа выполнена на 84 крысах — самцах линии Wistar массой 240 ± 20 г, которые случайным образом разделены на 4 группы: группа 1 ($n = 12$) — интактный контроль, группа 2 ($n = 30$) — животные с ТТ, группа 3 ($n = 21$) — животные с ТТ и наложением ДП с МТ на область ожога, группа 4 ($n = 21$) — животные с ТТ и внутрибрюшинным введением МТ. Для моделирования ТТ IIIA степени и относительной площадью 3,5% изолированный участок кожи межлопаточной области погружали в дистиллированную воду с температурой 98–99°C на 12 с. ДП с МТ (в концентрации 0,005 г/г) площадью 12 см² в группе 3 наносили сразу после ТТ, закрепляя асептической повязкой, перевязку осуществляли ежедневно в течение 5 суток. Внутрибрюшинно вводили МТ ежедневно в дозе 10 мг/кг в течение 5 суток. На 5-е, 10-е и 20-е сутки от момента индукции ТТ вычисляли площадь раны, скорость эпителизации в сыворотке определяли концентрацию интерлейкина-4 (IL-4), фактора некроза опухолей альфа (TNF α), интерферона-гамма (IFN γ). При экспериментальной ТТ в динамике наблюдений от 5-х к 20-м суткам уменьшается абсолютная и относительная площадь раневого дефекта, в связи с чем прогрессивно увеличивается скорость эпителизации раны и доля уменьшения ее площади, на 5-е, 10-е и 20-е сутки в сыворотке увеличивается концентрация TNF α и IL-4 с максимальными значениями на 10-е сутки наблюдения. Локальное применение МТ в составе ДП при ТТ ускоряет заживление ожоговой раны и снижает в

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сыворотке концентрацию $TNF\alpha$ и IL-4 на 5-е, 10-е и 20-е сутки. Внутрибрюшинное применение МТ при ТТ ускоряет заживление ожоговой раны и снижает в сыворотке концентрацию $TNF\alpha$ на 10-е и 20-е сутки. Ускоряющий репарацию эффект МТ при ТТ более выражен при локальном применении в составе ДП по сравнению с внутрибрюшинным введением.

Ключевые слова: термическая травма, мелатонин, репарация, цитокины, $TNF\alpha$, $IFN\gamma$, IL-4

BLOOD CYTOKINE PROFILE AND LESION SITE REPAIR IN DYNAMICS OF EXPERIMENTAL THERMAL TRAUMA AFTER LOCAL AND SYSTEMIC MELATONIN ADMINISTRATION

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Abstract. Burn injuries are one of the key medical and social problems. Despite the significant achievements in combustiology, the slow healing and the appearance of infection are the key problems in burn patients, which lead to a longer hospitalization period, to reduction of life quality and to emotional disorders. Up to 70% of all complications after thermal trauma (TT) are connected with infection – first of all, pneumonia, infections of urinal tract. The forming of infectious complications, including sepsis, after TT is associated with excessive immunosuppressive reactions, as compensation for a long, stable proinflammatory response, in particular, owing to hyperproduction and effects of IL-10, IL-4, $TGF-\beta$. Aim: to study the influence of systemic and local usage of MT with original dermal film (DF) on reparation and serum cytokine concentration indicators in dynamics of experimental TT. The study was conducted using 84 rats – males of Wistar line, which were divided randomly into 4 groups: 1st group (n = 12) – intact monitoring, 2nd group (n = 30) – animals with TT, 3rd group (n = 21) – animals with TT and DF with MT use on the region of burn, 4th group (n = 21) – animals with TT and intraperitoneal injection of MT. To model TT of IIIA degree and relative area 3,5%, isolated skin area of interscapular area was immersed in distilled water at a temperature of 98-99 °C at 12 s. The DF with MT (at a concentration of 0.005 g/g) on 12 cm² – area in 3rd group was used daily for 5 days. The MT was injected intraperitoneally daily at the dose of 10 mg/kg for 5 days. The wound area was calculated, the interleukin-4 (IL-4), tumor necrosis factor alpha ($TNF\alpha$), interferon-gamma ($IFN\gamma$) were determined in serum on 5th, 10th and 20th day from the moment of TT induction in each group. During experimental TT in dynamic monitoring from 5th to 20th day the absolute and relative areas of wound defect are reduced, because of that the epithelization speed and its part of area reduction are progressively increasing, on 5th, 10th and 20th day the concentration of $TNF\alpha$ and IL-4 in serum is increasing with maximum values on 10th day of monitoring. Local usage of MT in DF during TT accelerates the healing of burn wound and lowers the $TNF\alpha$ and IL-4 concentration in serum on 5th, 10th and 20th day. Intraperitoneal use of MT during TT accelerates the healing of burn wound and lowers the $TNF\alpha$ and IL-4 concentration in serum on 5th and 20th day. The reparation accelerating effect of MT during TT is more expressed in locale usage in DF rather than using intraperitoneal injection.

Keywords: thermal trauma, melatonin, reparation, cytokine, $TNF\alpha$, $IFN\gamma$, IL-4

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Introduction

Burn injuries are one of the key medical and social problems. Despite the significant achievements in combustiology, slow healing and appearance of infection are the key issues in burn patients, which

lead to prolonged hospitalization period, lowered quality of life and emotional disorders [1]. Up to 70% of all complications after thermal trauma (TT) are connected to infections primarily pneumonia and urinal tract infections. Development of infectious complications, including sepsis, after TT is associated with excessive immunosuppressive reactions to compensate for long-lasting stable proinflammatory response particularly owing to overproduced IL-10, IL-4, $TGF-\beta$ [2]. Immune reactions hold the key place in TT pathogenesis at each stage, the main factors of TT mortality reduction are due to knowledge of burn pathophysiology, development of pathogenetical substantiation of therapy methods, as well as methods

of safe necrectomy and wound closures [3]. In this regard, endogenous homeostasis regulators, which can interfere in mounting immune response to TT and to be involved as immunocorrectors attract special interest. Earlier, we showed that erythropoietin applied locally and systemically as well as local EGF administration in TT accelerate healing of burn injury and restoration of the immune status in clinical and experimental conditions [4, 5]. Melatonin (MT) regulates homeostasis exerting pleiotropic effects: regulation of sleep-wake cycle (change of the MT secretion rhythm in TT is considered as the key factor of sleep disorders), regulation of redox-factor with predominant antioxidant effect, pro- and anti-inflammatory, immunomodulatory, anti-apoptogenic, regulating cell proliferation and differentiation [6]. Skin cells produce MT, so that its metabolites are found in keratinocytes, melanocytes, dermal fibroblasts [7]. MT₁, MT₂, ROR α receptors were found in keratinocytes, skin fibroblasts, the cells of the hair follicle and dermal blood vessels, and melanocytes [8]. Nowadays, the pharmaceutical MT forms are not available in the RF for local use in skin injuries, and oral MT forms are not indicated for use in TT. **Aim:** to study an influence of systemic and local MT application with original dermal film (DF) on repair and dynamic serum cytokine profile in experimental TT.

Materials and methods

The study was conducted using 84 males Wistar rats, weighted 240 ± 20 g, reared on standard diet in experimental and biological clinic (vivarium) FSBEI HE SUSMU MOH Russia, under strict compliance with the requirements for the care and maintenance of laboratory animals. The research is approved by the

ethics committee of FSBEI HE SUSMU MOH Russia (Protocol No. 10, dated of 15.11.2019). The animals were randomized into 4 groups: group 1 ($n = 12$) – intact monitoring, group 2 ($n = 30$) – animals with TT, group 3 ($n = 21$) – animals with TT and DF, MT applied on the region of burn, group 4 ($n = 21$) – animals with TT and intraperitoneally inoculated MT. To model IIIA degree TT and relative area 3,5%, isolated skin area of interscapular area was immersed in distilled water at temperature of 98-99 °C for 12 sec. The burn depth was measured by morphological methods. The preparation “Zoletil-100” (Virbac Sante Animale, France) at dose of 20mg/kg was used for anesthesia. The aseptic dressing was applied on the area of burn every day for 20 days in group 2, 3 and 4. The DF with MT on 12 cm² area in group 3 was used immediately after TT, sealed with aseptic bandage, the dressing was carried out daily for 5 days. In preliminary studies, the composition of the DF sodium carboxymethylcellulose-based containing MT at concentration of 0.005 g/g, was developed and evaluated in accordance with pharmacological parameters: organoleptic parameters (appearance, color, transparency, elasticity, presence of impurities and microcracks), adhesive ability, mechanical tensile strength, thickness (patent application № 2020118766 from 29.05.2020). The MT (FLAMMA S.P.A., Italy) was inoculated intraperitoneally daily at dose of 10 mg/kg for 5 days. The wound area was calculated by digital planimetric method with camera “Nikon Coolpix S2800” (China) and software package “Microsoft Office Visio”. Epithelialization speed (VS) was calculated by using formula: $VS = S - S_n / t$ using S as the initial wound area before treatment (hereinafter, the area in the previous measurement); S_n – the area in the subsequent measurement; t – the

TABLE 1. INDICATORS OF INJURY REPARATION IN EXPERIMENTAL THERMAL TRAUMA (TT) IN CASE OF LOCAL (DF) AND SYSTEMIC USE OF MELATONIN (MT), Me ($Q_{0.25}$ - $Q_{0.75}$)

Indicators	Group 2 TT 5 th day	Group 2 TT 10 th day	Group 2 TT 20 th day	Group 3 TT + MT DF 5 th day	Group 3 TT + MT DF 10 th day	Group 3 TT + MT DF 20 th day	Group 4 TT + MT 5 th day	Group 4 TT + MT 10 th day	Group 4 TT + MT 220 th day
Burn area, cm ²	11.66 (11.50-11.94)	9.48 (9.28-9.93)	7.59 (7.23-7.84)	10.33 (10.17-10.56) [#]	8.34 (8.19-8.51) [#]	5.54 (5.24-5.88) [#]	10.92 (10.73-11.03) [#]	9.11 (8.53-9.50)	6.27 (6.18-6.42) [#] ^
Relative area, %	3.34 (3.25-3.39)	3.17 (3.10-3.29)	2.99 (2.94-3.12)	3.36 (3.23-3.42)	3.02 (2.91-3.13) [#]	1.98 (1.87-2.23) [#]	3.43 (2.91-3.46)	3.10 (3.01-3.15)	2.54 (2.17-2.86) [#] ^
Epithelization speed, % / day	0.89 (0.86-0.89)	1.90 (1.88-1.95)	2.26 (2.14-2.55)	1.33 (1.29-1.35) [#]	6.57 (5.92-6.93) [#]	14.30 (13.38-15.17) [#]	1.15 (1.04-1.22)	2.90 (2.62-3.27) [#] ^	8.89 (7.80-9.90) [#] ^
Injury area reduction, %	2.61 (2.59-2.64)	3.68 (3.53-4.23)	11.49 (11.43-11.64)	9.80 (9.64-10.08) [#]	16.10 (14.62-17.73) [#]	19.98 (19.30-20.38) [#]	3.31 (3.17-3.58) [^]	9.48 (8.81-10.22) [#] ^	15.09 (13.39-16.87) [#] ^

Note. [#], significant ($p < 0.05$) differences with group 2. [^], with group 3 on the corresponding day.

number of days between measurements. The wound area in the subsequent measurements was estimated in percentage %, taking the day 1 area as 100%, the data were expressed as % per day. On day 5, 10 and 20 after the TT onset blood sampling was conducted under anesthesia after thoracotomy by heart left ventricle puncture into vacuum tubes "Vacuette" (Greiner Bio-One, Austria). In immunoassay "Personal LAB" (Italy) with specific test-systems for rat (Bender Medsystems, Austria) the interleukin-4 (IL-4), tumor necrosis factor-alpha (TNF α), interferon-gamma (IFN γ) concentration were measured (pg/ml) in the serum. Statistic data processing was performed by using IBM SPSS Statistics v. 19 software. Characteristics of the samples are presented as "Me (Q_{0.25}-Q_{0.75})", Me – median, Q_{0.25} and Q_{0.75} – the values of the lower and higher quartile, respectively. The testing of statistical hypothesis in groups was conducted by using non-parametric criteria Kruskal–Wallis and Mann–Whitney. To assess a relationship between parameters studied, a Spearman correlation coefficient (R) was used. The differences were considered at significance level of $p < 0.05$.

Results and discussion

The repair indicators in experimental TT are presented in Table 1. On day 10 vs day 5 after TT burn absolute and relative area were decreased ($p < 0.05$), the epithelization speed and reduced wound area were increased ($p < 0.05$). Similar tendency was found on day 20 vs day 10 after TT: the skin burn absolute and relative area were decreased ($p < 0.05$), the epithelization speed and the reduced wound area were increased ($p < 0.05$). In addition, on day 20 after TT the absolute burn area, the rate of epithelialization and reduced wound area significantly ($p < 0.05$) differed from those on day 5, suggesting a progressive decrease in the burn absolute square, increased rate of

epithelialization and reduced wound size in dynamics monitoring.

The concentration of serum cytokines after TT is presented in Table 2. On day 5, 10 and 20 after TT an increased concentration of TNF α , IL-4 without significant changes in IFN γ concentration was found. In dynamics of TT, the concentration of TNF α and IL-4 in serum on day 10 vs day 5 was higher ($p < 0.05$) and didn't differ from those on day 20, that allows to note about peak level of TNF α and IL-4 on day 10 of monitoring. Use of MT in DF during experimental TT leads to significantly reduced burn area in absolute values on day 5, 10 and 20 vs day 10 and 20 (Table 1). On day 5, 10 and 20 of monitoring the epithelization speed and relative wound area decline was increased. Maximal differences were found on day 20, whereas absolute wound area decreased by 54%, and median epithelization speed increased more than by 10-fold compared to animals with TT without use of DF with MT. Analyzing serum cytokine concentration after use of DF with MT it was found that TNF α and IL-4 level was decreased on day 5, 10 and 20 as well as on day 10 and 20, respectively. At the same time, the concentration of TNF α on day 5 did not differ, whereas on day 10 and 20 it was increased in intact animals, the concentration of IL-4 at all timepoints was higher than in the intact animals. Comparing effects of MT in DF vs intraperitoneally injected MT, it was found that absolute and relative burn area on day 20 were higher, on day 5, 10 and 20 epithelization speed was lowered, day 10 and 20 the relative decrease in wound area was lowered compared to animals with TT applied with MT in DF. We found no significant differences in concentration of TNF α , IFN γ and IL-4 in TT after using MT intraperitoneally vs use of MT in DF. The change of gene expression, cytokines production and secretion in the TT focus were noticed in many studies for pro- (IL-1 β , IL-6, IL-8, IL-22,

TABLE 2. CONCENTRATION OF CYTOKINES IN SERUM IN EXPERIMENTAL THERMAL TRAUMA (TT) IN CASE OF LOCAL (DF) AND SYSTEMIC USE OF MELATONIN (MT), Me (Q_{0.25}-Q_{0.75})

Indicators	Group 1 Intact	Group 2 TT 5 th day	Group 2 TT 10 th day	Group 2 TT 20 th day	Group 3 TT + MT DF 5 th day	Group 3 TT + MT DF 10 th day	Group 3 TT + MT DF 20 th day	Group 4 TT + MT 5 th day	Group 4 TT + MT 10 th day	Group 4 TT + MT 20 th day
TNF α , pg/ml	1.18 (0.84- 1.96)	2.25 (2.02- 2.92)*	3.42 (2.72- 6.14)*	3.48 (1.77- 5.05)*	1.77 (1.07- 2.30)#	1.96 (1.35- 4.32)* #	1.57 (1.35- 2.69)* #	2.33 (1.85- 3.03)*	2.69 (2.19- 3.03)* #	1.91 (1.35- 3.03)* #
IFN γ , pg/ml	0.83 (0.29; 1.24)	0.73 (0.12- 1.15)	0.98 (0.59- 1.35)	1.06 (0.65- 1.74)	0.94 (0.54- 1.24)	0.78 (0.44- 1.34)	1.19 (0.37- 1.44)	0.84 (0.51- 1.49)	0.76 (0.52- 1.08)	1.08 (0.66- 1.22)
IL-4, pg/ml	0.79 (0.50- 1.75)	1.93 (1.36- 2.51)*	3.26 (2.58- 5.65)*	2.97 (1.57- 4.97)*	1.72 (1.36- 1.86)*	2.36 (1.29- 4.22)* #	2.08 (1.29- 2.65)* #	2.11 (1.79- 2.86)*	2.79 (2.43- 3.36)*	2.18 (1.79; 3.01)*

Note. #, significant ($p < 0.05$) differences with group 1. ^, with group 2 on the corresponding day.

IFN γ , TNF α , G-CSF, GM-CSF, MIP-2 α , MSR-1) and anti-inflammatory (IL-4, IL-10) cytokines depending on depth, area and localization of burn, age, concomitant pathology, and other factors [2]. The sources of cytokines in the TT focus are presented by activated neutrophils, macrophages (Langerhans cells, M1 and M2 subset), dendritic cells, and diverse lymphocyte populations. The release of cytokines from TT focus into systemic blood circulation leads to activation of circulated leukocytes, platelets and endotheliocytes as an additional source of cytokines. It is noteworthy that dysfunction of specified cells, their altered phenotype lead to controversial results in different studies and require further investigation. Heterogeneity of neutrophil phenotype, function and plasticity in TT, as well as the dysfunction after concomitant secondary infection, largely determine the pathogenesis scenario, likelihood of complications and the outcome. The presence of PGE2 in TT focus leads to shift in neutrophil pro- to anti-inflammatory and pro-reparative phenotype [9]. The M1 dysfunction on early stage of TT restricts the scar healing, however the dysfunction of M2 caused long-lasting oedema and alteration, delayed wound closure, however, excessive M2 activity caused excessive scarring, increased fibroblast proliferation, and angiogenesis [10]. Long-lasting increase of serum pro-inflammatory cytokine levels after TT is coupled to adverse outcome as a result of SIRS, shock. As a result, the serum and TT focus IL-4 and IL-10 concentration increase is a post-burn immunosuppression marker. We suspect that local use of MT in DF with lipophilic properties allowed to diffuse rapidly in intracellular space and enter into the cells in the lesion site via passive diffusion, as well as glucose transporters (GLUT1) and oligopeptides (PEPT1/2) [11]. The possible mechanism of MT effect is provided by at least 18 specific receptors. Plasma membrane receptors, associated with G-peptides are MT1 (Mel1a), MT2 (Mel1b), GPR50 (Mel1c), cytosolic receptor MT3 (chinoxidoreductase-2), metalloproteinase-9, protein-phosphatase-2, the loci of connection of MT in nucleus, as well as ROR α , vitamin D receptors (VDR) [11]. MT is considered as key skin antioxidant [7]. Due to direct absorption of APK, stimulated glutathione synthesis, activation of glutathione peroxidase, glutathione reductase, SOD, catalase, hemoxidase-1 activation of quinoxidoreductase-2, NOS-1 was decreased. Antioxidant effect of MT is enabled by ingesting mitochondria via the PEPT1/2 transporter, maintaining the mitochondrial membrane potential, and increasing oxidative phosphorylation, the production of ATP rather than APK. All of them contributed to restricted area of secondary alteration and subsequently decreased activation of neutrophils, monocytes/macrophages, endotheliocytes, lowered synthesis and

secretion of autokoides (что это), including pro-inflammatory cytokines.

Protective effect of MT in TT focus may be caused by its anti-apoptogenic effect, decrease in proapoptotic protein expression in keratinocytes due to reduced cytochrome C release from mitochondria, as well as caspase 9, 3 and 7 activation. DNA repair in skin cells is regulated by MT both indirectly due to the antioxidant effect mentioned above, and directly due to increased p53 expression [13]. The anti-inflammatory effect of MT is associated with the restriction of NF- κ B-dependent intracellular signaling pathways. Moreover, after ultraviolet B irradiation of keratinocytes, which leads to the activated NF- κ B, MT inhibits the expression of many pro-inflammatory factors: iNOS, COX-2, TNF α [14]. MT inhibits the NLRP3-dependent signaling pathway activated by mitochondrial oxidative damage and associated with the expression of pro-apoptogenic factors IL-1 β and IL-18. An important mediator of the anti-inflammatory MT effect is sirtuin SIRT1, which suppresses the activation of NF- κ B-, NLRP3-dependent pathways [15]. Consequently, protective effect of MT in skin after thermal injury may be caused by antioxidative and anti-apoptogenic action that reduces the area of secondary alteration and shortened inflammatory phase, alternative and vascular-exudative reactions selected to cell proliferation and repair reactions at the repair phase and the remodeling phase. This as well as direct restriction of NF- κ B-dependent and NLRP3-dependent intracellular signaling pathways, leads to decreased activity of cytokine-producing cells in the lesion site and systemic blood flow. A more pronounced effect of MT within DP, which stimulates repair and reduces the serum concentration of TNF α and IL-4 compared with the systemic use of MT, may be associated with the autonomy of the TT focus, which restricts the MT influx from the bloodstream.

Conclusion

Dynamic monitoring of experimental TT from day 5 to day 20 revealed that the absolute and relative wound areas were reduced due to the epithelization speed and its part of area reduction progressively increased, whereas serum concentration of TNF α and IL-4 on day 5, 10 and 20 increased with peak values on day 10. Local usage of MT in DF during TT accelerates the burn wound healing and lowers the serum TNF α and IL-4 concentration on day 5, 10 and 20. Intraperitoneal use of MT during TT accelerates the burn wound healing and lowers the serum TNF α and IL-4 concentration on day 5 and 20. The repair accelerating effect of MT during TT is more evident after locally vs intraperitoneally used DF.

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РЕГУЛЯЦИЯ ДИФФЕРЕНЦИРОВКИ ПЕРИФЕРИЧЕСКИХ В-ЛИМФОЦИТОВ ПРИ ПРИВЫЧНОМ НЕВЫНАШИВАНИИ БЕРЕМЕННОСТИ

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Резюме. Доказана важная роль иммунных нарушений в привычном невынашивании беременности. Уточнение характера дифференцировки В-лимфоцитов и факторов ее регуляции у женщин с угрожающим выкидышем и привычным невынашиванием беременности в анамнезе является актуальной проблемой, поскольку позволит выявить иммунные механизмы патогенеза данной патологии.

Цель: установить особенности дифференцировки В-лимфоцитов и факторов ее регуляции у женщин с привычным невынашиванием беременности в анамнезе и угрожающим самопроизвольным выкидышем на момент обследования.

Были обследованы беременные женщины в возрасте 18-40 лет в сроке гестации 5-12 недель. Основную группу составили 60 беременных женщин с угрожающим самопроизвольным выкидышем на момент обследования и привычным невынашиванием в анамнезе. В качестве контроля обследовано 35 беременных женщин с неосложненным течением беременности. Группу сравнения составили 25 первобеременных женщин с угрожающим самопроизвольным выкидышем на момент обследования. Материалом исследования служила периферическая венозная кровь. Методом проточной цитофлуориметрии определяли субпопуляции В-лимфоцитов CD19⁺, CD19⁺IgD⁺, CD20⁺IgM⁺, CD20⁺IgG⁺; CD19⁺CD20⁺CD38⁺, CD19⁺CD27⁻, CD19⁺CD27⁺. Сывороточный уровень BAFF и APRIL оценивался методом иммуноферментного анализа.

В основной группе зарегистрировано увеличение в периферической крови доли В-клеток, CD20⁺IgM⁺ лимфоцитов и клеток памяти, наряду со снижением уровня наивных клеток и плазмочитов. В группе сравнения зарегистрировано увеличение доли незрелых IgM⁺В-клеток, циркулирующих клеток памяти наряду со снижением наивных В-лимфоцитов. В основной группе наблюдалось выраженное снижение сывороточного уровня BAFF по сравнению с группой контроля и сравнения. Анализ содержания APRIL показал выраженную тенденцию к снижению в группах с угрожающим выкидышем относительно здоровых беременных. Таким образом, угрожающий привычный и спорадический выкидыши были ассоциированы со сдвигом дифференцировки В-лимфоцитов в сторону незрелых форм и недостатком регулирующего влияния BAFF и APRIL, что выражается в нарушении В-клеточного гомеостаза и ослаблении гуморальных эффекторных механизмов на системном уровне.

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Выявленные изменения могут свидетельствовать о едином механизме развития угрожающего самопроизвольного выкидыша, выраженность которого увеличивается при повторной потере беременности. Эти изменения могут приводить к усилению эффекторных цитотоксических механизмов и увеличению провоспалительных цитокинов, что может привести к развитию повреждающих реакций в фетоплацентарном комплексе, отражением чего может являться клиническая картина угрозы прерывания беременности.

Ключевые слова: беременность, угроза прерывания беременности, привычное невынашивание, В-лимфоциты, BAFF, APRIL

REGULATION OF PERIPHERAL B-LYMPHOCYTE DIFFERENTIATION IN RECURRENT MISCARRIAGE

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Abstract. The important role of immune disorders in recurrent miscarriage has been proven. Clarification of the character of B-lymphocyte differentiation and its regulation factors in women with threatened miscarriage and recurrent miscarriage in history is an urgent problem, since it will reveal the immune mechanisms of the pathogenesis of this pathology. Purpose: to establish the features of B-lymphocyte differentiation and factors of its regulation in women with a history of recurrent miscarriage and threatening spontaneous miscarriage at the time of examination.

Were examined pregnant women aged 18-40 years at a gestation period of 5-12 weeks. The main group consisted of 60 pregnant women with a threatening spontaneous miscarriage at the time of examination and a history of recurrent miscarriage. As a control, 35 pregnant women with uncomplicated pregnancy were examined. The comparison group consisted of 25 primary pregnant women with threatened spontaneous miscarriage at the time of examination. The material for the study was peripheral venous blood. Subpopulations of B-lymphocytes CD19⁺, CD19⁺ IgD⁺, CD20⁺ IgM⁺, CD20⁺ IgG⁺ were determined by flow cytometry; CD19⁺ CD20⁺ CD38⁺, CD19⁺ CD27⁻, CD19⁺ CD27⁺. Serum levels of BAFF and APRIL were assessed by enzyme-linked immunosorbent assay.

In the main group, an increase in the proportion of B-cells, CD20⁺ IgM⁺-lymphocytes and memory cells was recorded in the peripheral blood, along with a decrease in the level of naive cells and plasma cells. In the comparison group, an increase in the proportion of immature IgM⁺ B-cells, circulating memory cells, along with a decrease in naive B-lymphocytes, was registered. In the main group there was a pronounced decrease in the serum BAFF level compared with the control and comparison groups. Analysis of the APRIL content showed a pronounced downward trend in groups with threatened miscarriage relative to healthy pregnant women. Thus, threatening habitual and sporadic miscarriages were associated with a shift in the differentiation of B-lymphocytes towards immature forms and a lack of regulatory influence of BAFF and APRIL, which is reflected in the disruption of B-cell homeostasis and weakening of humoral effector mechanisms at the systemic level. The revealed changes may indicate a single mechanism for the development of a threatening spontaneous miscarriage, the severity of which increases with repeated loss of pregnancy. These changes can lead to an increase in effector cytotoxic mechanisms and an increase in proinflammatory cytokines, which can lead to the development of damaging reactions in the fetoplacental complex, which can be reflected in the clinical picture of the threat of termination of pregnancy.

Keywords: pregnancy, threatened abortion, recurrent miscarriage, B-lymphocytes, BAFF, APRIL

The problem of the etiology and pathogenesis of recurrent miscarriage remains of high priority, despite the attention paid to it. Currently, the attention is attracted by the immune mechanisms of recurrent pregnancy loss. Recurrent miscarriage in 50% cases is associated with pathological changes in various arms of the immune system and an inadequate maternal response to a semi-allogeneic fetus [2]. Pregnancy

is a complex multi-stage process that includes fertilization, implantation, placentation and further embryonic development. Such processes are regulated by hormones, cytokines and many other intercellular interactions. The mother-fetus immune relationship is currently viewed as a two-way interplay process. On the one hand, fetal antigens must be presented to the maternal immune system, and on the other hand, the

maternal immune system must recognize them and respond. [1].

B-lymphocytes play an important role during uncomplicated pregnancy as they produce antibodies involved in humoral immune responses. Recently, the autoantibody-producing B-lymphocytes has been studied in complicated pregnancy, such as spontaneous miscarriage, preeclampsia, intrauterine growth retardation, stillbirth and premature birth [10]. Currently, there are sporadic data on changes in B-lymphocyte subpopulations during recurrent miscarriage. According to Baturina et al., recurrent pregnancy loss was paralleled with decreased primary naive IgD⁺CD38⁺B-cell number in peripheral blood [3]. In addition, it was evidenced about increased number of CD19⁺CD5⁺-cells with simultaneously decreased CD19⁺-lymphocytes in women with a history of recurrent miscarriage [4]. These data suggest that the character of B-lymphocyte differentiation changes significantly in recurrent miscarriage.

Differentiation of B-lymphocytes is regulated by various factors. The most significant factors are soluble ligands BAFF (B-cell activation factor) and APRIL (proliferation-inducing ligand) [5]. Elevated serum levels of BAFF and APRIL are associated with autoimmune diseases. The severity of diseases and the level of pathogenic autoantibodies directly depend on the serum level of BAFF and APRIL [13]. An increased level of BAFF and APRIL was observed in patients with autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, diabetes [12], systemic scleroderma, etc. [14].

Currently, only sporadic data on the level of BAFF in recurrent pregnancy loss are available. The BAFF level was significantly reduced in patients with repeated spontaneous decidual and trophoblast abortion compared with healthy pregnant women [8]. Serum BAFF was reduced in pregnant women with fetal growth retardation [6]. Currently, no literature data on the APRIL level during pregnancy are found.

The aim of our study was to establish the characteristics of B-lymphocyte differentiation and factors of its regulation in women with former recurrent miscarriage and threatened termination of pregnancy at the time of examination.

Materials and methods

The study was carried out at the V. Gorodkov Ivanovo Research Institute of Maternity and Childhood of Ministry of Health of Russia in the years 2018-2020. Pregnant women aged 18-40 years were examined at the gestation period of 5-12 weeks. The main group consisted of 60 women with threatened spontaneous miscarriage upon examination and former recurrent miscarriage. The control group consisted of 35 women with uncomplicated course of pregnancy. The comparison group included 25

pregnant women with threatened sporadic miscarriage upon examination. The inclusion criteria for the study were: spontaneous singleton uterine viable pregnancy confirmed by ultrasound and signs of threatened miscarriage. The exclusion criteria were: a miscarriage in progress, structural changes in the karyotypes of spouses established at the pregravid stage, anomalies in development of the reproductive system, anembryonia, pregnancy with the help of assisted reproductive technologies, autoimmune diseases, endocrine dysfunctions, acute and exacerbated inflammatory chronic diseases, allergic reaction upon examination, cancer processes. Peripheral blood samples were used in the study.

Using a flow cytometer FACSCanto II (BD Biosciences, USA), we determined percentage of CD19⁺B-cells, expression of immunoglobulins CD19⁺IgD⁺, CD20⁺IgM⁺, CD20⁺IgG⁺; level of plasma cells (CD19⁺CD20⁺CD38⁺) and naive cells (CD19⁺CD27⁻); memory cells CD19⁺CD27⁺. We determined the percentage of subpopulations in the pool of CD19⁺ or CD20⁺-lymphocyte gate cells. We used the following monoclonal antibodies: anti-CD19 conjugated with PE (Beckman Coulter, France), anti-CD19 conjugated with APC (Beckman Coulter, France), anti-CD20 conjugated with FITC (Beckman Coulter, France), anti-CD27 and CD38 conjugated with PE (Beckman Coulter, USA) and anti-IgD conjugated with FITC (Beckman Coulter, USA), anti-IgM conjugated with PE (eBioscience, USA), anti-IgG conjugated with PE-Cy7 (Becton, Dickinson and Company, USA). There were analyzed at least 10,000 cells in each sample by using the BDFACS Diva Software (Becton Dickinson, USA). The level of serum BAFF and APRIL was measured by ELISA (eBioscience, Austria).

Statistical data processing was carried out using licensed software packages, e.g., Microsoft Office 2010, Statistica for Windows 13.0. The data are presented as mean (M) and standard error of the mean (m). Statistical significance between differences was assessed by the Student's test, because the data followed the criteria of normal distribution. Differences were considered statistically significant at $p < 0.05$.

Results and discussion

While assessing the count of peripheral B-lymphocytes, it was found that women with threatened recurrent miscarriage had it significantly increased among CD19⁺-cell subset compared to remaining pregnant women (Table 1) ($p < 0.05$ in all cases). Assessing the level of IgM- and IgG-positive CD20⁺B-lymphocytes in the peripheral blood in main group found increased percentage of CD20⁺IgM⁺-lymphocytes compared to the control group ($p < 0.05$) that tended to increase while compared to pregnant

TABLE 1. CHARACTERISTICS OF THE CONTENT OF CD19⁺B-LYMPHOCYTES, IgM⁺ AND IgG⁺ CELLS IN THE POOL OF CD20⁺ LYMPHOCYTES, NAIVE B-CELLS, PLASMA CELLS, MEMORY B-CELLS IN THE POOL OF CD19⁺ PERIPHERAL BLOOD LYMPHOCYTES IN WOMEN WITH TREATED SPONTANEOUS ABORTION AND RECURRENT MISCARRIAGE IN HISTORY

Indicator, % (M±m)	Control group (n = 35)	Main group (n = 60)	Comparison group (n = 25)
CD19⁺B-cells	4.6±0.4	6.00±0.47 p ₁ = 0.02	5.21±1.00
Immature B-cells CD20⁺IgM⁺	30.0±4.0	40.43±3.24 p ₁ = 0.05	36.77±4.73
Mature B-cells CD20⁺IgG⁺	8.0±1.9	5.05±0.98	6.01±1.14
Plasmacytes CD19⁺CD38⁺CD20⁻	3.1±0.5	1.96±0.25 p ₁ = 0.03	6.90±1.75 p ₂ = 0.05 p ₃ = 0.01
B memory cells CD19⁺IgD⁺	33.9±2.3	50.06±4.28 p ₁ = 0.01	43.98±3.02 p ₂ = 0.02

Note. p₁ is the level of statistical significance of the differences between the main and control groups; p₂ is the level of statistical significance of the differences between the comparison group and the control group; p₃ is the level of statistical significance of differences between the main group and the comparison group.

TABLE 2. CHARACTERISTICS OF THE FACTORS REGULATING THE DIFFERENTIATION OF B-LYMPHOCYTES IN WOMEN WITH THREATENING SPONTANEOUS MISCARRIAGE AND RECURRENT MISCARRIAGE IN HISTORY

Indicator, % (M±m)	Control group (n = 35)	Main group (n = 60)	Comparison group (n = 25)
BAFF	1.5±0.1	1.30 ±0.05 p ₁ = 0.03	1.90±0.21 p ₂ = 0.01
APRIL	1.2±0.8	0.34±0.06	0.31±0.43

Note. p₁ is the level of statistical significance of the differences between the main and control groups; p₂ is the level of statistical significance of the differences between the comparison group and the control group.

women with threatened sporadic miscarriage. The number of CD20⁺IgG⁺B-lymphocytes was reduced in groups with threatened recurrent and sporadic miscarriage compared with healthy pregnant women (p < 0.05).

Analyzing differentiation features of peripheral B-lymphocytes showed that the number of naive CD19⁺CD27⁺B-lymphocytes in the main group and the comparison group was lower than in the control group. The number of plasma cells CD19⁺CD20⁻CD38⁺-cells in the main group was significantly lower than in remaining subjects (p < 0.05 in all cases). In the peripheral blood of patients of the main group and the comparison group, the number of CD19⁺CD27⁺B-memory cells was higher than in those lacking threatened termination of pregnancy.

Evaluation of the factors regulating B-lymphocyte differentiation showed a significant decrease in the serum BAFF level in the study group compared to other pregnant women (Table 2). The difference in serum BAFF concentration in comparison vs. control group was statistically significant. Analysis of the serum APRIL level showed that in the main group it

prominently tended to decrease in pregnant women with threatened termination of pregnancy compared with healthy pregnant women.

According to the literature, physiological pregnancy is accompanied by hormone-mediated B-cell lymphopenia and increased level of circulating BAFF, which is necessary for differentiation of immature transitional B-cells into naive mature B-cells [10]. Here, we estimated the number of circulating B-lymphocytes and subset composition, as well as serum concentration of factors regulating B-cell differentiation. The data obtained showed that women with threatened habitual miscarriage were characterized by pronounced changes in B-lymphocyte subset composition. According to our data, threatened recurrent miscarriage was associated with B-cell lymphocytosis, increased percentage of immature CD20⁺IgM⁺ and memory B-cells, along with decreased count of naive and plasma cells.

CD20⁺IgM⁺B-cells are characterized as immature, short-lived B-lymphocytes that undergo antigen-independent positive selection leading to elimination of cells exerting high affinity to autoantigens. This

B-cell subpopulation is characterized by long-term inactivation after contacting cognate antigen and reduced potential for further clonal proliferation and differentiation. In addition, immature B-cells exhibit reduced affinity to antigens, immature phenotype and lowered expression of receptors for major regulatory ligands [11].

Similar changes in B-lymphocyte differentiation were observed among women at risk of terminated first pregnancy. We observed increased percentage of immature IgM⁺-cells, circulating memory cells and decreased naïve B-lymphocytes. We assume that the data obtained may suggest a single mechanism for developing threatened miscarriage. Moreover, the magnitude of skewed B-lymphocyte differentiation progress along with recurrent loss of pregnancy.

BAFF and APRIL signal through the three receptors: BAFF specific receptor (BAFF-R), L-TACI (transmembrane activator-1 and calcium modulator and cyclophilin ligand interacting with CD267) and BCMA (B-cell maturation antigen, CD269). The BAFF-BCMA interaction plays an important role in the survival of plasma cells. The BAFF-BAFFR interaction leads to activation of the NFB- B pathway and the transcription of the anti-apoptotic factor Bcl-2. This promotes the survival of marginal, follicular zones, as well as IL-10-producing B-lymphocytes [9]. Signaling through TACI and BCMA is important for survival of APRIL-induced IgA⁺Breg [5].

BAFF plays a critical role in the homeostasis of the entire B-lymphocyte pool under physiological conditions, contributing to survival and maturation of B-cells. It is known that increased activation of B-cells leads to excessive generation of plasma cells and potentiation of humoral reactions, which can lead to the development of autoimmune processes [11]. In addition, the BAFF / BAFF-R interaction activates the PI3K-Akt signaling pathway as well as increases viability and activation of T-lymphocytes, which are involved in recurrent miscarriage [9]. Thus, BAFF is involved in maintaining B-cell homeostasis and is an essential factor in the pathogenesis of recurrent miscarriage.

It has been shown that exposure to BAFF and APRIL in vitro and in vivo enhances the expression of the immunosuppressive cytokine IL-10 by regulatory

B-cells via the TACI receptor [5]. Deficiency in IL-10 production by B-lymphocytes interferes with full proliferation, survival and differentiation of Breg. This disrupts the maturation of plasma cells – the main antibody producers. Plasmocytes are a source of blocking aIgG antibodies. These asymmetric antibodies bind other Igs as well as monocytes and macrophages at the mother-fetus border [7]. Consequently, aIgG deficiency leads to weakened protective mechanisms of the semi-allogenic fetus against cellular cytotoxicity and phagocytosis of the maternal immune system.

APRIL acts later in B-cell differentiation and modulates function and survival of B-cells after contact with antigen being involved in generation and survival of bone marrow long-lived plasma cells [13]. In addition, APRIL promotes the differentiation of naïve human B-cells into IL-10-producing IgA⁺B-cells. These APRIL-induced IgA⁺B-cells exhibit the Breg phenotype and inhibit effector T-cells and macrophages via IL-10 and PD-L1 [5].

The decrease in serum BAFF concentration recorded by us in women with threatened habitual miscarriage may indicate both accelerated ligand binding to the receptor and impaired homeostasis of both B- and T-cell immunity. A decrease in the serum APRIL level among women with threatened pregnancy loss (both in primary pregnant women and group of recurrent miscarriage) may point at lowered survival of mature high-effector B-lymphocytes, as well as Breg. These changes can lead to enhanced cytotoxic effects and cytokine profile skewed to pro-inflammatory response against fetal antigens, which can lead to developing reactions damaging placenta and the fetus being reflected in the clinical picture of the threatened termination of pregnancy.

Thus, threatened recurrent miscarriage is associated with disturbed regulatory effect of BAFF and APRIL on B-lymphocyte differentiation. These changes at systemic level are manifested by increased number of immature subsets and deficiency in highly efficient cells. The changes revealed in B-cell homeostasis and compromised regulation of humoral effector mechanisms lead to insufficient execution of the maternal immune tolerance to semi-allogeneic fetus.

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ВЛИЯНИЕ ПОЧЕЧНОЙ ДИСФУНКЦИИ НА УРОВЕНЬ СЫВОРОТОЧНЫХ АНГИОПОЭТИН-ПОДОБНЫХ БЕЛКОВ И АНТИТЕЛ К ФОСФОЛИПИДАМ У БОЛЬНЫХ РЕВМАТОИДНЫМ АРТРИТОМ С МЕТАБОЛИЧЕСКИМ СИНДРОМОМ

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Резюме. Ревматоидный артрит (РА) является частым фоном для развития почечной патологии. Хроническая болезнь почек (ХБП) определяется более чем у 30% пациентов с РА. Наряду с воспалением и другими факторами прогрессирования основного заболевания развитию почечного поражения при РА способствует наличие метаболического синдрома (МС).

Цель исследования — оценить взаимосвязь сывороточных концентраций ангиопоэтин-подобных белков (АППБ) и антител к фосфолипидам (аФЛ) с развитием почечной дисфункции у больных РА.

Было обследовано 158 пациентов с РА (91,8% — женщин и 8,2% — мужчин) в возрасте от 21 до 80 лет и средней длительностью заболевания — 9 (4-15) лет. Преобладали пациенты серопозитивные по ревматоидному фактору и по антителам к циклическому цитруллинированному пептиду, с развернутой клинической стадией и умеренной активностью ($3,2 < DAS28 \leq 5,1$) патологического процесса.

ELISA-тест был использован для количественного определения в сыворотке крови больных РА ангиопоэтин-подобного белка 3-го типа и 4-го типа и антител к фосфолипидам (аФЛ-IgG/IgM) для суммарного выявления антител к кардиолипину, фосфатидилсерину, фосфатидилинозитолу, фосфатидиловой кислоте и комплексу отрицательно заряженного фосфолипида и β_2 -гликопротеина-I.

Более половины обследованных больных РА имели расчетную скорость клубочковой фильтрации (pСКФ) в пределах от 89 до 60 мл/мин/1,73 м² (распределение по стадиям ХБП: C1 — 21,5%; C2 — 58,9%; C3 — 19,6%). Признаки МС (сочетания повышенного АД, повышения уровня триглицеридов и нарушений углеводного обмена на фоне центрального ожирения) были диагностированы у 68 (43%) больных РА.

Был выполнен многофакторный дисперсионный анализ по сравнению изучаемых показателей (АППБ3, АППБ4, аФЛ) в зависимости от pСКФ в группах больных РА без признаков метаболи-

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ческого синдрома и больных РА с МС. Между больными РА с различной степенью выраженности метаболических нарушений были установлены существенные различия в уровне АППБ3 ($F = 8,86$, $p = 0,0034$) и АППБ4 ($F = 29,6$, $p < 0,001$), но не аФЛ ($p > 0,05$). Проведение многофакторного дисперсионного анализа показало достоверное увеличение АППБ4 в сыворотке крови больных РА со сниженной рСКФ (< 89 мл/мин) ($F = 18,5$, $p < 0,001$) и выраженными метаболическими изменениями ($F = 24,2$, $p < 0,001$). Таким образом, на содержание АППБ4 у больных РА непосредственное влияние оказывали только два фактора (почечная дисфункция и наличие МС), способные более чем в 30% случаев описать изменчивость данного признака. Квадрат множественного коэффициента корреляции (R^2) в данной модели составил 0,33. АППБ 4-го типа следует рассматривать в роли ключевого фактора, связывающего развитие почечной дисфункции и метаболические изменения, вызванные ревматоидным воспалением.

Ключевые слова: ревматоидный артрит, почечная дисфункция, ангиопоэтин-подобные белки, метаболический синдром

INFLUENCE OF RENAL DYSFUNCTION ON THE LEVEL OF SERUM ANGIOPOIETIN-LIKE PROTEINS AND ANTI-PHOSPHOLIPID ANTIBODIES IN PATIENTS WITH RHEUMATOID ARTHRITIS AND METABOLIC SYNDROME

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Abstract. Rheumatoid arthritis (RA) is a frequent background for the development of renal pathology. Chronic kidney disease (CKD) is determined in more than 30% of patients with RA. Along with inflammation and other factors in the progression of the underlying disease, the development of renal damage in RA is facilitated by the presence of metabolic syndrome (MetS).

The aim of this study is to assess the relationship of serum concentrations of angiopoietin-like proteins (ANGPTL) and antiphospholipid antibodies (aPL) with the development of renal dysfunction in patients with RA.

We examined 158 patients with RA (91.8% – women and 8.2% – men) aged 21 to 80 years old and an average duration of the disease – 9 (4-15) years. The majority of patients were seropositive for rheumatoid factor and for antibodies to cyclic citrullinated peptide, with an advanced clinical stage and moderate activity ($3.2 < DAS28 \leq 5.1$) of the pathological process.

The ELISA test was used for the quantitative determination of angiopoietin-like protein type 3 and type 4 and antibodies to phospholipids (aPL-IgG/IgM) for total detection of antibodies to cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylacetic acid and a complex of negatively charged phospholipid and β_2 -glycoprotein-I.

More than half of the examined RA patients had the calculated glomerular filtration rate (eGFR) ranging from 89 to 60 ml/min/1.73 m² (allocation by CKD stages: C1 – 21.5%; C2 – 58.9%; C3 – 19.6%). Signs of MetS (a combination of increased blood pressure, increased triglyceride levels and carbohydrate metabolism disorders against the background of central obesity) were diagnosed in 68 (43%) RA patients.

Multivariable analysis of variance was performed to compare the studied parameters (ANGPTL3, ANGPTL4, aPL) depending on eGFR in groups of RA patients without signs of metabolic syndrome and RA patients with MetS. Significant differences in the level of ANGPTL3 ($F = 8.86$, $p = 0.0034$) and ANGPTL4 ($F = 29.6$, $p < 0.001$), but not aPL ($p > 0.05$) were found between RA patients with varying degrees of severity of metabolic disorders. Multivariable analysis of variance showed a significant increase in ANGPTL4 in the blood serum of RA patients with reduced eGFR (< 89 ml/min) ($F = 18.5$, $p < 0.001$) and pronounced metabolic changes ($F = 24.2$, $p < 0.001$). Thus, only two factors (renal dysfunction and the presence of MetS) had a direct

effect on the ANGPTL4 content in RA patients, which could describe the variability of this sign in more than 30% of cases. The squared multiple correlation coefficient (R^2) in this model was 0.33. ANGPTL type 4 should be considered as a key factor linking the development of renal dysfunction and metabolic changes caused by rheumatoid inflammation.

Keywords: rheumatoid arthritis, renal dysfunction, angiotensin-like proteins, metabolic syndrome

Introduction

Rheumatoid arthritis (RA) is a frequent comorbidity for developing renal pathology [5, 15]. Chronic kidney disease (CKD) is determined in more than 30% of patients with RA, and the hyperfiltration (C1) stage predominates in such patients, being considered as the most important indicator of the subsequent progression of renal dysfunction and an increase in related cardiovascular risk [14].

Along with inflammation and other factors in the progression of the underlying disease, the development of renal damage in RA is facilitated by the presence of metabolic syndrome (MetS). The prevalence of MetS among patients with RA according to Hallajzadeh J. et al. (2017) is 30.65%, but it ranges from 14.32 to 37.83%, depending on factors related to the characteristics of the studied population and the method used for determining MetS [9].

Obviously, there is a close relationship between MetS course and the severity of articular syndrome in RA, especially in newly diagnosed and untreated patients, as well as evidence to consider MetS as an independent risk factor for chronic kidney disease [7].

The improvement of the methods of nephroprotective strategy aimed at inhibiting the progression of CKD in RA should include not only the study of hemodynamic mechanisms of CKD progression, but also immunoinflammatory aspects. In addition, the asymptomatic course in the early stages of CKD accounts for to search for new biomarkers that can predict the progression of kidney damage in RA.

Antiphospholipid antibodies (aPL) – a heterogeneous group of autoantibodies directed against negatively charged phospholipids (cardiolipin), protein-phospholipid complexes or plasma proteins (β_2 -glycoprotein-I, β_2 -GP-I) – are characteristic of antiphospholipid syndrome, but they may also be present in diseases not associated with thrombosis (hypertension, diabetic nephropathy) [3, 12].

According to a systematic review by El Hasbani G. et al. (2021), antiphospholipid antibodies can be detected on average in 14% of patients with inflammatory and autoimmune rheumatic and musculoskeletal diseases (in addition to systemic lupus erythematosus) [6]. According to Olech E. et al., the incidence of antiphospholipid antibodies (aPL) in patients with RA is 28% (median – 22%) [13] being slightly lower in systemic scleroderma (> 30%) [6].

Angiotensin-like proteins of types 3 and 4 (ANGPTL3 and ANGPTL4), included in the group of adipokines and participating in the regulation of homeostasis of fat, lipid and glucose metabolism, can become a promising object for studying the pathogenetic mechanisms that determine the manifestations of comorbid pathology in RA. There are indications on the key role of such proteins in the regulation of physiological and multiple pathophysiological processes (regulation of lipid and carbohydrate metabolism, inflammation, hematopoiesis, etc.) [2, 11], which makes them attractive target markers for studying cardiorenal and metabolic complications of RA.

The aim of this study is to assess a relationship between serum concentrations of ANGPTL and aPL and development of renal dysfunction in patients with RA.

Materials and methods

We examined 158 patients with RA (91.8% – women and 8.2% – men) aged 21 to 80 years old, with average duration of the disease – 9 (4–15) years. The majority of patients were seropositive for rheumatoid factor (RF) and for antibodies to cyclic citrullinated peptide (ACPA), at advanced clinical stage and moderate activity ($3.2 < \text{DAS28} \leq 5.1$) of the pathological process (Table 1).

There were not included RA patients with signs of acute bacterial and viral infection at the time of the study, with detected malignant neoplasm of any localization and severe concomitant pathology (myocardial infarction, vascular thrombosis, type 1 or 2 diabetes mellitus) in history, as well as never treated with biological drugs.

Physical examination consisted of interviewing complaints, collecting anamnesis, studying the medical patient documentation, assessing the general condition, measuring blood pressure on both arms, anthropometry, calculating body mass index (BMI) in kg/m², waist/hip ratio (W/H) and calculating the DAS28 index.

The ELISA test was used to quantitate angiotensin-like protein type 3 (Human Angiotensin-like Protein 3 ELISA; Bio Vendor, Czech Republic) and type 4 (RayBio Human ANGPTL4 ELISA; RayBiotech, USA), antibodies to cyclic citrullinated peptide (ACPA) (Anti-CCP hs; Orgentec Diagnostika, Ger-

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS WITH RA

Studied indicator	Value
Age, years, M±SD	55.4±10.7
Duration of RA, n (%)	
< 4 years	41 (25.9)
5-9 years	39 (24.7)
> 10 years	78 (49.4)
Seropositivity for RF, n (%)	91 (57.6%)
Seropositivity for ACPA, n (%)	95 (60.1%)
Clinical stage of RA, n (%)	
0 – provisional RA	3 (1.9)
1 – early	16 (10.1)
2 – moderate	38 (24.1)
3 – severe	72 (45.6)
4 – terminal	29 (18.3)
R° stage, n (%)	
I	16 (10.1)
II	61 (38.6)
III	68 (43.1)
IV	13 (8.2)
The presence of erosion, n (%)	
+	126 (79.7)
–	32 (20.3)
RA activity according to DAS28 index, n (%)	
Remission of RA (DAS28 < 2,6)	26 (16.5)
Low (2.6 ≤ DAS28 ≤ 3.2)	26 (16.5)
Moderate (3.2 < DAS28 ≤ 5.1)	92 (58.2)
High (DAS28 > 5.1)	14 (8.8)
Functional class, n (%)	
0	1 (0.6)
I	24 (15.2)
II	108 (68.4)
III	24 (15.2)
IV	1 (0.6)

many) and antibodies to phospholipids (aPL-IgG/IgM) (Anti-Phospholipid Screen IgG/IgM; Orgentec Diagnostika, Germany) for total detection of antibodies to cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol and a complex of negatively charged phospholipid and β_2 -glycoprotein I. All serum samples from patients with RA were analyzed simultaneously (in one session) in accordance with the manufacturer's instructions.

A combination of increased blood pressure ($\geq 140/90$ mmHg), increased triglyceride levels (≥ 1.7 mmol/L) and disorders of carbohydrate metabolism (increased fasting plasma glucose ≥ 6.1 mmol/L) along with central obesity (waist volume > 94 cm in men and > 80 cm in women)

served as a rationale for inclusion in the group of RA patients with signs of metabolic syndrome.

To assess renal function in RA patients, the calculated glomerular filtration rate (GFR) was used according to the CKD-EPI formula (Chronic Kidney Disease Epidemiology Collaboration, 2009), taking into account the height and weight of any patient without indexing by body surface area (CKD-EPI_{height / weight}). According to the recommendations of KDIGO (Kidney Disease: Improving Global Outcomes, 2012), GFR values < 60 ml/min/1.73 m² were regarded as a certain decrease, and GFR values from 60 to 89 ml/min/1.73 m² – as a slight decrease in global kidney function.

Statistical analysis of the obtained sample data was carried out using the computer programs Microsoft

Office Excel 2010 (Microsoft Corp., USA) and STATISTICA 10.0 (StatSoft Inc., USA). Depending on the distribution of the studied variables, the data are presented as mean $\pm$ standard deviation of the mean ($M \pm SD$) or median and interquartile interval ($Me (Q_{0.25}-Q_{0.75})$). While comparing two independent groups, the methods of analysis of variance were used: with a normal distribution of characteristics – analysis of variance ANOVA, with a non-normal distribution – the Kruskal–Wallis analysis (H-test). The relationship between quantitative traits, the distribution of which obeyed the normal law, was determined by the Pearson correlation coefficient (r). In case of abnormal and/or rank distribution of features, the data of correlation analysis by Spearman's coefficient (r_s) were used. The results were considered statistically significant at $p < 0.05$.

Results and discussion

More than half of the examined RA patients had eGFR ranging from 89 to 60 ml/min/1.73 m² (distribution by CKD stages: C1 – 21.5%; C2 – 58.9%; C3 – 19.6%). There was no sharply decreased renal function (GFR < 30 ml/min/1.73 m²), corresponding to CKD C4-5 stages.

Signs of MetS (a combination of increased blood pressure, increased triglyceride levels and carbohydrate metabolism disorders along with central obesity) were diagnosed in 68 (43%) RA patients.

The level of ANGPTL3 in the blood serum of patients with RA ($n = 158$) was 641.9 ± 224.5 ng/ml, and the level of ANGPTL4 ($n = 158$) was $3.15 (0.77-12.1)$ ng/ml. 74.7% of patients with RA ($n = 118$) were found to be positive for ANGPTL3, and 49.4% ($n = 78$) for ANGPTL4.

The upper limit of normal concentration ($< M + 3SD$) for ANGPTL3 (472 ng/ml) and for ANGPTL4 (3.24 ng/ml) was established after determining these parameters in the serum of 33 healthy individuals.

Correlations of varying strength were found between ANGPTL3 and age ($r = 0.23, p = 0.03$), CRP level ($r = 0.21, n = 100, p = 0.031$), blood calcium level ($r = 0.32, p = 0.014$); between ANGPTL4 and clinical stage of RA ($r_s = 0.21, p = 0.008$), the number of swollen joints ($r_s = 0.19, p = 0.03$), CRP level ($r_s = 0.29, n = 100, p = 0.002$), the level of circulating immune complexes ($r_s = 0.31, n = 126, p = 0.01$).

The level of aPL-IgG in the blood serum of RA patients ($n = 158$) was $5.91 (3.47-7.25)$ U/ml; aPL-IgM – $6.62 (4.37-9.11)$ U/ml. Positive aPL-IgG of low or medium level (ranged 10 – 40 U/ml) were found in 14 (8.9%), aPL-IgM – in 23 (14.6%) patients with RA. Moreover, 7 (4.4%) patients had an increased level of both aPL-IgG and aPL-IgM. There was a correlation of average strength between aPL-IgG and the duration (number of months) of glucocorticosteroid intake ($r_s = 0.31, n = 78, p = 0.006$), as well as a weak strength correlation between aPL-IgM and ANGPTL4 level ($r_s = 0.26, p = 0.037$), RF-IgM level ($r_s = 0.28, p = 0.007$), DAS28 index ($r_s = 0.19, p = 0.015$).

We studied a relation between the indicators (ANGPTL3, ANGPTL4 and aPL-IgG/IgM) and the incidence of renal dysfunction (according to the level of $eGFR_{\text{growth/weight}}$) in RA patients with varying degrees of severity of metabolic changes. $eGFR_{\text{growth/weight}}$ indices in RA patients ($n = 158$) had a normal distribution (K-S $d = 0.064, p > 0.2$; Lilliefors $p < 0.15$; Shapiro–Wilk $W = 0.98573, p = 0.1$).

The average glomerular filtration rate in RA patients was 74.0 ± 18.6 ml/min. Negative correlations of average strength were revealed between the eGFR indices and the level of ANGPTL3 ($r = -0.32, p < 0.001$) and ANGPTL4 ($r_s = -0.31, p < 0.001$), as well as with age ($r_s = -0.28, p < 0.001$), duration of RA ($r_s = -0.22, p = 0.005$) and an increase in systolic blood pressure ($r_s = -0.25, p = 0.001$).

A negative correlation of weak strength was found between the level of eGFR and the intake (at the time

TABLE 2. CONCENTRATION OF ANGPTL3, ANGPTL4 AND aPL-IgG/IgM IN RA PATIENTS WITH DIFFERENT GFR

Measure	RA patients with different GFR		
	Group I ($n = 34$)	Group II ($n = 93$)	Group III ($n = 31$)
ANGPTL3, ng/ml	$533.4 \pm 161.7^{I-III}$	650.0 ± 223.9	733.2 ± 244.1
ANGPTL4, ng/ml	$0.77 (0.28-3.60)^{I-II, I-III}$	$3.3 (0.93-12.10)$	$6.48 (1.52-19.30)$
aPL-IgG, U/ml	$5.70 (3.46-7.15)$	$6.18 (3.69-7.89)$	$4.97 (2.68-7.08)$
aPL-IgM, U/ml	$5.02 (3.98-8.87)^{I-III}$	$6.37 (4.55-8.98)$	$8.85 (5.67-9.97)$

Note. Upper case indicates intergroup differences at $p < 0.05$.

of the study) of glucocorticoids ($n = 158$, $r_s = -0.16$, $p = 0.048$), but there was no correlation with the dose and frequency of receiving non-steroidal anti-inflammatory drugs ($n = 133$, $p = 0.099$ and $n = 132$, $p = 0.784$).

The patients were divided into three groups according to the measured $eGFR_{\text{growth/weight}}$: group I – optimal renal function, > 90 ml/min; group II – a slight decrease in renal function, from 89 to 60 ml/min; group III – reduced renal function, < 59 ml/min (Table 2).

Significant differences were noted in the level of ANGPTL3 in patients from the first group with RA patients in whom $eGFR_{\text{growth/weight}} < 59$ ml/min (groups I-III: H-test = 6.55, $p = 0.032$). There were no other intergroup differences in the level of ANGPTL3. Significant differences in the level of ANGPTL4 in patients with normal renal function (group I) with groups of RA patients with reduced $eGFR$ (groups I-II: H-test = 10.7, $p = 0.001$; groups I-III: H-test = 20.1, $p < 0.001$) were identified. ANGPTL4 indices also had intergroup differences (groups II-III: H-test = 7.2, $p = 0.007$) with $eGFR_{\text{growth/weight}}$ less than 90 ml/min (Table 2).

ANGPTL types 3 and 4 acting along with C-reactive protein (CRP) as markers of systemic inflammation in RA may indicate a direct effect of chronic inflammation on renal function. It has been reported that inflammation promotes glomerular damage through infiltration of inflammatory cells (monocytes and macrophages), which stimulate the proliferation of mesangial cells. A persistently high CRP level for at least 6 months is a significant risk factor for the development of chronic kidney disease [10]. In addition, Clement L.C. et al. (2014) found that ANGPTL4 is a link between proteinuria and hypertriglyceridemia in nephrotic syndrome [4].

There were no intergroup differences in the concentration of aPL-IgG ($p = 0.23$). The aPL-IgM level in group III was significantly higher than in the group of RA patients with $eGFR_{\text{growth/weight}} > 90$ ml/min (groups I-III: H-test = 5.49, $p = 0.02$) (Table 2).

According to Couderc M. et al. (2016) the development of renal failure in RA is more likely associated with cardiovascular risk factors, which are more often observed in RA (age, gender, smoking, hypertension, hypercholesterolemia) and are risk factors for chronic kidney disease, but not associated with the activity or severity of the disease [5].

Antiphospholipid antibodies can cause prolongation of phospholipid-dependent coagulation disorders, although patients with rheumatic diseases are at higher risk of thromboembolic complications rather than bleeding. The clinical significance of aPL in RA is not determined, although the frequency of detected antibodies to cardiolipin is often higher than

in healthy people. The presence of these antibodies is considered to be a nonspecific marker of the activated immune system [8]. We were unable to establish a reliable relationship between aPL-IgG/IgM and the presence of venous thrombosis in patients with RA in the anamnesis ($p > 0.05$). However, according to Yusuf H.R. et al. (2014), autoimmune diseases, including RA, may be associated with an increased risk of venous thromboembolism among hospitalized patients [16], which may presumably be associated with the intake of glucocorticoids.

At the final stage of the study, a multivariable analysis of variance was performed to compare the studied parameters (ANGPTL3, ANGPTL4, aPL) depending on $eGFR$ in groups of RA patients with/without signs of metabolic. Significant differences in the level of ANGPTL3 ($F = 8.86$, $p = 0.0034$) and ANGPTL4 ($F = 29.6$, $p < 0.001$), but not aPL ($p > 0.05$) were found between RA patients with varying degrees of severity of metabolic disorders.

The study of the influence of several factors (the presence of MetS and renal dysfunction) on the level of ANGPTL3 in RA patients showed that these factors and their interactions can explain an insignificant variability of ANGPTL3 ($R^2 = 0.11$), which indicates a low quality of the model.

While studying the influence of the selected factors on the level of ANGPTL4, a more pronounced difference in the level of ANGPTL4 was noted in the presence of metabolic disorders in groups of RA patients with varying degrees of renal dysfunction. Nevertheless, the considered factors and their interactions, although allowing to explain a significant part of the variability in ANGPTL4 ($R^2 = 0.32$), also did not make this model significant ($p = 0.1$).

We differentiated RA patients into patients with high or optimal $eGFR$ (≥ 89 ml/min) and patients with reduced $eGFR$ (< 89 ml/min) when combining groups of RA patients with varying degrees of renal dysfunction (group II and group III). Multivariable analysis of variance using the new characteristics showed a significant increase in serum ANGPTL4 of RA patients with reduced $eGFR$ ($F = 18.5$, $p < 0.001$) and pronounced metabolic changes ($F = 24.2$, $p < 0.001$). The presence of MetS did not affect the serum ANGPTL4 level in RA patients with normal renal function. The decrease in renal function was accompanied by significantly increased ANGPTL4 that was more noticeable in the group of RA patients with MetS ($F = 5.76$, $p = 0.176$).

Thus, only two factors (renal dysfunction and the presence of MetS) had a direct effect on the ANGPTL4 level in RA patients, which could describe the variability of this sign in more than 30% of cases. The squared multiple correlation coefficient (R^2) in this model was 0.33.

Conclusion

Chronic rheumatoid inflammation and the combination of RA with MetS potentiate development of renal dysfunction, noted according to our data in 78.5% of patients, being accompanied by increased level of serum ANGPTL types 3 and 4 of patients.

ANGPTL type 4 should be considered as a key factor bridging the development of renal dysfunction and metabolic changes caused by rheumatoid inflammation. A better understanding of the actions and mechanisms of ANGPTL may be of high priority

for developing effective therapeutic methods lowering the progression of arthritis, metabolic syndrome and cardio-renal complications, thereby improving the quality of life of RA patients .

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СООТНОШЕНИЕ НАИВНЫХ CD45RA⁺ И CD31⁺Т-КЛЕТОК У ПАЦИЕНТОВ С РЕВМАТОИДНЫМ АРТРИТОМ ПРИ СТИМУЛЯЦИИ ГОМЕОСТАТИЧЕСКИМИ ФАКТОРАМИ *IN VITRO*

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Резюме. Большое внимание исследователей направлено на изучение роли факторов гомеостатической пролиферации в патогенезе аутоиммунных заболеваний, однако до сих пор недостаточно изучено влияние IL-7 и IL-15 на фенотип наивных Т-клеток при патологии. Целью данного исследования стало изучение соотношения CD45RA⁺ и CD31⁺ наивных клеток среди CD4⁺ и CD8⁺-лимфоцитов здоровых индивидуумов и пациентов с ревматоидным артритом (РА) при стимуляции IL-7, IL-15 *in vitro*. В периферической крови мы не обнаружили отличий по числу CD45RA⁺ и CD31⁺-клеток между группами доноров и пациентов. Стимуляция комбинацией IL-7 с IL-15 способствовала увеличению числа CD4⁺CD45RA⁺ и CD8⁺CD45RA⁺ у доноров относительно их уровня в периферической крови. Тогда как у пациентов с РА число CD8⁺CD45RA⁺-клеток снижалось под действием IL-15, комбинации IL-15 с IL-7 по сравнению с контролем без стимуляции, и достоверно отличалось, как и число CD4⁺CD45RA⁺-клеток, от содержания данных клеток у доноров в тех же условиях. Достоверных отличий по содержанию CD31⁺-клеток и числу CD31⁺-клеток, отвечающих пролиферацией на цитокины, как между группами доноров и пациентов с РА, так и различными вариантами культивирования не было выявлено. Таким образом, можно говорить о том, что под влиянием факторов гомеостатической пролиферации происходит пропорциональное увеличение CD31⁺Т-клеток как у доноров, так и пациентов с РА. При этом наивные Т-клетки доноров при культивировании сохраняют экспрессию CD45RA, а наивные Т-клетки пациентов частично ее утрачивают. Полученные данные говорят о том, что под действием факторов гомеостатической пролиферации при РА происходит конверсия фенотипа наивных Т-клеток в фенотип Т-клеток памяти.

Ключевые слова: наивные Т-клетки, CD31, гомеостатическая пролиферация, IL-7, IL-15, ревматоидный артрит

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PROPORTION OF NAIVE CD45RA⁺ AND CD31⁺T-CELLS IN PATIENTS WITH RHEUMATOID ARTHRITIS UNDER STIMULATION WITH HOMEOSTATIC FACTORS *IN VITRO*

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Abstract. Much attention of researchers is directed to study the role of factors of homeostatic proliferation in the pathogenesis of autoimmune diseases, however, the effect of IL-7 and IL-15 on the phenotype of naive T-cells has not yet been sufficiently investigated in the pathology. The aim of this study was to investigate the proportion of CD45RA⁺ and CD31⁺ naive cells among CD4⁺ and CD8⁺-lymphocytes from healthy individuals and patients with rheumatoid arthritis (RA) upon stimulation with IL-7, IL-15 *in vitro*. In peripheral blood, we did not find any differences in the number of CD45RA⁺ and CD31⁺-cells between the group of donors and group of RA patients. In donors, stimulation by a combination of IL-7 with IL-15 promoted an increase in the proportion of CD4⁺CD45RA⁺ and CD8⁺CD45RA⁺ relative to their level in the peripheral blood. Whereas in RA patients the number of CD8⁺CD45RA⁺-cells decreased under IL-15 and the combination of IL-15 with IL-7 compared to the control without stimulation, and it, as a proportion of CD4⁺CD45RA⁺-cells, significantly differed from the content of these cells under the same conditions in donors. There were no significant differences in the content of CD31⁺-cells and the number of CD31⁺-cells proliferating to cytokines, both between the groups of donors and patients with RA, and between different culture conditions. Thus, we can say that under the factors of homeostatic proliferation, there is a proportional increase in CD31⁺T-cells number, both in donors and in patients with RA. At the same time, naive T-cells from donors retain the expression of CD45RA during cultivation, while naive T-cells from patients partially lose it. The data obtained indicate that in RA, under factors of homeostatic proliferation the phenotype of naive T-cells is converted into the phenotype of memory T-cells.

Keywords: naive T-cells, CD31, homeostatic proliferation, IL-7, IL-15, rheumatoid arthritis

Introduction

Homeostasis of T-cells is contributed by two mechanisms: the generation of naive T-cells in the thymus and homeostatic proliferation (HP) on the periphery. The process of HP is based on signalling through the T-cell receptor and the receptors for IL-7, IL-15 [13, 14]. Markers of recent thymic emigrants (RTE) are presented by the surface molecule CD31 and the amount of T-cell excision circles (TREC) formed during T-cell receptor (TCR) gene rearrangement in the thymus [9]. At the periphery, RTEs undergo maturation and become naive T-cells. Despite the thymus involution and decline in migrating RTEs, the number of naive T-cells in the periphery does not undergo significant age-related changes suggesting about a more intensive HP of naive T-cells [6]. Also, the intensity of HP increases after lymphopenia induced by various factors, while the degree of HP intensity is directly related to the filling of lymphatic niches [6, 11, 12].

As a result of lymphopenia-induced proliferation, T-cell clones bearing TCRs recognizing self peptides expand, that can lead to the accumulation of potentially autoreactive cells [9]. The negative conse-

quences of HP also include conversion of naive T-cell phenotype into surrogate memory cells, that suggests the involvement of HP in developing autoimmune diseases, including rheumatoid arthritis (RA) [7, 13].

Over the past decade, more data have been accumulated confirming the active role for IL-7 and IL-15 in the pathogenesis of autoimmune diseases. Elevated levels of IL-7, IL-15 are detected in the blood serum and bone marrow of patients with RA. TNF-induced synoviocytes produce IL-15, which, in turn, promotes production of TNF by macrophages, as well as indirectly contributes to the production of IFN γ , IL-1 β , IL-17 by T-cells during inflammation [15]. It has been shown that under the influence of IL-7, T-cells predominantly produce cytokines related with Th1 and Th17-cells [10]. IL-7 mediates T-dependent activation of macrophages, dendritic cells and B-cells, which is accompanied by increased expression of differentiation factors, chemokines, adhesive and costimulatory molecules, catabolic enzymes [2].

Based on the data presented above, we examined *in vitro* effect of homeostatic factors IL-7 and IL-15 on percentage of CD45RA⁺ and CD31⁺ naive cells

among CD4⁺ and CD8⁺-lymphocytes from healthy individuals and patients with RA.

Materials and methods

Object of investigation

There were enrolled patients with rheumatoid arthritis ($n = 13.48 \pm 2.4$ years) with moderate and high disease activity, healthy individuals ($n = 13.45 \pm 2.1$ years), examined at the Department of Rheumatology, Clinic of Immunopathology of RIFCI (Novosibirsk) with the participation of rheumatologists. Written informed consent was obtained from all participants included in the study prior to collection of peripheral blood samples.

T-cell phenotyping by flow cytometry

Phenotyping of naive T-cells among PBMCs, isolated on the Ficoll-Urographin density gradient from the peripheral blood of donors and patients with RA, and in cell cultures was performed by staining with antibody panels. Peripheral blood cells were stained with fluorochrome-conjugated monoclonal antibodies to antigens: CD3-FITC (Sorbent, Russia), CD4-PE (Sorbent, Russia), CD45RA-PE/Cy7 (Beckman Coulter, USA), CD31-APC (BioLegend, USA). For staining culturing cells the following panel was used: CD3-PerCP/Cy5.5 (BioLegend, USA), CD4-PE (Sorbent, Russia), CD45RA-PE/Cy7 (Beckman Coulter, USA), CD31-APC (BioLegend, USA). Analysis of T-cell phenotype was performed on a flow cytometer FACS CantoII (BD, USA) using the FACS Diva software (BD, USA).

Cell culturing

PBMCs isolated from the peripheral blood of donors and patients with RA were cultured in the 24-well plates (TPP, Switzerland) in the culture medium RPMI-1640 (HyClone, USA) supplemented with 10% FCS (Gibco, USA) and antibiotics 50 µg/ml gentamicin (Dalchimpharm, Russia), 25 µg/ml thienam (Merck Sharp & Dohme Corp., USA), with/without IL-7 (50 ng/ml, PeproTech, USA) and IL-15 (50 ng/ml, PeproTech, USA), in humid atmosphere, with 5% CO₂, at 37 °C for 7 days. Before culturing, cells were stained with the vital dye CFSE (4 µM, Molecular Probes, USA) to assess the proliferative response of T-cells to cytokines. Non-proliferating cells have the highest intensity of fluorescent by CFSE; during cell division, the intensity of fluorescent dye decreases proportionally to the number of completed cell divisions.

Statistical analysis

Statistical data processing was carried out by using the software "Statistica 6.0" (StatSoft, USA) and "GraphPadPrism 9.0" (GraphPad, USA), using nonparametric statistics methods (Mann-Whitney test). For multiple comparisons within the group of donors and patients with RA, the Friedman method with posterior pairwise comparison was used to identify differences between the culture conditions.

Differences were considered statistically significant at $p < 0.05$.

Results and discussion

It was shown that no significant differences in percentage of naive CD45RA⁺, CD31⁺T-cells in the peripheral blood between the groups of donors and patients were observed. Treatment with IL-15 or IL-7 combined with IL-15 (IL-7+15) revealed that percentage of CD4⁺CD45RA⁺ and CD8⁺CD45RA⁺ in the group of donors was significantly increased compared to group of patients (Figure 1, M-U test, $p < 0.05$). In addition, the stimulation with IL-15, IL-7+15 in the group of donors resulted in increased number of CD4⁺CD45RA⁺-cells compared to that in the peripheral blood (Figure 1A). In donors, an increase in percentage of CD8⁺CD45RA⁺-cells was observed under stimulation with IL-7 combined with IL-15 (Figure 1C). In RA patients, no significant changes in the number of CD45RA⁺-cells cultured with the homeostatic factors were found (Figure 1B), excepting CD8⁺45RA⁺-cells, which decreased after exposure to IL-15 and IL-7+15 vs control intact T-cells (Figure 1D).

No significant differences in the CD31 expression on T-cells were found between the groups of donors and patients, as well as under the different culture conditions (Table 1). We estimated the number of proliferated CD31⁺-cells in the cultures (Table 2), and found no significant differences between the groups of donors and patients with RA. However, the number of CD4⁺CD31⁺-cells proliferating in response to IL-15 in the group of patients with RA compared with the group of donors tended to increase ($p = 0.07$). The peak response of naive T-cells to stimulation was observed by combining both cytokines IL-7, IL-15.

Thus, it may be concluded that under the influence of HP factors, percentage of CD31⁺T-cells, both in donors and in patients with RA was increased. However, donor cells during culturing retain CD45RA expression, the percentage of such cells increased under the influence of IL-15 as well as IL-15 combined with IL-7. In RA patients, the percentage of CD45RA-expressing cells is reduced under the same conditions. The data obtained suggested that in RA, under the influence of HP factors resulted in conversion of naive-to-memory T-cell phenotype.

Our data about the effect of HP factors on the number of CD31⁺T-cells, proliferation with sustained phenotype are consistent with the published data. It was shown that *in vitro* IL-7-induced proliferation of CD4⁺CD31⁺ naive T-cells does not affect the stable surface CD31 expression [1]. Loss of the CD31 molecule can occur after receiving a signal through the T-cell receptor [8]. CD31⁺T-cells from RA patients showed a proliferation level comparable to that in donor cells in response to IL-7, IL-15. Typically, peripheral blood T-cells showed low responsiveness

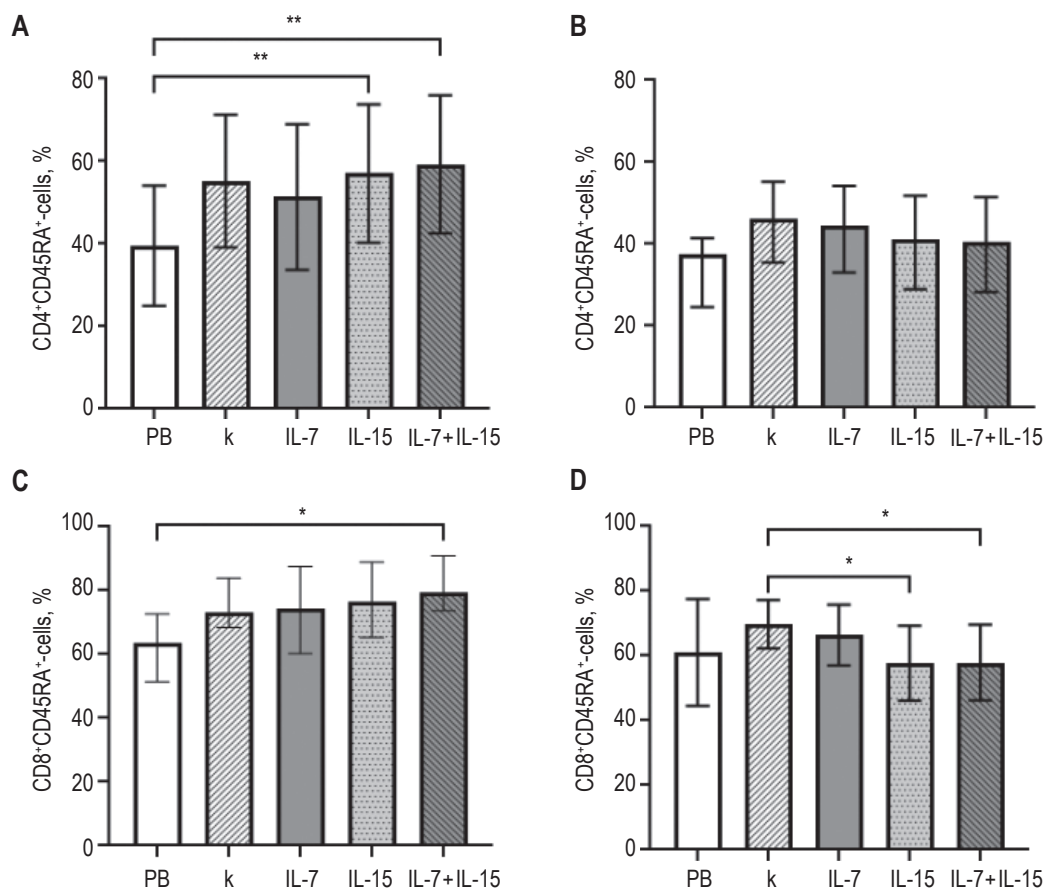


Figure 1. Proportion of CD4⁺CD45RA⁺ and CD8⁺45RA⁺-cells in cultures *in vitro* and in the peripheral blood of healthy volunteers and patients with rheumatoid arthritis

Note. A, percentage of CD4⁺CD45RA⁺-cells from donors. B, percentage of CD4⁺CD45RA⁺-cells from patients with RA. C, percentage of CD8⁺45RA⁺-cells from donors. D, percentage of CD8⁺45RA⁺-cells from patients with RA. Data are present as median with interquartile range (25th and 75th percentiles); PB, peripheral blood; k, culture without stimulation. *, significant differences, $p < 0.05$; **, significant differences, $p < 0.01$

TABLE 1. CONTENT OF CD31⁺ CELLS AMONG CD4⁺ AND CD8⁺-LYMPHOCYTES IN THE PERIPHERAL BLOOD AND *IN VITRO* CULTURES IN HEALTHY INDIVIDUALS AND PATIENTS WITH RA, Me (Q_{0.25}-Q_{0.75})

	CD4 ⁺ CD31 ⁺ , %	CD8 ⁺ CD31 ⁺ , %
PB_donors	32.9 (20.1-44.0)	75.5 (64.1-83.1)
PB_RA patients	26.4 (22.9-32.4)	74.7 (72.9-78.9)
k_donors	32.5 (19.3-45.0)	76.8 (65.5-82.2)
k_RA patients	26.0 (19.1-28.0)	75.6 (61.0-76.7)
IL-7_donors	30.0 (22.6-45.8)	78.6 (71.8-80.3)
IL-7_RA patients	23.6 (22.9-37.4)	77.9 (72.0-79.1)
IL-15_donors	29.7 (18.3-44.2)	75.4 (65.0-81.8)
IL-15_RA patients	22.1 (20.2-30.8)	77.6 (72.1-80.4)
IL-7+15_donors	37.3 (24.0-45.7)	77.8 (67.7-82.9)
IL-7+15_RA patients	28.0 (22.9-37.3)	77.1 (74.6-82.3)

Note. PB, peripheral blood; k, culture without stimulation.

TABLE 2. RESPONSIVENESS OF CD4⁺CD31⁺ AND CD8⁺CD31⁺-CELLS FROM HEALTHY INDIVIDUALS AND PATIENTS WITH RA TO HOMEOSTATIC CYTOKINES *IN VITRO*, Me (Q_{0.25}-Q_{0.75})

Cell subset	Proliferated cells, %			
	k	IL-7	IL-15	IL-7 + IL-15
Donors CD4 ⁺ CD31 ⁺	1.8 (1.2-4.8)	12.5 (6.8-39.5)*	8.7 (5.5-18.7)*	27.5 (13.4-59.2)*
RA patients CD4 ⁺ CD31 ⁺	1.7 (0.8-2.2)	14.4 (5.4-27.7)*	18.2 (5.8-36.6)*	38.9 (11.0-43.4)*
Доноры CD8 ⁺ CD31 ⁺	1.4 (0.6-2.9)	30.9 (21.4-54.7)*	54.2 (37.8-76.6)*	68.4 (58.3-88.4)*
RA patients CD8 ⁺ CD31 ⁺	1.4 (0.8-2.8)	27.8 (16.3-48.5)*	70.6 (44.7-77.6)*	75.2 (39.7-78.5)*

Note. *, significant differences compared to cells without stimulation (k), p < 0.05.

to mitogenic stimulation, while synovial CD4⁺-lymphocytes are hyper-reactive *in vitro*, including the high response to IL-7 stimulation [14]. In addition, IL-7 is able to enhance the response of T-cells to phytohemagglutinin in patients with RA under the clinical remission, confirming its involvement in the disease pathogenesis [5].

In RA, the decrease in the percentage of CD45RA⁺-cells was revealed in cultures added with IL-15 or IL-15 combined with IL-7, suggesting that under the influence of HP factors, an increased percentage of CD45RA⁺CD31⁺-cells may occur. It has been shown that, under the action of proinflammatory cytokines, IL-7 promotes accumulation of cultured central memory T-cells *in vitro*, which can be differentiated from naive T-lymphocytes under inflammatory

conditions [3]. IL-15 itself can act as a pro-inflammatory cytokine, promoting the production of TNF by macrophages and other pro-inflammatory cytokines by T-cells [15].

Conclusion

Thus, this study demonstrated that HP factors, individually or in combination, promote the proliferation of CD31⁺-positive cells and sustained their percentage both in health and RA. However, taking into account the lower number of cells expressing CD45RA in patients with RA, the proliferation of CD4⁺31⁺ and CD8⁺CD31⁺-cells treated with IL-15 and IL-15 combined with IL-7 occurs along with losing phenotype of naive T-cells in autoimmune pathology.

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СУБКЛЕТОЧНАЯ ЛОКАЛИЗАЦИЯ ТРАНСКРИПЦИОННОГО ФАКТОРА FoxP3 У ПАЦИЕНТОВ С ОСТРЫМ КОРОНАРНЫМ СИНДРОМОМ: СРАВНИТЕЛЬНЫЙ АНАЛИЗ И ПРОСПЕКТИВНОЕ НАБЛЮДЕНИЕ

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Резюме. Ключевые клеточные и молекулярные факторы, вовлеченные в разрешение воспаления после острого инфаркта миокарда, остаются изученными недостаточно. Т-регуляторные (Treg) лимфоциты характеризуются значительным потенциалом к регуляции силы и направления иммунных ответов при повреждении миокарда. Функциональная активность Treg-лимфоцитов зависит от транскрипционного фактора forkhead box protein P3 (FoxP3). Он также может экспрессироваться в конвенционных Т-лимфоцитах на этапе их активации. Ядерная локализация FoxP3 является обязательным условием, определяющим способность FoxP3 вносить вклад в регуляцию супрессорной активности Treg-лимфоцитов.

Целью данной работы стала сравнительная оценка частоты и абсолютного количества FoxP3⁺Т-лимфоцитов, в сочетании с определением субклеточной локализации FoxP3, у пациентов с острым инфарктом миокарда и хроническим коронарным синдромом, а также исследование изменений этих параметров в краткосрочный период наблюдения пациентов с инфарктом миокарда. Исследование включало 14 пациентов с хроническим коронарным синдромом (8 мужчин; 6 женщин; средний возраст 63,2±9,0 лет) и 5 пациентов с острым передним инфарктом миокарда с подъемом сегмента ST (4 мужчины; 1 женщина; средний возраст 61,4±11,2 лет), обследованных на 1-е, 3-и и 7-е сутки после события. Частота FoxP3⁺ конвенционных и регуляторных Т-лимфоцитов оценивалась во фракции мононуклеарных лейкоцитов периферической крови, наряду с определением уровня ядерной локализации FoxP3, методом проточной цитометрии с визуализацией.

Пациенты с инфарктом характеризовались снижением абсолютного количества FoxP3⁺Treg-лимфоцитов, по сравнению с пациентами с хроническим коронарным синдромом, и демонстрировали еще более выраженное снижение абсолютного количества FoxP3⁺Treg-клеток к 7-му дню после инфаркта, в то время как относительное количество Treg и конвенционных Т-лимфоцитов значимо не различалось. Уровень ядерной локализации FoxP3 был ниже как в Treg, так и в конвенционных Т-лимфоцитах пациентов с инфарктом в первый день, по сравнению с пациентами с хроническим коронарным синдромом. Абсолютное количество FoxP3⁺Treg с ядерной локализацией оставалось

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значимо сниженным на 1-е и 7-е сутки после инфаркта по сравнению с пациентами с хроническим коронарным синдромом.

Таким образом, в нашем исследовании мы показали, что ядерная локализация FoxP3 снижается при остром инфаркте миокарда и может являться более чувствительным маркером изменения функционирования Treg, по сравнению с обычной оценкой их относительного количества.

Ключевые слова: FoxP3, T-регуляторные лимфоциты, T-конвенционные лимфоциты, субклеточная локализация, острый коронарный синдром, хронический коронарный синдром

SUBCELLULAR LOCALIZATION OF FoxP3 TRANSCRIPTION FACTOR IN PATIENTS WITH ACUTE CORONARY SYNDROME: COMPARATIVE ANALYSIS AND PROSPECTIVE OBSERVATION

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Abstract. The key cellular and molecular factors being involved in the resolution of inflammation following acute myocardial infarction remain poorly understood. T-regulatory (Treg) lymphocytes are characterized by the extreme potential to regulate the strength and direction of immune responses during the myocardial injury. The functional activity of Treg-lymphocytes depends upon the transcription factor forkhead box protein P3 (FoxP3). It may be also expressed in conventional T-lymphocytes at the stage of their activation. Nuclear localization of FoxP3 is a prerequisite factor determining its ability to impact the suppressive functions of Treg-lymphocytes.

The aim of the present study was comparative evaluation of FoxP3⁺T-lymphocytes frequency and counts, combined with estimation of FoxP3 subcellular localization, in patients with acute myocardial infarction and chronic coronary syndrome and examination of changes of these parameters in the short-term follow-up of patients with myocardial infarction. The study included 14 patients with chronic coronary syndrome (8 males; 6 females; 63.2±9.0 y.o.) and 5 patients with acute anterior ST-segment elevation myocardial infarction (4 males; 1 female; 61.4±11.2 y.o.) at days 1, 3 and 7 after the event. The frequency of FoxP3⁺ conventional and regulatory T-lymphocytes was evaluated in peripheral blood mononuclear cells together with estimation of the level of FoxP3 nuclear localization by imaging flow cytometry.

Patients with infarction were characterized by the decreased counts of FoxP3⁺Treg-lymphocytes compared to patients with chronic coronary syndrome, and exhibited even further decrease in the counts of FoxP3⁺Treg-cells at day 7 after infarction, while frequency of Treg and conventional T-lymphocytes did not differ significantly. The level of FoxP3 nuclear translocation was lower both in Treg and conventional T-lymphocytes in patients at day 1 post-infarction compared to patients with chronic coronary syndrome. Absolute counts of FoxP3⁺Tregs with nuclear FoxP3 localization remained significantly lower both at days 1 and 7 post-infarction compared to patients with chronic coronary syndrome.

Thus, here we demonstrated that FoxP3 nuclear localization experiences decrease in the course of acute myocardial infarction and may serve as a more sensitive marker of changes in Treg-lymphocyte functioning than simple evaluation of their frequency.

Keywords: FoxP3, T-regulatory lymphocytes, T-conventional lymphocytes, subcellular localization, acute coronary syndrome, chronic coronary syndrome

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Introduction

Even though the current therapeutic approaches allowed to make a prominent breakthrough in management of patients with acute myocardial in-

farction (AMI), the burden of heart failure (HF) developing further still poses the major problem worldwide. It is associated with unfavorable left ventricle remodeling following improper resolution of the post-infarct inflammation. In the course of myocardial injury the reparative phase is expected to be initiated after the day 4 post-MI. However, the key cellular and molecular factors being involved

in the regulation of shift between development of inflammation and its resolution remain obscure. T regulatory (Treg) lymphocytes represent the most perspective cellular population to impact the heart healing after AMI [7].

Treg-lymphocytes are characterized by the immense potential to regulate the magnitude and modality of immune responses during the acute myocardial injury able to suppress the activity of T-conventional (Tconv) cells, govern differentiation of macrophages towards M2 phenotype and limit recruitment of pro-inflammatory leukocytes towards the site of infarction [9, 10, 11]. A whole body of surface markers have been evaluated to distinguish Tregs among remaining CD4⁺T-lymphocytes. Unfortunately, the majority of them have been confirmed to be unspecific. Until recently, transcription factor forkhead box protein P3 (FoxP3) was considered as a unique marker and master regulator of the Treg subset program. Later, an evidence emerged that FoxP3 may also be expressed by the Tconv-lymphocytes upon activation. However, FoxP3 in Tconv-cells was primarily confined to the cytoplasm [6].

The subcellular localization of FoxP3 holds a great importance in functioning of Treg-lymphocytes. Only upon nuclear translocation, FoxP3 is able to stabilize the transcription of the Treg-associated genes (*Ctla4*, *IL2ra*) and suppress transcription of the genes specific to Tconv-lymphocytes (*IFN γ* , *IL2* etc.). The process of nuclear translocation is rather complex, and depends on cell functional status [8]. The data on the FoxP3 subcellular localization in Treg and Tconv-lymphocytes in patients with chronic and acute coronary syndromes are unexplored.

The aim of the present study was to compare percentage and number of FoxP3⁺T-lymphocytes along with estimation of FoxP3 subcellular localization, in patients with acute myocardial infarction and chronic coronary syndrome and examine their changes in the short-term follow-up of patients with myocardial infarction.

Materials and methods

The study was conducted at the Cardiology Research Institute of the Federal State Budgetary Institution "Tomsk National Research Medical Center of the Russian Academy of Sciences". The protocol was approved by the local ethics committee and designed according to the principals of the Helsinki Declaration. There were enrolled 14 patients with chronic coronary syndrome (CCS) (8 males; 6 females; 63.2 $\pm$ 9.0 y.o.) and 5 patients with acute anterior ST-segment elevation myocardial infarction (STEMI) (4 males; 1 female; 61.4 $\pm$ 11.2 y.o.) at days 1, 3 and 7 after the onset. All the patients signed an informed consent to participate in the study.

All patients underwent selective coronary angiography on an Artis one angiographic complex and

Digitron-3NAC computer system (Siemens Shenzhen Magnetic Resonance Ltd., Shenzhen, China). The severity of the atherosclerosis was assessed by calculating the Gensini Score, that, on average, was 27.5 (16.0-44.0) [3]. All CCS patients received optimal therapy. Reperfusion of the infarct-related artery (IRA) has been achieved in all STEMI patients (the mean time of recanalization constituted 5 hours), and coronary angioplasty and IRA stenting were performed. Health status was evaluated and functional class of chronic heart failure was assessed according to the 6-minute walk test. Exclusion criteria were as follows: acute coronary events earlier than 6 months before the study (transitory ischemic attack, acute coronary syndrome, acute myocardial infarction); coronary artery bypass graft earlier than 6 months prior the study; obesity class III and higher (BMI > 40); confirmed symptomatic forms of arterial hypertension; severe comorbidity (hepatic, kidney failure, oncological diseases); refusal from participation in the study.

The peripheral blood mononuclear cells (PBMCs) were obtained from the fasting heparinized blood via centrifugation on density gradient (Histopaque 1077, Sigma Aldrich, USA). FoxP3⁺T-regulatory lymphocytes were identified among CD4⁺-cells by high CD25 expression and detected intracellular transcription factor FoxP3. FoxP3⁺T-conventional lymphocytes were identified among CD4⁺-cells by low CD25 expression and detected intracellular transcription factor FoxP3. Monoclonal antibodies used were as follows: FITC-anti-CD4, APC-anti-CD25, PE-anti-FoxP3 (BD eBiosciences, USA).

After the surface staining cells were fixed and permeabilized with specific buffer solutions for detection of FoxP3 (BD eBiosciences, USA). Next, cells were incubated with PE-anti-FoxP3 antibodies. After the final wash and fixation cells were incubated with 7-Amino-Actinomycin D (7-AAD) for nuclear staining. Samples were analyzed on imaging flow cytometer AMNIS FlowSight using INSPIRE software (MD Millipore, Seattle, USA). The Nuclear Localization Wizzard was used for CD4⁺CD25^{high} and CD4⁺CD25^{lo} subsets to calculate Similarity Morphology between PE-anti-FoxP3 and 7-AAD which corresponded to the frequency of Treg and Tconv-lymphocytes with nuclear FoxP3 localization. The absolute count of FoxP3⁺-cells was calculated by using the hemocytometer data.

Statistical analysis was performed by using Statistica 10 ("StatSoft" Inc., USA) software. The Mann–Witney U-test was used to estimate the differences between independent groups. Wilcoxon matched pairs signed rank test was used to evaluate the differences between dependent groups. Spearman's rank correlation coefficient (r) was used to estimate statistical significance between variables. Difference between groups were considered significant at level of p value below 0.05.

Results and discussion

STEMI patients on day 1 post-MI were characterized by insignificantly decreased frequency of Treg-lymphocytes and insignificantly elevated frequency of Tconv-lymphocytes compared to CCS patients (Table 1). Meanwhile, the level of FoxP3 nuclear translocation was lower both in Treg and Tconv-lymphocytes in STEMI patients on day 1 post-MI compared to CCS patients (Table 1).

We performed consecutive comparisons between the STEMI and CCS patients regarding the FoxP3⁺-lymphocytes' frequency and the level of FoxP3 nuclear translocation during the patient follow-up. Frequency of Treg-cells insignificantly increased on day 3, while frequency of Tconv-lymphocytes insignificantly decreased (Table 1). The difference of FoxP3 nuclear translocation between STEMI and

CCS patients remained on day 3, but disappeared on day 7 (Table 1).

In STEMI patients we revealed neither differences in FoxP3⁺-cells frequency nor at the level of FoxP3 nuclear translocation at different time points which might be due to insufficient number of patients in STEMI group.

We calculated an absolute number of FoxP3⁺T-lymphocytes and T-lymphocytes with FoxP3⁺ nuclear translocation by using hemocytometer data. STEMI patients were characterized by decreased count of FoxP3⁺Treg-lymphocytes compared to CCS patients, continued to further decrease on day 7 after infarction (Table 2). FoxP3⁺Tconv-lymphocytes' counts also tended to insignificantly decrease (Table 2). The absolute count of cells with FoxP3 nuclear localization insignificantly increased by day 7 after STEMI. But

TABLE 1. SUBSETS OF FoxP3⁺T-LYMPHOCYTES AND FoxP3 NUCLEAR TRANSLOCATION IN PATIENTS WITH CHRONIC AND ACUTE CORONARY SYNDROMES

Parameters	Patients with chronic coronary syndrome (n = 14)	Patients with acute coronary syndrome (n = 5)		
		day 1	day 3	day 7
FoxP3 ⁺ T-regulatory lymphocytes (Treg), %	7.2 (6.2-8.4)	6.7 (3.8-7.0) p = 0.297	7.7 (5.3-7.8) p = 0.687	7.0 (4.9-7.4) p = 0.444
FoxP3 ⁺ T-conventional lymphocytes (Tconv), %	1.6 (1.3-1.8)	2.1 (1.0-2.8) p = 0.754	2.6 (1.7-2.8) p = 0.186	1.5 (1.4-2.3) p = 0.893
Treg with nuclear FoxP3 localization, %	98.2 (96.8-98.7)	74.8 (64.9-92.9) p = 0.026	82.3 (54.7-95.2) p = 0.007	92.9 (92.6-95.7) p = 0.056
Tconv with nuclear FoxP3 localization, %	88.3 (73.1-96.9)	58.7 (33.9-67.7) p = 0.034	63.6 (17.4-77.3) p = 0.026	72.2 (67.5-79.4) p = 0.087

Note. The frequency of Treg and Tconv-lymphocytes is represented as percentage of all CD4⁺-lymphocytes; the frequency of lymphocytes with FoxP3 nuclear localization is represented as percentage of all FoxP3⁺-lymphocytes; p indicates the level of significance compared to the group of patients with chronic coronary syndrome.

TABLE 2. ABSOLUTE COUNTS OF FoxP3⁺T-LYMPHOCYTES AND T LYMPHOCYTES WITH FoxP3⁺ NUCLEAR TRANSLOCATION IN STEMI PATIENTS AT DIFFERENT TIME POINTS

Parameters	CCS patients	STEMI patients		p
		day 1	day 7	
FoxP3 ⁺ T-regulatory lymphocytes (Treg), × 10 ⁷ /l	7.79 (5.34-9.48)	4.64 (2.49-4.83)	3.21 (2.21-3.65)	p ₁ = 0.056 p ₇ = 0.007 p ₁₋₇ = 0.043
FoxP3 ⁺ T-conventional lymphocytes (Tconv), × 10 ⁷ /l	1.50 (0.74-2.54)	1.46 (1.02-1.76)	1.01 (0.72-1.07)	p ₁ = 0.687 p ₇ = 0.156 p ₁₋₇ = 0.225
Treg with nuclear FoxP3 localization, × 10 ⁷ /l	7.35 (5.29-9.41)	1.00 (0.07-3.47)	3.12 (1.71-3.38)	p ₁ = 0.007 p ₇ = 0.010 p ₁₋₇ = 0.893
Tconv with nuclear FoxP3 localization, × 10 ⁷ /l	0.98 (0.57-2.46)	0.28 (0.03-0.99)	0.60 (0.52-0.73)	p ₁ = 0.129 p ₇ = 0.391 p ₁₋₇ = 0.225

Note. p₁ indicates the level of significance between CCS patients and STEMI patients at day 1; p₇ indicates the level of significance between CCS patients and STEMI patients at day 7; p₁₋₇ indicates the level of significance between ATEMI patients at day 1 and day 7.

absolute counts of FoxP3⁺Tregs with nuclear FoxP3 localization remained significantly lowered both on day 1 and day 7 post-STEMI compared to patients with CCS (Table 2).

According to the data of the correlation analysis, absolute count of the FoxP3⁺Treg-lymphocytes on day 1 post-infarction inversely correlated with the end-systolic and end-diastolic volumes of the left ventricle both on days 3 and 7 of patient follow-up ($r_s = -0.900$; $p = 0.037$ for each correlation).

In our study we have revealed decreased absolute count of Tregs on day 1 post-STEMI compared to the CCS patients, which decreased even further on day 7, without significant change in their frequencies. Earlier, it was shown that Tregs are recruited into the heart as early as within the first day following the onset, and persist for up to 2 weeks [1]. We suppose that such changes in the absolute Treg numbers in our study are associated with their redistribution from the periphery to myocardial tissue, which leads to no changes in Treg frequencies. However, Banasal S.S. et al. (2019) found expression of pro-inflammatory cytokines and dysfunctional characteristics in expanded Tregs after AMI. Of note, FoxP3⁺Tconv-cells and FoxP3 subcellular localization were not examined in this study [1]. It is possible that increased proinflammatory Tregs in the abovementioned study may be due to expansion of activated FoxP3⁺Tconv-lymphocytes. The fact that echocardiographic features of the unfavorable left ventricle remodeling inversely correlated with absolute count of Treg-lymphocytes supports an existence of pro-resolving protective functions for genuine FoxP3⁺Treg subpopulation.

To our knowledge, our study is the first to evaluate the level of FoxP3 nuclear translocation both in Treg and Tconv-lymphocytes in patients after STEMI compared to CCS patients and during the 7-day follow-up. We found decreased FoxP3 nuclear translocation on day 1 after STEMI compared to CCS patients that disappeared by day 7 after the onset.

According to Magg T. et al. (2012) the nuclear translocation of FoxP3 may also be affected by the cell maturity. Thus, naïve CD45RA⁺-cells predominantly expressed FoxP3 in the nucleus, whereas memory CD45RA⁻-cells were characterized by the cytoplasmic FoxP3 localization [6]. Meanwhile, another study showed decreased ratio between the naïve and memory Treg-cells in non-ST-elevation myocardial infarction patients, with memory Tregs displaying impaired suppressive properties [9]. We may assume that such differences in FoxP3 nuclear localization both in Tregs and Tconv-lymphocytes may be accounted for not by the changes of its translocation level, but rather due to high number of memory T-lymphocytes entered the circulation at the onset of myocardial infarction. Also, existing FoxP3 isoforms lacking nuclear export sequences and tending to be restricted to nucleus has been described [6]. In particular, low expression of FOXP3Δ2 was associated with atherosclerotic plaque instability [5]. The possibility of FoxP3 alternative splicing in the course of STEMI should not be excluded. The question remains, whether Treg-lymphocytes with FoxP3⁺ nuclear localization post-STEMI preserve their suppressive functions? It has been shown that interaction of FoxP3 with nuclear galectin-1 may interfere with its binding to DNA and abrogating FoxP3 suppressive activity [2].

Thus, in the present study we demonstrated that FoxP3 nuclear localization dampens in the course of STEMI and may be a more sensitive marker of altered Treg-lymphocyte functioning compared to assessing Treg frequency. Novel approaches in flow cytometry allow rather low labor-consuming approaches to evaluate this parameter. The data obtained also reveal an importance to take into consideration existence of the FoxP3⁺ conventional T-lymphocyte subset. Further studies on the kinetics of FoxP3⁺T-cells redistribution in humans and its impact on the course of the disease are required.

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КОРРЕЛЯЦИОННЫЙ АНАЛИЗ МЕЖДУ ЛАБОРАТОРНЫМИ ИММУННЫМИ И МЕТАБОЛИЧЕСКИМИ ПАРАМЕТРАМИ ПРИ ХРОНИЧЕСКОЙ ИШЕМИИ МОЗГА I И II СТАДИИ НА ФОНЕ ГИПЕРТОНИЧЕСКОЙ БОЛЕЗНИ ПОСЛЕ ЛЕЧЕНИЯ

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Резюме. Проведено определение взаимосвязей между нарушениями лабораторных показателей иммунного и метаболического статуса у пациентов с хронической ишемией головного мозга I-II стадии. В исследование было включено 104 пациента, из которых 76 больных женского пола и 28 мужского, с ХИМ на фоне гипертонической болезни II степени, из которых 52 больных были с I стадией и 52 со II стадией в возрасте 50 ± 5 лет. Кроме того, изучены клинические и лабораторные показатели у 22 здоровых доноров того же возраста. Оценку клинико-лабораторных данных осуществляли в начале лечения и через 2 недели после его окончания. Определяли в плазме крови и эритроцитах сорбционную способность эритроцитов и сорбционную емкость гликокаликса (СЕГ), активность процессов перекисного окисления липидов, состояние антиоксидантной системы, выявляли уровень стабильных метаболитов оксида азота (SMNO), неоптерина, С-реактивного белка, цитокинов (TNF α , IL-1 β , IL-8, IFN γ , IL-18, G-CSF, IL-4, IL-10), иммуноглобулинов (IgM, IgG, IgA), компонентов системы комплемента (C3, C4, C5, C5a), фагоцитарную и кислородзависимую активность полиморфноядерных лейкоцитов крови. Проводя сравнительную оценку результатов корреляционного, факторного и кластерного анализов оценки показателей иммунного и метаболического статуса у пациентов с ХИМ I стадии выявлены наиболее значимые лабораторные параметры, необходимые для определения в клинике с целью объективной оценки выраженности иммунных и метаболических нарушений: TNF α , IL-8, IL-10, SMNO и СЕГ. У больных ХИМ II стадии с целью объективной оценки выраженности иммунных и метаболических нарушений рекомендованы TNF α , IL-8, IL-17, IL-10, фагоцитарное число нейтрофилов и СЕГ.

Ключевые слова: хроническая ишемия мозга, взаимосвязи параметров иммунометаболического статуса

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CORRELATION ANALYSIS BETWEEN LABORATORY IMMUNE AND METABOLIC PARAMETERS IN CHRONIC BRAIN ISCHEMIA STAGES I AND II ALONG WITH HYPERTENSION AFTER TREATMENT

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Abstract. Determination of interrelationships between impairments of laboratory parameters of immune and metabolic status in patients with chronic cerebral ischemia of I-II stages was carried out. The study included 104 patients, of which 76 were female and 28 males, with CCI on the background of II degree hypertension, of which 52 patients were with stage I and 52 with stage II at the age of 50 ± 5 years. Also, clinical and laboratory parameters were studied in 22 healthy donors of the same age. Evaluation of clinical and laboratory data was carried out at the beginning of treatment and 2 weeks after its end. The sorption capacity of erythrocytes and the sorption capacity of the glycocalyx (SEG), the activity of lipid peroxidation processes, the state of the antioxidant system were determined in blood plasma and erythrocytes, the level of stable metabolites of nitric oxide (SMNO), neopterin, C-reactive protein, cytokines (TNF α , IL-1 β , IL-8, IFN γ , IL-18, G-CSF, IL-4, IL-10), immunoglobulins (IgM, IgG, IgA), complement system components (C3, C4, C5, C5a), the phagocytic and oxygen-dependent activity of polymorphonuclear blood leukocytes. Comparative assessment of the results of correlation, factorial and cluster analyzes for assessing the parameters of the immune and metabolic status in patients with stage I CCI revealed the most significant laboratory parameters necessary for determination in the clinic for objective assessment of the severity of immune and metabolic disorders: TNF α , IL-8, IL-10, SMNO and NEG. In patients with CCI stage II, to objectively assess the severity of immune and metabolic disorders, TNF α , IL-8, IL-17, IL-10, the phagocytic number of neutrophils and SEG are recommended.

Keywords: chronic cerebral ischemia, interrelationships of parameters of immunometabolic status

Introduction

In the world and in Russia, cerebrovascular disease (CEH), ranked second in the structure of mortality from circulatory diseases and total mortality, and the prevalence of chronic cerebral ischemia (CHEM) is 60-75% of all CEH. Now up to 10-15% of the population is over 65 years old, it is predicted that in 20 years this figure will approach 20-25% and the number of people over 80 years old will grow most rapidly, and their number may increase 3 times in the next decade. Since 2000, there has been a steady trend towards "rejuvenation" of the population of patients with various types of CVD, which is associated with a steady increase in the impact of adverse external factors and insufficient effectiveness of prevention programs for socially significant diseases, primarily for atherosclerosis and arterial hypertension [1, 3, 4].

Materials and methods

The relationship between violations of laboratory indicators of immune and metabolic status in patients with stage I-II chronic brain ischemia during treatment was determined.

The study included 104 patients, 76 female and 28 male patients, who formed the main group of hospitalized in the neurological Department of the Kursk regional clinical hospital, with correcting level of blood pressure, with CHEM against the background of grade II hypertension, of which 52 patients were stage I and 52 with stage II at the age of 50 ± 5 years.

All patients during their stay in the hospital for 14 days received daily basic pharmacological therapy: an angiotensin-converting enzyme inhibitor – enalapril maleate (Berlipril) (supportive hypotensive therapy) and a vasoactive drug – Vinpocetin (Cavinton).

All patients with CHEM stage I and II were equally divided into 4 groups: within 14 days, all patients of the first groups received Ceretone and Actovegin, 2-4 groups – Cerakson and Mexicor. Patients of the third and fourth groups additionally received immunomodulatory drugs, respectively, Glutoxim and Polyoxidonium.

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The criteria for inclusion in the main group were: age from 40 to 60 years; absence of concomitant diseases in the acute stage, presence of CHEM against the background of grade II, stage II hypertension, risk 2, diagnosed 5 or more years ago in accordance with the recommendations of the world health organization and the International society for arterial hypertension (MOG, 1999).

The exclusion criteria were: symptomatic arterial hypertension; severe or moderate atherosclerotic changes in the fundus vessels; heart rhythm and conduction disorders; chronic heart failure of more than II FC in accordance with the classification of the new York heart Association (NYHA); hemodynamically significant stenoses of brachiocephalic and cerebral vessels, heart defects; myocardial infarction, postinfarction cardiosclerosis and progressive angina or indications thereof a history of diabetes or impaired glucose tolerance.

All patients underwent a comprehensive clinical and instrumental examination according to generally accepted standards, and in all cases, the diagnosis of stage I and II CHEM was verified. The treatment followed the principles of evidence-based medicine.

Evaluation of clinical and laboratory data in groups with CHEM was performed at the beginning of treatment and 2 weeks after its end. From 10 ml of heparinized blood, plasma and red blood cell mass were obtained by centrifugation, with which the sorption capacity of red blood cells (SSE) and the sorption capacity of glycocalyx (SEG) were immediately determined.

The intensity of lipid peroxidation processes was assessed by the content of acyl hydroperoxides and malondialdehyde (MDA and AGP) in blood plasma and erythrocytes.

The state of the antioxidant system was determined by direct/competitive solid-phase enzyme immunoassay using ready-made commercial kits: the activity of superoxide dismutase (SOD) “Bender

MedSystems” (Austria) and catalase “Cayman Chemical” (USA). Total antioxidant activity (OAA) was detected by a method based on the degree of inhibition of ascorbate – and ferroinduced oxidation of twin-80 to MDA. The level of stable metabolites of nitric oxide (SMNO) was detected using a set for solid-phase enzyme immunoassay by R&D (England).

In addition, plasma levels of neopterin “IBL” (Germany), endothelin-1 “Biomedica” (Slovakia) and erythropoietin “Biomerica” (USA) were detected by enzyme immunoassay. Ceruloplasmin (CP) was determined by immunoturbidimetry using the sentinel kit (Spain), and C-reactive protein (CRP) using the Vector-best kit (Russia) using the BTS-350 semi-automatic analyzer (BioSystems, Spain).

Cytokines (TNF α , IL-1 β , IL-6, IL-8, IFN γ , IL-2, IL-17, IL-18, G-CSF, IL-4, IL-10, IL-1ra) were detected by solid-phase enzyme immunoassay using sets of Vector – Best CJSK (Russia), components of the complement system (C3, C3a, C4, C5, C5a) and factor H-diagnostic set of Cytokine LLC (Russia). The activity of the C1-inhibitor was determined by chromogenic method based on the ability to inhibit C1-esterase. Registration of all the results of enzyme immunoassay was performed using a microplate photometer “Sunrise”, Tecan (Austria).

The phagocytic activity of polymorphonuclear blood leukocytes after their isolation from the blood on the phycoll-urographin density gradient ($d = 1.077$) was evaluated by determining the phagocytic index, phagocytic number, and phagocytosis activity index. The activity of oxygen-dependent neutrophil systems was evaluated using a PD 303 SApel spectrophotometer (Japan) based on the nitroblue tetrazolium reduction reaction (NBT-test), spontaneous and stimulated by zymosan, the stimulation index and the functional reserve of neutrophils.

In statistical processing of the research results, the χ^2 (Chi-square) criterion was used to compare the qualitative parameters. The Shapiro–Wilk test was used to assess whether quantitative traits belong to a distribution type. The student’s t-test was used to compare normally distributed values. The statistical significance of differences in quantitative values with an abnormal distribution was assessed using the Mann–Whitney U-test and the Wilcoxon test (when comparing dependent groups). The values of normally distributed quantitative parameters are represented by the arithmetic mean (M) with the error of the arithmetic mean (m), and abnormally distributed ones are represented by the median (Me) with an interquartile interval ($Q_{0.25}$ – $Q_{0.75}$). Relationships were established based on factor analysis, cluster analysis, and Spearman’s rank correlation coefficient.

TABLE 1. COMPARATIVE EVALUATION OF CORRELATION AND FACTOR ANALYSIS AND CLUSTER ANALYSIS OF THE ASSESSMENT OF INDICATORS OF THE IMMUNE SYSTEM AND METABOLIC STATUS IN STAGE I CHEM

Indicators	Correlation analysis	Factoral analysis	Clustal analysis
TNF α	+	+	+
IL-1 β	+	+	
IL-6	+	+	+
IL-8	+	+	+
IL-17			
IL-18			
IL-4			
IL-10	+++	+	+
IL-1ra	++		
IFN γ			
IL-2	+++		
G-CSF			
C3	++		
C3a			+
C4			+
C5	+++		
C5a			
C ₁ -inhib.	++		+
Factor N	++++		+
FP			
FCH			
NBT-sp	+++		+
NBT-st.	+		
MDA		+	
AGP		+	
OAA			
SOD			
Catalase			
Cerulo-plasmin			
Neopterin			
Endothe-lin-1		+	
SRB		+	
SMNO	+	+	+
Erythro-poietin	+	+	
*MDA		+	
*AGP		+	
*OAA			
*SOD		+	
*Catalase		+	
*SMNO		+	+
SEG	+	+	+
SSJ		+	+

TABLE 2. COMPARATIVE EVALUATION OF CORRELATION AND FACTOR ANALYSIS AND CLUSTER ANALYSIS OF THE ASSESSMENT OF INDICATORS OF THE IMMUNE SYSTEM AND OXIDANT STATUS IN STAGE II CHEM

Indicators	Correlation analysis	Factoral analysis	Clustal analysis
TNF α	+	+	+
IL-1 β		+	
IL-6		+	
IL-8	+	+	+
IL-17	++	+	+
IL-18		+	
IL-4	+		
IL-10	+++	+	+
IL-1ra			
IFN γ	+++		
IL-2	+		
G-CSF			
C3	++		
C3a			+
C4			
C5			
C5a	+		
C ₁ -ing.			
Factor H	++		+
FP			
FCH	+++	+	+
NBT-sp.	++		
NBT-st.			+
MDA		+	
AGP		+	
OAA			
SOD		+	
Catalase			
Cerulo-plasmin			
Neopterin			
Endothe-lin-1		+	
SRB		+	
SMNO			
Erythro-poietin		+	
*MDA		+	
*AGP		+	
*OAA			
*SOD		+	
*Catalase		+	
*SMNO	+		
SEG	+	+	+
SSJ		+	

Differences were considered statistically significant at $p < 0.05$.

Results and discussion

To identify independent factors in the maintenance of immune and metabolic homeostasis in the studied groups of patients with CHEM, we conducted a factor analysis.

In stage I and II CHEM, the presence of approximately the same factors in importance in each of the groups of patients takes place.

In patients with stage I, the most significant are at the systemic level – TNF α , IL-1 β , IL-6, IL-8, IL-10, MDA, AGP, endothelin-1, CRP, SMNO, erythropoietin, and within red blood cells-MDA, AGP, SOD, catalase, SMNO, SEG, and CSE.

In patients with stage II of the disease, according to factor analysis, the most significant indicators are the following in the blood – TNF α , IL-1 β , IL-6, IL-8, IL-17, IL-18, IL-10, FH, AGP, SOD, endothelin-1, CRP, erythropoietin, inside red blood cells – MDA, AGP, SOD, SEG and CSE.

A certain proof of integrative processes between laboratory status terms is the presence of reliable correlations between them [2, 5, 6]. After the correlation analysis between the components of the immune and metabolic status in patients with stage I and II CHEM, we analyzed the types of connections: intra-system and intersystem.

The control group documented the presence of 73 reliable correlations, 32 of which are intrasystem, and 41 – between-system. In patients with CHEM I, there are 53 such connections (23 and 30, respectively). In the

group of patients CHEM stage I receiving Cereton and Actovegin, such relations 54 (22 and 32 respectively), patients after application Ceraxon and Mexicor such ties 59 (25 and 34 respectively), in patients treated with Ceraxon, Mexico and Glutoxim – 70 (28 and 42 respectively) and in patients who were administered Ceraxon, Mexicor and Polyoxidonium, the maximum observed number of such relationships – 76 (31 and 45 respectively).

The use of cluster analysis revealed the grouping of a number of indicators of the immune status and metabolic link in patients with stage I and II CHEM. Thus, patients of the first and second groups have 4 grouped clusters of indicators, while there are some significant differences in the presented indicators within each of the clusters in groups with CHEM I and CHEM II.

The constructed dendrograms clearly indicate a pronounced maladaptation of the mechanisms for maintaining immune and metabolic homeostasis in the early and later stages of the disease.

By conducting a comparative assessment of the results of correlation, factor and cluster analyses of the assessment of immune and metabolic status indicators in patients with stage I CHEM, it is possible to identify the most significant indicators of immunometabolic status necessary for determining the clinical severity of immune and metabolic disorders: TNF α , IL-8, IL-10, SMNO and SEG (Table 1).

In patients with stage II CHEM, the following parameters can be recommended for determination in the clinic in order to objectively assess the severity of immune and metabolic disorders: TNF α , IL-8, IL-17, IL-10, FH and SEG (Table 2).

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ПАРАМЕТРЫ КЛЕТОЧНОГО ИММУНИТЕТА У БОЛЬНЫХ РЕМИТТИРУЮЩЕЙ ФОРМОЙ РАССЕЯННОГО СКЛЕРОЗА

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Резюме. Целью настоящей работы стало изучение некоторых параметров клеточного иммунитета у больных рассеянным склерозом (РС). В исследование были включены 10 пациентов с ремиттирующей формой РС в возрасте от 32 до 50 лет. Диагноз был установлен клинически и подтвержден магнитно-резонансным исследованием. Пациенты не получали иммуносупрессивную терапию в течение по крайней мере 6 месяцев до начала исследования. Неврологический статус всех обследованных пациентов оценивался по функциональной шкале Куртцке с использованием расширенной шкалы инвалидизации (EDSS) и составил в среднем $4,0 \pm 0,67$ балла. Среднее число обострений в год было $1,25 \pm 0,25$. При исследовании таких параметров иммунного статуса, как количество Т-, В-, НК-клеток, содержание иммуноглобулинов, фагоцитарная активность моноцитов и гранулоцитов, продукция ими активных видов кислорода, у пациентов с РС существенных различий в сравнении с нормальным донорским уровнем не наблюдалось. Вместе с тем, нами было отмечено усиление пролиферативного ответа мононуклеарных клеток крови на миелиновый антиген в 2,35 раза. Содержание $CD4^+CD45RO^+CD62L^+$ и $CD8^+CD45RO^+CD62L^+$ центральных Т-клеток памяти, а также $CD8^+CD45RO^+CD62L^-$ эффекторных Т-клеток памяти в крови больных РС значительно превышало контрольные значения ($p < 0,05$). Также у больных РС, по сравнению со здоровыми лицами, имело место повышенное содержание наивных $CD4^+CD45RO^-$ и $CD8^+CD45RO^-$ Т-клеток, несущих $IFN\gamma$ ($p < 0,01$), и увеличение $CD4^+CD45RO^+$ и $CD8^+CD45RO^+$ Т-клеток памяти, продуцирующих в ответ на активацию $IFN\gamma$ или $IFN\gamma$ вместе с IL-4 ($p < 0,01$). С этими данными согласуется значительное увеличение сывороточных уровней $IFN\gamma$ и IL-17 и отсутствие изменений уровня IL-4. Относительное содержание «наивных» $CD4^+CD25^+FoxP3^+$, а также индуцированных $CD4^+CD25^+FoxP3^+$ регуляторных Т-клеток у больных РС существенно не изменялось по сравнению с донорскими значениями. Результаты оценки некоторых показателей иммунного статуса у больных РС свидетельствуют о функциональной перестройке иммунной системы в сторону Th1 типа иммунного ответа. Очевидно, что иммуноотропное лечение РС должно быть направлено на инактивацию аутоиммунных Т- и В-лимфоцитов, подавление продукции провоспалительных медиаторов и усиление активности естественных и индуцированных регуляторных Т-клеток.

Ключевые слова: клеточный иммунитет, Т-лимфоциты, цитокины, клетки памяти, рассеянный склероз, Т-клеточная вакцина

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CELLULAR IMMUNITY PARAMETERS IN PATIENTS WITH REMITTING MULTIPLE SCLEROSIS

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Abstract. The aim of this work was to study some parameters of cellular immunity in patients with multiple sclerosis (MS). The study included 10 patients with relapsing-remitting MS aged 32 to 50 years. Diagnosis was clinically established and confirmed by magnetic resonance imaging. Patients did not receive immunosuppressive therapy for at least 6 months prior to study entry. The neurological status of all examined patients was assessed using the Kurtzke functional scale using the Extended Disability Scale (EDSS) and averaged 4.0 ± 0.67 points, the mean number of exacerbations per year was 1.25 ± 0.25 . While studying such parameters of the immune status such as the number of T, B, NK-cells, the content of immunoglobulins, the phagocytic activity of monocytes and granulocytes, their production of reactive oxygen species, no significant differences were observed in patients with MS in comparison with the normal donor level. At the same time, we have noted an increase in the proliferative response of mononuclear blood cells to myelin antigen by 2.35 times. The content of $CD4^+CD45RO^+CD62L^+$ and $CD8^+CD45RO^+CD62L^+$ central memory T-cells, as well as $CD8^+CD45RO^+CD62L^-$ effector memory T-cells in the blood of MS patients significantly exceeded the control values ($p < 0.05$). Also, in MS patients, compared with healthy individuals, there was an increased level of naive $IFN\gamma$ -positive $CD4^+CD45RO^-$ and $CD8^+CD45RO^-$ T-cells ($p < 0.01$), and an increase in $CD4^+CD45RO^+$ and $CD8^+CD45RO^+$ memory T-cells producing $IFN\gamma$ or $IFN\gamma$ together with IL-4 in response to the activation ($p < 0.01$). Consistent with these data, there were significantly increased serum $IFN\gamma$ and IL-17 levels and no changes in IL-4 levels. The relative level of naive $CD4^+CD25^+FoxP3^+$, as well as induced $CD4^+CD25^+FoxP3^+$ regulatory T-cells in MS patients did not significantly change compared to donor values. The results of assessing some parameters of the immune status in MS patients indicate a functional reshaping of the immune system towards the Th1 type of immune response. It is obvious that immunotropic treatment of MS should be aimed at inactivating auto-immune T- and B-lymphocytes, suppressing the production of proinflammatory mediators, and enhancing the activity of natural and induced regulatory T-cells.

Keywords: cellular immunity, T-cells, cytokines, memory cells, multiple sclerosis, T-cell vaccine

Introduction

Multiple sclerosis is a neurodegenerative disease with an autoimmune pathogenesis. In 90% of cases, disease onset proceeds in a remitting form, so that each exacerbation aggravates severity of neurological symptoms. Over time, multiple sclerosis acquires a secondary progressive course in about 50% of cases. Primary progressive disease course occurs in about 10% of patients.

A great number of experimental and clinical studies were dedicated to MS pathogenesis and treatment and indicate that immune system plays a dominant role in its pathogenesis. [1]. The ability of the human immune system to respond to vast array of antigens poses a risk that some T-cells might recognize host own antigens, such as myelin antigens of the central nervous system (CNS). Most autoreactive T-lymphocytes are eliminated in the thymus via negative selection (central tolerance). However, some of these T-cells escape from thymus to peripheral sites usually being controlled by peripheral

tolerance mechanisms. If case such mechanisms fail it might be due to weakened regulatory T-cells activity and/or increased effector T and B-cell resistance to suppression, autoreactive T-cells may be activated and become a sufficient cause for developing autoimmune disease [1, 5]. It was shown, that CNS in MS patients was infiltrated by activated T-cells, B-cells, plasma cells, dendritic cells and macrophages suggesting that both cellular and humoral immune responses, as well as various immunopathological effector mechanisms contribute to tissue damage. Type 1 $CD4^+$ -helper T (Th1) cells produce cytokines ($IFN\gamma$, $TNF\alpha$, IL-2) resulting in recruitment of resident macrophages and effector cytotoxic cells to inflammation site, secretion of proinflammatory cytokines, enhanced activity of antigen-presenting cells, as well as increased production of reactive oxygen and nitrogen species creating favourable conditions for developing inflammation [8]. Type 17 $CD4^+$ -helper T-cells (Th17), producing IL-17 and IL-22, increase permeability of blood-brain barrier, activate myelin-reactive T- and B-cells. In addition

to releasing proinflammatory mediators, CD8⁺T-cells directly attack oligodendrocytes and neurons, causing their death through secreted granzymes and perforin involved in pore formation and triggering programmed cell death [1]. This process leads to loss of myelin and damage to axons. It should be noted that entry of epitopes into the bloodstream may lead to a broader autoimmune response involving additional autoantigens [4, 6, 10].

Modern methods of treatment for multiple sclerosis are based on long-term immunosuppressants and disease-modifying drugs, which lower rate of relapses and severity of inflammation in the central nervous system. They decrease activity of B and T-cells as well as blood-brain barrier permeability. Obviously, it is also necessary to develop approaches for specific disease suppression not associated with systemic suppression of the immune system, but selectively suppressed immune responses to host own antigens [14]. The aim of this study was to examine some parameters of cellular immunity in patients with multiple sclerosis in order to search for and develop new methods of specific therapy and assess efficacy.

Materials and methods

The study was carried out within the framework of the Exploratory Scientific Research, approved by the Scientific Council and the Ethics Committee of the Federal State Budgetary Scientific Institution Research Institute of Fundamental and Clinical Immunology, Novosibirsk. The study included 10 patients with relapsing-remitting MS aged 32 to 50 years. Informed consent was obtained from each patient. The diagnosis was made clinically and confirmed by magnetic resonance imaging. Patients received no immunosuppressive therapy for at least 6 months prior to study entry. The neurological status of all examined patients was assessed using the Kurtzke functional scale, the Extended Disability Scale (EDSS) that averaged 4.0 ± 0.67 points, the average annual number of exacerbations was 1.25 ± 0.25 . The immune status of patients (count of CD4⁺CD3⁺CD45⁺ and CD8⁺CD3⁺CD45⁺T-cells, CD16⁺CD56⁺CD45⁺NK-cells, CD19⁺CD45⁺B-lymphocytes, serum immunoglobulins, phagocytic tests) was assessed in the clinical and immunological laboratory of the Institute by using standard methods.

To assess the antigen-induced proliferative response, PBMC were cultured in a 96-well plate at a concentration of 2×10^5 cells in complete culture medium RPMI 1640, with/without myelin basic protein (MBP) for 5 days in a humid atmosphere with 5% CO₂. Cell proliferation was evaluated by [³H] thymidine incorporation.

The relative number of memory cells was determined by flow cytofluorimetry using phycoerythrin (PE)-labeled monoclonal antibodies (MA) LT4 (CD4)

and LT8 (CD8) (Sorbent, Moscow) conjugated with fluorescein isothiocyanate (FITC) MA to CD45RO (eBioscience, USA) labeled with allophycocyanin (APC) anti-CD62L MA (eBioscience, USA). The following cell populations were determined based on surface marker staining: naive cells (CD45RO⁻CD62L⁺), central memory cells (CD45RO⁺CD62L⁺), effector memory cells (CD45RO⁺CD62L⁻). The data are depicted as the percentage of each population out of total lymphocyte count.

Surface markers of regulatory (CD4⁺CD25⁺FoxP3⁺) cells were determined by using anti-CD4 MA labeled with APC; MA to IL-2 receptor (CD25) conjugated to FITC. FoxP3 expression was assessed by using PE-labeled MA (all reagents purchased from Becton Dickinson, USA). The percentage of positive cells was determined on a FACS Calibur immunocytometer.

The level of IFN γ and IL-4-producing lymphocytes was assessed by an intra-cellular cytokine staining in 4-hour PBMC cultures treated with 30 ng/ml phorbol ester, 1 μ g/ml ionomycin, and 1 μ g/ml brefeldin A (all ICN reagents, USA). After cultivation, the cells were treated with MA against surface markers CD4 and CD8 labeled with peridinin chlorophyll protein (PerCP) and conjugated with APC MA to CD45RO (eBioscience, USA), fixed, permeabilized and incubated with MA against IFN γ (FITC) and IL-4 (PE) (Becton Dickinson, USA). Next, the cells were washed and analyzed on a FACSCaliburTM flow cytometer (BD Biosciences, USA). Data are presented as the percentage of each cell population out of total lymphocyte count.

Serum cytokines IFN γ , IL-4, IL-17 were quantified by using the enzyme-linked immunosorbent assay using commercial kits (Vector-Best, Russia).

Results and discussion

We investigated parameters of immune status in MS patients and observed no significant differences compared to volunteers. T, B, NK-cell count, immunoglobulin level, phagocytic activity of monocytes and granulocytes as well as production of reactive oxygen species were within normal range (Table 1).

We noted proliferative response of MNCs to myelin antigen that increased by 2.35-fold, which may indicate at increased number or functional activity of myelin-reactive lymphocytes in patient blood samples.

The number of blood CD4⁺CD45RO⁺CD62L⁺ and CD8⁺CD45RO⁺CD62L⁺ central memory T-cells in MS patients, as well as CD8⁺CD45RO⁺CD62L⁻ effector memory T-cells were increased significantly. Control values ($p < 0.05$). Our studies indicate at accelerated differentiation of naive cells into central CD4⁺ and CD8⁺ memory T-cells and cytotoxic effector CD8⁺T-cells in MS. This effect promotes

TABLE 1. IMMUNOLOGICAL CHARACTERISTICS IN PATIENTS WITH REMITTING MULTIPLE SCLEROSIS, M±m

Immunological parameters	MS Patients
CD4 ⁺ CD3 ⁺ CD45 ⁺ T-cells, percentage of total lymphocytes	49.75±4.29
CD8 ⁺ CD3 ⁺ CD45 ⁺ T-cells, percentage of total lymphocytes	29.75±4.02
CD16 ⁺ CD56 ⁺ CD45 ⁺ NK-cells, percentage of total lymphocytes	7.00±0.91
CD19 ⁺ CD45 ⁺ B-lymphocytes, percentage of total lymphocytes	8.50±0.64
IgM serum	2.34±0.48
IgA serum	2.16±0.58
IgG serum	10.93±0.60
CIC level of circulating immune complexes in serum	27.50±10.04
Monocytes phagocytic, %	90.25±4.50
Granulocytes phagocytic, %	93.50±3.27
Monocytes producing reactive oxygen species, %	76.25±4.80
Granulocytes producing reactive oxygen species, %	91.25±3.12
Proliferative response of MNC from patients, cpm medium mielin	2306±466 5416±965
Serum cytokine content IFN γ , pg/ml IL-4, pg/ml IL-17, pg/ml	144.0±46.7 11.70±2.98 8.3±3.7
Memory T-cells CD4 ⁺ CD45RO ⁺ CD62L ⁺ CD4 ⁺ CD45RO ⁺ CD62L ⁻ CD4 ⁺ CD45RO ⁻ CD62L ⁺ CD8 ⁺ CD45RO ⁺ CD62L ⁺ CD8 ⁺ CD45RO ⁺ CD62L ⁻ CD8 ⁺ CD45RO ⁻ CD62L ⁺	4.89±0.56 7.64±1.38 6.55±0.54 0.37±0.10 1.28±0.30 4.67±1.37
Memory T-cells producing intracellular IFN γ и IL-4 CD4 ⁺ /CD45RO ⁺ /IFN γ ⁺ /IL-4 ⁻ CD4 ⁺ /CD45RO ⁺ /IFN γ ⁺ /IL-4 ⁺ CD4 ⁺ /CD45RO ⁺ /IFN γ ⁻ /IL-4 ⁺ CD4 ⁺ /CD45RO ⁻ /IFN γ ⁺ /IL-4 ⁻ CD4 ⁺ /CD45RO ⁻ /IFN γ ⁺ /IL-4 ⁺ CD4 ⁺ /CD45RO ⁻ /IFN γ ⁻ /IL-4 ⁺ CD8 ⁺ /CD45RO ⁺ /IFN γ ⁺ /IL-4 ⁻ CD8 ⁺ /CD45RO ⁺ /IFN γ ⁺ /IL-4 ⁺ CD8 ⁺ /CD45RO ⁺ /IFN γ ⁻ /IL-4 ⁺ CD8 ⁺ /CD45RO ⁻ /IFN γ ⁺ /IL-4 ⁻ CD8 ⁺ /CD45RO ⁻ /IFN γ ⁺ /IL-4 ⁺ CD8 ⁺ /CD45RO ⁻ /IFN γ ⁻ /IL-4 ⁺ CD8 ⁺ /CD45RO ⁻ /IFN γ ⁻ /IL-4 ⁻	8.60±3.64 2.68±1.09 8.69±4.91 1.93±0.90 0.96±0.29 0.48±0.22 11.10±4.23 4.39±2.48 1.47±0.84 7.48±2.09 1.43±0.56 0.74±0.25

antigen-specific T-cell expansion in response to repeated, systemic antigen effects [12].

It is believed that IFN γ -producing T-cells contribute to developing MS, whereas IL-4 production by T-cells may have a neuroprotective effect [7, 13]. We assessed blood T-cell count capable of producing IFN γ and/or IL-4. The data are presented in the Table 1 indicating that count of naive CD4⁺CD45RO⁻

and CD8⁺CD45RO⁻T-cells carrying IFN γ ($p < 0.01$) and CD4⁺CD45RO⁺ and CD8⁺CD45RO⁺ memory T-cells, producing IFN γ with/without IL-4 activated in MS patients vs. healthy individuals was significantly increased ($p < 0.01$). It indicates at systemic skewing of T-cell function towards pro-inflammatory immune responses.

These data show consistently a significantly increased serum levels of IFN γ and IL-17 without affecting IL-4 levels, which also indicates a shift towards dominance of Th1 immune reaction, which is a prerequisite for triggering specific autoimmune response.

A body of evidence on relative number of regulatory T-cells showed that the count of naive CD4⁺CD25⁺FoxP3⁺ (2.58 ± 1.22), as well as induced CD4⁺ CD25⁺FoxP3⁺ (14.13 ± 5.89) T-cells in MS patients did not change significantly compared to donor values (2.72 ± 1.08 and 17.4 ± 5.9 , respectively).

Assessing some parameters of T-cell immunity in patients with multiple sclerosis indicates a functionally skewed immune system towards Th1 type immune response. It is believed that autoimmune T-helper (Th)-1 and Th17-cells specific for myelin-associated antigens play a major role in MS pathogenesis, regardless of initial triggering event [11]. Activated encephalitogenic T-cells invade the CNS, and, by producing pro-inflammatory cytokine recruit macrophages, CD8⁺T-cells and NK-cells in nervous tissue-destructive processes [7]. It is assumed that activation of CD4⁺T-cells (including memory cells) is associated with MS exacerbation, whereas activation of CD8⁺T-cells reflect systemic immunological dysregulation in MS patients [2, 12]. IFN γ , TNF α and perforin produced by CD8⁺T-cells are involved in macrophages activation and maintenance of chronic inflammation as well as demyelinating process.

It is obvious that immunotropic treatment of MS should be aimed at inactivating auto-immune T and B

lymphocytes, suppressing proinflammatory mediator production, and enhancing activity of natural and induced regulatory T-cells. One of the most promising approaches for pathogenetic treatment of multiple sclerosis is based on vaccination with inactivated autoimmune myelin-reactive T-lymphocytes. Such an immunization leads to generation of anticolonotypic cytolytic CD8⁺T-cells specifically recognizing idiotypic T-cell receptor structures involved in the autoimmune process [9]. Moreover, T-cell vaccination causes the generation of anti-clonotypic and anti-ergotypic CD4⁺T-cells, producing anti-inflammatory cytokines IL-4 and IL-10 in activated state, thus preventing the development of the tissue destructive process. Also, T-cell vaccination stimulates functional activity of regulatory CD4⁺CD25⁺T-cells, induces anti-ergo-typical T-cell response and production of anti-idiotypic antibodies [15]. In our institute (NIIFKI), there was developed a new two-stage technology for producing a T-cell vaccine (TCV): i) cultured antigen-specific selection of the patient T-cells, and ii) T-cell growth for propagating cell number via nonspecific stimulation. A final vaccine is dominated by the most autoreactive T-cells, since in the presence of a complex of neuronal antigens, these cells receive the peak growth advantages *in vitro*. It implies that anti-idiotypic immune response induced by the polyclonal T-cell vaccine should primarily target those antigen-reactive cells mostly involved in the pathological process. We believe that T-cell vaccination can produce tangible results, especially in the early stages of the disease.

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ИССЛЕДОВАНИЕ ЦИТОКИНСИНТЕЗИРУЮЩЕЙ ФУНКЦИИ МОНОНУКЛЕАРНЫХ КЛЕТОК КРОВИ БОЛЬНЫХ РАССЕЯННЫМ СКЛЕРОЗОМ ПОД ДЕЙСТВИЕМ ОЛИГОПЕПТИДА КОНСЕРВАТИВНОГО РЕГИОНА ЭНДОГЕННОГО РЕТРОВИРУСА HERV-E λ 4-1

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Резюме. Учитывая данные об ассоциации эндогенного ретровируса человека I класса субгруппы HERV-E λ 4-1 с рассеянным склерозом, аутоиммунным заболеванием, сопровождающимся нейровоспалением, изменением уровня нейротрансмиттеров, прогрессирующей неврологической дисфункцией, а также способность данного ретровируса к репликации и продукции протеинов в потенциальными иммуномодулирующими свойствами, целью данной работы было сравнительное исследование цитокинсинтезирующей функции иммунных клеток крови условно здоровых лиц и больных рассеянным склерозом под действием синтетического 17-аминокислотного олигопептида, гомологичного консервативному региону гидрофобного трансмембранного протеина p15E HERV-E λ 4-1. Объектом исследования были 40 больных, 17 мужчин в возрасте 38,0 (31,0-47,0) лет и 23 женщины в возрасте 39,0 (31,0-50,0) лет с установленным диагнозом рассеянного склероза (G35, МКБ-10), удовлетворяющим критериям McDonald 2005, в модификации 2010 г., непрерывно-прогредиентным типом течения заболевания и длительностью 17,0 (14,0-18,0) лет и 30 условно-здоровых лиц, 12 мужчин в возрасте 32,0 (23,0-43,0) лет и 18 женщин в возрасте 36,0 (29,0-46,0) лет. Было проведено открытое обсервационное одноцентровое когортное контролируемое рандомизированное исследование. Обнаружено, что под действием ретровирусного олигопептида стимулировалась спонтанная продукция IL-1 β , IL-6, TNF α , IFN γ и IL-2 МНК доноров в культуре, но не изменялась таковая IL-4 и IL-10. В то же время спонтанная и митоген-стимулированная продукция всех исследуемых цитокинов не изменялась под действием контрольного олигопептида. Культивирование РНА-стимулированных МНК доноров в присутствии ретровирусного олигопептида, по сравнению с контрольным, сопровождалось увеличением высвобождения IL-1 β , IL-6 и TNF α в культуральный супернатант. Больные рассеянным склерозом характеризовались более высоким содержанием IL-1 β , IL-6 и IFN γ в культуральном супернатанте нестимулированных митогеном МНК, по сравнению с условно-здоровыми лицами, а также более высокой продукцией IL-6 и IFN γ в ответ на стимуляцию РНА. У больных рассеянным склерозом

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зом ретровирусный олигопептид, в отличие от контрольного, стимулировал спонтанную продукцию IL-1 β , IL-6, TNF α и IFN γ , не изменяя таковой IL-4 и IL-10. Полученные результаты свидетельствуют о провоспалительных свойствах синтетического олигопептида, гомологичного консервативному региону гидрофобного трансмембранного протеина p15E HERV-E λ 4-1, что, вероятно, является одним из механизмов реализации патологических свойств эндогенного ретровируса человека HERV-E λ 4-1 при рассеянном склерозе.

Ключевые слова: рассеянный склероз, непрерывно-прогредиентное течение, эндогенный ретровирус человека, олигопептид, мононуклеарные клетки крови, функциональная активность, цитокины

INVESTIGATION OF BLOOD MONONUCLEAR CELLS CYTOKINE-PRODUCTION FUNCTION FROM PATIENTS WITH MULTIPLE SCLEROSIS TREATED WITH THE ENDOGENOUS RETROVIRUS HERV-E λ 4-1 CONSERVATIVE REGION SYNTHETIC OLIGOPEPTIDE

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Abstract. Considering to the data of class I human endogenous retrovirus HERV-E λ 4-1 subgroup association with multiple sclerosis, an autoimmune disease accompanied by neuroinflammation, changes in the neurotransmitters level, progressive neurological dysfunction, as well as the ability of this retrovirus to replicate and to produce proteins with potential immunomodulatory properties, the aim of this work was a comparative study of the blood immune cells cytokine synthesizing function in conventionally healthy individuals and multiple sclerosis patients under the synthetic 17 – amino acid oligopeptide homologous to the hydrophobic transmembrane protein p15E HERV-E λ 4-1 conserved region influence. The 40 patients, 17 male persons aged 38.0 (31.0–47.0) years old and 23 female persons aged 39.0 (31.0–50.0) years old with an established diagnosis of multiple sclerosis (G 35, ICD-10), corresponding to the McDonald 2005, modified in 2010 criteria with a continuously progressive disease course and the disease duration of 17.0 (14.0–18.0) years, and 30 conditionally healthy individuals, 12 male persons aged 32.0 (23.0–43.0) years old and 18 female persons aged 36.0 (29.0–46.0) years old were the objects of the study. An open-label, observational, single-center, cohort, controlled, randomized trial was conducted. It was found that the donor's blood mononuclear cells IL-1 β , IL-6, TNF α , IFN γ and IL-2 spontaneous production in culture was stimulated, but that of IL-4 and IL-10 did not change under the retroviral oligopeptide influence. At the same time, the spontaneous and mitogen-stimulated production of all studied cytokines did not change under the control oligopeptide influence. The PHA-stimulated donor's blood mononuclear cells cultivation in presence of the retroviral oligopeptide, as compared to the control one, was accompanied by an increase in the IL-1 β , IL-6 and TNF α release into the culture supernatant. The multiple sclerosis patients were characterized by IL-1 β , IL-6 and IFN γ higher content in the mitogen-unstimulated blood mononuclear cells culture supernatant, compared with conditionally healthy individuals, as well as by a higher production of IL-6 and IFN γ in response to PHA stimulation. The retroviral oligopeptide, in contrast to the control one, stimulated the IL-1 β , IL-6, TNF α and IFN γ spontaneous production without altering that of IL-4 and IL-10 in multiple sclerosis patients. The obtained results indicate that the synthetic oligopeptide homologous to the conserved region of the hydrophobic transmembrane protein p15E HERV-E λ 4-1 has the pro-inflammatory properties, which is probably the one of human endogenous retrovirus HERV-E λ 4-1 pathological abilities realization mechanism in multiple sclerosis.

Keywords: multiple sclerosis, continuously progressive disease course, human endogenous retrovirus, oligopeptide, blood mononuclear cells, functional activity, cytokines

Introduction

An increased incidence has been observed in most economically developed countries in recent decades for the multiple sclerosis (MS), an autoimmune demyelinating disease of the nervous system, accompanied by neuroinflammation, changes in the neurotransmitters level, progressive neurological dysfunction, cognitive impairment, affective disorders, as well as the irreversibility of neurological symptoms which are characteristic of this disease, insufficient relevance of modern methods for the pathological process activity monitoring, incomplete certainty and multifactorial nature of the etiology and pathogenesis mechanisms [1, 2, 3, 4, 7, 10, 13, 14], determine the relevance of new potential etiological factors and their involvement in the MS pathogenesis identification and research.

Among the MS autoimmune inflammation triggers in the nervous system, endogenous retroviruses (ER) are considered as some of the most relevant being capable of polyclonal T-lymphocytes activation [8, 9, 11, 12, 15]. These retroviruses are an integrated in the provirus form of exogenous retroviruses and are a type of the genome mobile elements – RNA retrotransposons, DNA sequences that make up to 8% of the human genome distributed in more than 700,000 discrete loci. Usually, the retroelement activity in the human genome is repressed by both genetic and epigenetic mechanisms. However, in the evolution process some of them acquired the pathogenic properties and the ability to replicate as a result of mutations and recombinations, to form the virion structure and produce viral proteins with immunomodulatory properties and fulfill the role of superantigens – to form antigenic epitopes by the molecular mimicry mechanism recognized by the immune system cells. Inflammation and the immune system activation, in turn, are factors that modulate ER transcription, since their promoter regions contain binding sites for transcription factors, involved in the formation of oxidative stress response, which inhibits the deacetylase activity stimulating simultaneously histone acetylation and activation of ER expression [5, 19]. ER RNA can be recognized by Toll-like receptors as pathogen-associated cues, which induces the type I interferon production. Taking into account our earlier data about the class I retrovirus of the HERV-E λ 4-1 subgroup (ER λ 4-1) association with the MS course, as well as its ability to replicate and produce proteins [6, 8, 15], **the aim of this work** was to compare blood immune cells cytokine-synthesizing function from apparently healthy individuals and MS patients treated with the synthetic 17-amino acid oligopeptide, the hydrophobic transmembrane protein p15E ER λ 4-1 conserved region homologous influence.

Materials and methods

The 40 patients, 17 males aged 38.0 (31.0–47.0) years old and 23 females aged 39.0 (31.0–50.0) years old with an established MS diagnosis (G 35, ICD – 10), corresponding to the McDonald 2005 criteria, modified in 2010, with a continuously progressive type of disease course and a disease duration of 17.0 (14.0–18.0) years, and 30 apparently healthy individuals, 12 males aged 32, 0 (23.0–43.0) years old and 18 females aged 36.0 (29.0–46.0) years old were enrolled to the study. An open-label, observational, single-center, cohort, controlled, randomized study to evaluate the synthesis of certain cytokines in vitro was conducted.

Cytokine production in culture supernatants of blood mononuclear cells (MNCs) from apparently healthy individuals and MS patients was carried out by the solid-phase enzyme-linked immunosorbent assay method using ELISA-BEST (Koltsovo, Novosibirsk, Russia) and Pro Con (St. Petersburg, Russia) test systems, according to the manufacturer's instructions. For that purpose, the patient MNCs were collected from venous blood added with heparin and isolated by centrifugation on a Ficoll density gradient (Lymphocyte separation medium, ICN Biomedicals Inc.) at 1500 rpm. within 40 minutes.

Cells harvested from the interphase were washed in 199 culture medium, pelleted by centrifugation and resuspended at a concentration of 20×10^6 / ml in RPMI-1640 culture medium containing 10% human blood serum AB (IV), 10 mM Hepes buffer, 4×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 40 μ g/ml gentamicin, and cultured at 37 °C and 5% CO₂ in an atmosphere for 24–72 hours, depending on the experimental conditions. The research protocol was developed in accordance with the Helsinki Declaration by the World Medical Association “Ethical principles for conducting of scientific medical research with human participation”, as amended in 2013 and with the “Rules of Good Clinical Practice” approved by the Russian Federation Ministry of Health Order No. 200n, dated of 01.04.2016.

The 17-amino acid retroviral or control (with the reverse amino acid sequence) oligopeptides at suboptimal concentration were introduced into the cell culture for 24 hours after the onset of cell culture with or without the suboptimal mitogen concentration (50 μ g/ml) (Phytohemagglutinin-L *Phaseolus vulgaris* (PHA), Sigma Aldrich), determined in a series of preliminary experiments.

Statistical data processing was carried out using descriptive statistics, comparative analysis, on the base of nonparametric Mann–Whitney U test, with the commercial software package “Statistica 10.0” (StatSoft, USA) use. Results were presented as a median and an interval between 1 and 4 quartiles (Me (Q_{0.25}–Q_{0.75}) %). Differences were considered as statistically significant at $p < 0.05$. Calculating

sample size principles: sample size was not calculated previously.

Results and discussion

The data obtained on ER λ 4-1 effect assessing blood donors MNC baseline cytokine production are presented in Table 1.

We found that under the retroviral oligopeptide exposure, the spontaneous production of IL-1 β , IL-6, TNF α , IFN γ and IL-2 by donor MNCs was stimulated, but that of IL-4 and IL-10 did not. At the same time, spontaneous production for all studied cytokines did not change after treating with control oligopeptide. The suboptimal PHA concentration

TABLE 1. RETROVIRAL OLIGOPEPTIDE EFFECT ON CONDITIONALLY HEALTHY PERSONS BLOOD MONONUCLEAR CELLS CYTOKINE PRODUCTION, Me ($Q_{0.25}$ - $Q_{0.75}$)

Cytokines	Spontaneous production, pg/ml			PHA-induced production, pg/ml		
	0.9% NaCl	Control oligopeptide	Retroviral oligopeptide	0.9% NaCl	Control oligopeptide	Retroviral oligopeptide
IL-1 β	24.3 (19.9-27.8)	26.7 (23.1-31.2)	171.0 (182.2-214.8)*	151.9 (57.4-245.1)^	134.4 (78.3-165.6)	190.9 (156.2-219.4)*
IL-6	15.1 (12.4-17.6)	17.8 (15.3-19.5)	20.5 (19.4-23.4)*	22.8 (18.5-23.4)^	19.1 (17.2-23.1)	257.3 (232.4-276.9)*
TNF α	5.4 (2.0-7.8)	2.4 (2.1-2.6)	37.6 (34.1-39.5)*	148.5 (143.1-154.7)^	144.2 (138.9-147.6)	215.5 (182.4-248.4)*
IFN γ	8.10 (7.6-8.9)	8.1 (7.6-8.4)	78.5 (41.3-105.8)*	16.0 (11.5-19.3)^	16.0 (13.3-19.6)	27.5 (16.9-30.4)
IL-4	1.0 (0.8-1.4)	1.2 (0.9-1.4)	2.3 (0.9-2.5)	12.4 (9.8-14.6)^	12.8 (11.8-14.9)	10.8 (8.4-11.8)
IL-10	1.5 (1.2-1.6)	1.6 (1.5-1.8)	1.2 (0.7-1.7)	37.9 (30.3-40.6)^	34.5 (31.0-36.8)	33.8 (29.0-35.1)
IL-2	0.6 (0.5-0.6)	0.6 (0.5-0.7)	9.4 (8.9-10.3)*	10.6 (9.5-11.7)^	9.8 (8.2-11.4)	10.8 (8.8-12.1)

Note. n = 30; * – p < 0.05 between the control and retroviral oligopeptide; ^ – p < 0.05 between the spontaneous and PHA-induced production (Mann–Whitney U test).

TABLE 2. MULTIPLE SCLEROSIS PATIENTS BLOOD MONONUCLEAR CELLS CYTOKINE PRODUCTION UNDER THE RETROVIRAL OLIGOPEPTIDE INFLUENCE, Me ($Q_{0.25}$ - $Q_{0.75}$)

Cytokines	Spontaneous production, pg/ml			PHA-induced production, pg/ml		
	0,9% NaCl	Control oligopeptide	Retroviral oligopeptide	0,9% NaCl	Control oligopeptide	Retroviral oligopeptide
IL-1 β	47.8 (29.3-77.1)	36.4 (15.8-42.2)	192.0 (164.2-246.1)*	122.8 (66.1-241.2)^	125.9 (82.1-166.0)	235.3 (143.7-250.0)*
IL-6	35.1 (25.2-48.2)	39.1 (22.3-49.4)	110.7 (67.1-133.5)*	123.4 (65.2-154.2)^	119.6 (95.8-124.4)	264.9 (219.8-293.2)*
TNF α	7.9 (4.0-11.5)	9.9 (7.3-13.9)	44.6 (25.2-58.8)*	139.5 (106.0-199.6)^	146.0 (117.2-158.9)	223.6 (180.0-250.0)*
IFN γ	18.1 (12.0-29.2)	22.3 (17.0-37.8)	78.7 (48.4-106.3)*	29.2 (16.3-37.4)^	28.1 (13.2-36.1)	34.4 (17.0-37.2)
IL-4	1.9 (0.7-3.4)	2.3 (0.9-3.2)	2.9 (1.2-4.6)	11.7 (8.2-16.7)^	12.4 (10.1-16.9)	10.2 (4.6-10.1)
IL-10	2.6 (1.3-3.6)	1.6 (0.4-2.7)	2.9 (0.7-3.9)	33.9 (27.5-41.4)^	32.0 (26.1-40.2)	37.4 (24.3-38.6)
IL-2	1.8 (0.6-3.4)	1.7 (0.5-2.9)	1.7 (0.8-1.5)	12.0 (8.4-14.6)^	9.2 (7.8-13.7)	12.1 (7.5-15.5)

Note. As for Table 1.

resulted in increased level for all cytokines studied. Exposure to control oligopeptide had no changes in PHA-stimulated cytokine production. However, the PHA-stimulated donor MNCs cultured with the retroviral oligopeptide were accompanied by increased release of the IL-1 β , IL-6 and TNF α into the culture supernatant.

The results of the MS patient MNCs cytokine-synthesizing function after exposure to retroviral oligopeptide are presented in Table 2.

MS patients were characterized by a higher level of IL-1 β , IL-6, and IFN γ in the of mitogen-unstimulated MNCs culture supernatant compared with those from apparently healthy individuals ($p < 0.05$, Mann–Whitney U test), as well as by higher production of IL-6 and IFN γ ($p < 0.05$, Mann–Whitney U test) in response to PHA stimulation.

The suboptimal PHA concentration was accompanied by the increased production of all cytokines studied in the culture supernatant. The retroviral vs control oligopeptide stimulated the IL-1 β , IL-6, TNF α and IFN γ spontaneous production,

without altering that of IL-4 and IL-10 in MS patients. The control oligopeptide did not affect the cytokines level in the supernatant of PHA-stimulated MNCs. However, the culture of MS patients PHA-stimulated MNCs with the retroviral oligopeptide was accompanied by further increase in production of IL-1 β , IL-6, and TNF α into the culture supernatant.

Thus, the results shown above indicate about sequence-specific pro-inflammatory properties of a synthetic oligopeptide homologous to the conserved region of the hydrophobic transmembrane protein p15E ER λ 4-1, which might serve as one of the pathological properties enabled by the human endogenous retrovirus HERV-E λ 4-1 in multiple sclerosis and accompanying its low-grade production and neuroinflammation. The detection of the increased cytokines production mainly by Th1, Th17 and macrophages involved in the MS pathogenesis after exposure to the retroviral oligopeptide confirms its potential role in the pathogenesis of this disease and can serve as a perspective target for pathogenetically justified therapy.

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ОСОБЕННОСТИ ПОЛЯРИЗАЦИИ МОНОЦИТОВ ПРИ РАЗЛИЧНЫХ ИСХОДАХ БЕРЕМЕННОСТИ У ЖЕНЩИН С ПРИВЫЧНЫМ НЕВЫНАШИВАНИЕМ

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Резюме. В настоящее время в патогенезе привычного невынашивания особую роль уделяют иммунологическим факторам, в частности роли врожденного иммунитета. Цель исследования — оценка относительного содержания моноцитов в периферической крови, продуцирующих IL-4, IL-6, IL-10, IFN γ , а также выявление новых критериев прогнозирования исхода беременности у женщин с угрозой прерывания в ранние сроки и привычным невынашиванием. Обследовано 88 беременных в сроке гестации 5-12 недель, основную группу составили 59 женщин с привычным невынашиванием и угрожающим выкидышем на момент исследования, группу контроля — 29 женщин с неосложненным течением беременности без привычного невынашивания. Основная группа в зависимости от исходов беременности была подразделена на подгруппы: I подгруппа — 42 женщины, чья беременность завершилась своевременными родами, II подгруппа — 8 женщин с преждевременными родами, III подгруппа — 9 женщин с прерыванием беременности в сроке до 22 недель (самопроизвольный выкидыш и неразвивающаяся беременность). В группе контроля у всех женщин произошли своевременные роды. Материал исследования — периферическая венозная кровь. На проточном цитофлуориметре FACSCanto II с использованием моноклональных антител оценивали относительное содержание IL-4⁺, IL-6⁺, IL-10⁺, IFN γ ⁺ моноцитов. Статистическая обработка данных проводилась с помощью пакета стандартных прикладных программ.

В группе женщин с привычным невынашиванием и угрожающим выкидышем было снижено относительное содержание IL-10⁺ и IL-4⁺ моноцитов и повышено содержание IL-6⁺ моноцитов по сравнению с группой контроля ($p = 0,0001$ во всех случаях). Статистически значимых различий в содержании IFN γ ⁺ моноцитов в сравниваемых группах не выявлено ($p = 0,069$). При относительном содержании IL-4⁺ моноцитов равном 26,7% или менее прогнозируют преждевременные роды. При относительном содержании IL-10⁺ моноцитов равном 27,0% или менее прогнозируют прерывание беременности (самопроизвольный выкидыш или неразвивающуюся беременность) в сроке геста-

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ции до 22 недель. Было выявлено повышение соотношения $IFN\gamma^+/IL-4^+$, $IFN\gamma^+/IL-10^+$, $IL-6^+/IL-4^+$, $IL-6^+/IL-10^+$ моноцитов в основной группе ($p < 0,0001$ во всех случаях).

У женщин с привычным невынашиванием во всех подгруппах уровень М1-моноцитов преобладал над уровнем М2-моноцитов. Полученные данные позволили разработать новые прогностические критерии прерывания беременности до 22 недель и преждевременных родов.

Ключевые слова: беременность, привычное невынашивание, моноциты

FEATURES OF MONOCYTE POLARIZATION AT DIFFERENT OUTCOMES IN WOMEN WITH RECURRENT MISCARRIAGE

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Abstract. Currently, in the pathogenesis of recurrent miscarriage, a special role is given to immunological factors, in particular the role of innate immunity. The aim of the study was to assess the relative content of monocytes in the peripheral blood producing IL-4, IL-6, IL-10, $IFN\gamma$, as well as to identify new criteria for predicting the outcome of pregnancy in women with the threat of early termination and recurrent miscarriage. Materials and methods. 88 pregnant women at 5-12 weeks' gestation were examined, the main group consisted of 59 women with recurrent miscarriage and threatened miscarriage at the time of the study, the control group — 29 women with uncomplicated pregnancy without recurrent miscarriage. The main group, depending on the outcomes of pregnancy, was subdivided into subgroups: subgroup I — 42 women whose pregnancy ended in timely delivery, subgroup II — 8 women with preterm labor, subgroup III — 9 women with abortion up to 22 weeks (spontaneous miscarriage and non-developing pregnancy). In the control group, all women had a timely delivery. Research material — peripheral venous blood. The relative content of $IL-4^+$, $IL-6^+$, $IL-10^+$, $IFN\gamma^+$ monocytes was assessed on a FACSCanto II flow cytometer using monoclonal antibodies. Statistical data processing was carried out using a package of standard applied programs. Results. In the group of women with recurrent miscarriage and threatened miscarriage, the relative content of $IL-10^+$ and $IL-4^+$ monocytes was reduced and the content of $IL-6^+$ monocytes was increased compared to the control group ($p = 0.0001$ in all cases). There were no statistically significant differences in the content of $IFN\gamma^+$ monocytes in the compared groups ($p = 0.069$). With a relative content of $IL-4^+$ monocytes equal to 26.7% or less, preterm labor is predicted. With a relative content of $IL-10^+$ monocytes equal to 27.0% or less, abortion (spontaneous miscarriage or miscarriage) is predicted in gestational age up to 22 weeks. An increase in the ratio of $IFN\gamma^+/IL-4^+$, $IFN\gamma^+/IL-10^+$, $IL-6^+/IL-4^+$, $IL-6^+/IL-10^+$ monocytes was found in the main group ($p < 0.0001$ in all cases). Conclusions. In women with recurrent miscarriage in all subgroups, the level of M1 monocytes prevailed over the level of M2 monocytes. The data obtained allowed the development of new prognostic criteria for termination of pregnancy up to 22 weeks and premature birth.

Keywords: pregnancy, recurrent miscarriage, monocytes

Introduction

Recurrent miscarriage (RM) is a complication of early pregnancy, which occurs in married couples with a frequency of 2-5% [6, 8]. The etiology of recurrent miscarriage is diverse and not fully understood. Currently, special attention is paid to immunological factors that play an important role in the pathogenesis of this complication of pregnancy [2]. It is known that from the earliest stages of pregnancy various immune cells create a special microenvironment

to support a semi-allogeneic fetus [7]. The innate cells play a special role in these mechanisms [4, 7]. Monocytes are an integral part of the innate immune system [3] exerting high plasticity and, depending on the microenvironment, can functionally differentiate into a population of classically activated (M1) or alternatively activated (M2) cells [7, 9]. Each of the monocyte populations is characterized by different functions and cytokine production. Studies show that during normal pregnancy, monocyte differentiation

shifts towards an anti-inflammatory immune response with produced corresponding cytokines typical to the M2 population [10]. Moreover, an imbalance towards classically activated monocytes (M1) and their production of pro-inflammatory cytokines significantly increases the risk of early pregnancy loss or complications such as premature birth [2, 10]. Despite the available data on the participation of monocytes in immunological processes during pregnancy, it is necessary to identify a role of imbalance in their polarization in developing recurrent miscarriage.

The aim of the study was to assess the percentage of monocytes in the peripheral blood producing IL-4, IL-6, IL-10, IFN γ , as well as to identify new criteria for predicting outcome of pregnancy in women with threatened early termination and recurrent miscarriage in history.

Materials and methods

A survey of 88 pregnant women at a gestational age of 5-12 weeks was carried out at the gynecological clinic of the V. Gorodkov Ivanovo Research Institute of Maternity and Childhood. The main group included 59 women with RM and threatened miscarriage at the time of the study. By retrospective analysis, this group of women was divided into subgroups depending on the pregnancy outcome. Subgroup I consisted of 42 women in whom pregnancy ended in timely delivery, subgroup II – 8 women with preterm labor, subgroup III – 9 women whose pregnancy ended in abortion at age up to 22 weeks of gestation, including spontaneous miscarriage and undeveloped pregnancy. The control group included 29 women with uncomplicated pregnancy and lacking recurrent miscarriage in history, who had a timely delivery. The groups are recruited uniformly. Exclusion criteria: spontaneous miscarriage in progress; multiple pregnancy; pregnancy resulting from the use of assisted reproductive technologies; anembryonia or other reliable signs of an unviable uterine pregnancy; abnormalities in the uterus development; structural changes in the karyotypes of spouses, established at the pre-gravid stage; active infection and presence of severe female extragenital pathology at the time of examination; severe allergic reactions at the time of the examination. The study complied with ethical standards. All women enrolled to the study signed an informed consent. The biological material was presented by whole peripheral venous blood collected before treatment, in a volume of 10 ml into a test tube added with heparin. FACSCanto II flow cytometer (Becton Dickinson, USA) was used with monoclonal antibodies anti-IL-10, anti-IL-6 (eBioscience), anti-IL-4, anti-IFN γ (Beckman Coulter) to assess percentage of IL-4⁺, IL-6⁺, IL-10⁺, IFN γ ⁺ monocytes. Statistical data processing was carried out by using “Statistica 13.0”, “MedCalc” software.

Before calculations, all parameters were tested for normality. The normal distribution was checked using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Taking into account the normality of the distribution, the data were presented as arithmetic mean and standard error of the mean ($M \pm m$). The threshold significance level of differences was set at $p < 0.05$. The search for the separation point, the calculation of sensitivity and specificity was carried out based on the construction of the receiver operating characteristic curve (ROC analysis).

Results and discussion

While analyzing the studied parameters, it was revealed that the relative percentage of IL-4⁺ and IL-10⁺ monocytes in the peripheral blood was lower in the main group with threatened termination in the first trimester with RM compared with those in uncomplicated pregnancy ($p = 0, 0001$ in both cases), whereas the level of IL-6⁺ monocytes in the main group was higher than in the control group ($p = 0.0001$). No significant differences in the percentage of IFN γ ⁺ monocytes in pregnant women from both groups were found ($p = 0.069$) (Table 1).

The population of M2 monocytes is characterized by the production of anti-inflammatory cytokines, which we assessed by the intracellular production of IL-4⁺ and IL-10⁺. A comparative analysis of percentages of IL-4⁺ M2 monocytes in the peripheral blood in women with threatened interruption with RM revealed that in all subgroups it was significantly decreased compared to control group ($p < 0.05$). Our retrospective analysis showed that the relative content of IL-4⁺ M2 monocytes in the group of women with RM differed depending on the outcome of pregnancy. In the subgroups of women whose pregnancy ended in abortion at age of up to 22 weeks and premature birth, it was lower than in the subgroup of women with timely birth ($p < 0.05$ in all cases). In subgroup I, the level of IL-4⁺ monocytes was $35.47 \pm 2.0\%$, subgroup II – $23.56 \pm 2.12\%$, subgroup III – $23.87 \pm 3.66\%$, and in the control group – $53, 23 \pm 2.3\%$. However, it should be noted that the level of IL-4⁺ monocytes was lower in the subgroup of women with threatened miscarriage with RM and timely delivery compared to the control group ($p < 0.05$).

According to our data, we have identified new prognostic criteria for preterm birth in women with threatened miscarriage and a history of recurrent miscarriage. So, with a relative content of IL-4⁺ monocytes comprising at least 26.7%, preterm labor may be predicted. According to the results of the ROC analysis, the area under the ROC curve (AUC) was 0.885, the sensitivity of the method was 77.8%, the specificity – 95.7%, and the accuracy – 90.6%. Based on these data, a “Method for predicting premature

TABLE 1. CONTENT OF IFN γ ⁺, IL-4⁺, IL-10⁺ AND IL-6⁺ MONOCYTES IN WOMEN WITH THE THREAT OF TERMINATION AND RECURRENT MISCARRIAGE

Indicator, %	Control group (n = 29)	Main group (n = 59)	
IL-4 ⁺	53.04±2.36	30.12±1.47	p = 0.0001
IL-6 ⁺	29.45±1.57	71.69±1.29	p = 0.0001
IL-10 ⁺	65.61±2.27	30.45±1.51	p = 0.0001
IFN γ ⁺	50.50±4.57	59.90±2.06	p = 0.069

birth in women with threatened early miscarriage with RM in history” was proposed.

A comparative analysis of percentage of IL-10⁺ monocytes in the peripheral blood in women with the threatened interruption with RM found that all subgroups had it significantly decreased *vs* control group ($p < 0.05$). In addition, we found that the level of IL-10⁺ monocytes in the group of women with RM was lower in the subgroups with terminated pregnancy at age of up to 22 weeks (23.7±1.42%) and preterm birth (24.12±2.33%) *vs* subgroup of women with timely delivery (34.45±1.67%) ($p = 0.0001$ and $p = 0.006$, respectively).

According to the data obtained, we have identified new prognostic criteria for termination of pregnancy before 22 weeks of gestation in women with threatened miscarriage with RM in history. Abortion (spontaneous miscarriage or miscarriage) is predicted at gestational age of up to 22 weeks in case of percentage of IL-10⁺ monocytes reaching at least 27.0%. Based on these data, a “Method for predicting abortion in women with threatened miscarriage and recurrent miscarriage in history” was proposed.

The level of M1 monocytes, which is characterized by the production of proinflammatory cytokines, was assessed by the intracellular production of IFN γ ⁺ and IL-6⁺. Analyzing the results obtained, we revealed no significant differences in the frequency of such cells in all subgroups of women with RM ($p > 0.05$ in all cases). However, the level of IL-6⁺ monocytes was higher in all subgroups of women with recurrent miscarriage compared with the control group ($p < 0.05$ in all cases), whereas the relative content of IFN γ ⁺ monocytes did not differ from that one in the control group and in all subgroups of women with RM ($p > 0.05$ in all cases).

We also found increased ratio for IFN γ ⁺/IL-4⁺, IFN γ ⁺/IL-10⁺, IL-6⁺/IL-4⁺, IL-6⁺/IL-10⁺ monocytes in the group of women with RM ($p < 0.0001$ in all cases), which indicates the predominance of inflammatory reactions in this pathology.

It is known that a successful pregnancy contributes to the maintenance of the immune balance, ensures the mother’s immunotolerance to the fetal antigens [3, 10]. IL-4 and IL-10 – producing monocytes are characterized by anti-inflammatory and immunosuppressive properties [1] being involved in

angiogenesis, placenta formation and maintenance of tolerance to semi-allogenic fetus, additionally regulating Treg function [1]. Local inflammation, mediated by cells of innate and acquired immunity, is necessary for successful implantation and tissue remodeling in the first trimester. However, an excessive inflammatory response leads to serious complications of pregnancy, including termination of pregnancy. According to the literature, unfavorable pregnancy outcome is coupled to higher level of proinflammatory cytokines [2]. IFN γ and IL-6 – producing monocytes are characterized by pro-inflammatory functions [5], but an excessive inflammatory response inhibits the anti-inflammatory response.

We found that women with recurrent miscarriage and threatened early miscarriage had reduced percentage of IL-10⁺ and IL-4⁺ monocytes with M2 functions, whereas frequency of IL-6⁺ monocytes was increased compared to the control group. According to our data, women with recurrent miscarriage in all subgroups had dominated M1 profile.

While assessing the study results, a similar dynamic of changes was found in women with RM, whose pregnancy ended in interruption at age of up to 22 weeks and premature birth. In this situation, it can be assumed that other mechanisms able to compensate for pathological processes in threatened termination might occur allowing to prolong pregnancy until some certain timepoint. According to the data obtained, it can be assumed that if pregnancy is terminated before 22 weeks, a persistent inflammatory reaction, impaired angiogenesis and placental development from early gestation develop most rapidly. Whereas a chronic inflammatory reaction in early pregnancy can probably lead to a decrease in the anti-infectious protection of the fetoplacental complex and amniotic fluid, which ultimately can lead to premature birth. Together, these data support the notion that the early absence of appropriate anti-inflammatory reactions and excessive inflammatory response can lead to unfavorable course of pregnancy. The data obtained allowed to identify new early predictive criteria for termination of pregnancy before 22 weeks and premature birth.

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ИЗМЕНЕНИЕ ПЕЧЕНОЧНОЙ ЭКСПРЕССИИ ГЕНА *SOD1* В ПАТОГЕНЕЗЕ НАЖБП ПРИ ОЖИРЕНИИ

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Резюме. Стеатоз в печени при ожирении увеличивает работу митохондрий по утилизации избыточных липидов. Перегрузка β -окисления жирных кислот, цикла трикарбоновых кислот и окислительного фосфорилирования приводит к снижению уровня АТФ и повышению образования активных форм кислорода. В норме, митохондрии способны эффективно удалять повышенный уровень активных форм кислорода с помощью антиоксидантной системы и метаболической адаптации клетки к измененным условиям. Целью данного исследования явилось изучение роли печеночной экспрессии *SOD* в патогенезе НАЖБП при ожирении. Было выявлено, что уровень экспрессии *SOD1* в печени у больных ожирением с и без СД 2 типа с ИМТ > 40 кг/м² был ниже, чем у здоровых доноров. Число копий митохондриальной ДНК (мтДНК) в печени у всех больных ожирением было ниже более чем в два раза относительно значений контрольной группы. В печени у больных ожирением без СД 2 типа уровень белка *SOD1* и число копий мтДНК были взаимосвязаны между собой и отрицательно коррелировали с площадью жировых включений. Таким образом, у больных ожирением снижение антиоксидантной защиты в печени приводит к уязвимости митохондрий, что, в свою очередь, способствует прогрессированию стеатоза и инсулинорезистентности.

Ключевые слова: ожирение, СД 2 типа, супероксиддисмутаза, мтДНК, печень, стеатоз

HEPATIC *SOD1* GENE EXPRESSION CHANGES IN THE NAFLD PATHOGENESIS IN OBESITY

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Abstract. Steatosis in the liver in obesity increases the work of mitochondria to utilize excess lipids. An overload of β -oxidation of fatty acids, the tricarboxylic acid cycle, and oxidative phosphorylation leads to a decrease in ATP and an increase in the formation of reactive oxygen species. Normally, mitochondria can efficiently remove elevated levels of reactive oxygen species using the cell's antioxidant system and metabolic

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adaptation to altered conditions. This study aimed to investigate the role of hepatic *SOD* expression in the pathogenesis of NAFLD in obesity. It was found that the level of *SOD1* expression in the liver in obese patients with and without type 2 diabetes with a BMI > 40 kg/m² was lower than in healthy donors. The copy number of mitochondrial DNA (mtDNA) in the liver in all obese patients was more than two times lower than in the control group. In the liver of obese patients without type 2 diabetes, the *SOD1* protein level and the mtDNA copy number were interrelated and negatively correlated with the area of fatty inclusions. Thus, in obese patients, a decrease in antioxidant defense in the liver leads to the vulnerability of mitochondria, which, in turn, contributes to the progression of steatosis and insulin resistance.

Keywords: obesity, T2DM, superoxide dismutase, mtDNA, liver, steatosis

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Introduction

Insulin resistance, oxidative stress, and inflammation play a central role in the non-alcoholic fatty liver disease (NAFLD) pathogenesis [3] liver cancer, and indications for orthotopic liver transplantation. Given its high prevalence, the absence of FDA-approved drugs for NAFLD is noticeable. In the pathogenesis of NAFLD, it is well known that mitochondrial dysfunction arises as a result of changes in ETC complexes and the membrane potential ($\Delta\psi_m$). Although the pathogenesis of lipid accumulation in the liver has been described, the molecular mechanisms behind transition of steatosis to inflammation and fibrosis are still poorly understood. Decreased ability to oxidize fatty acids, increased delivery and transport of free fatty acids (FFA), as well as increased fatty acid production in the liver are considered important factors of hepatocyte damage in obesity [3] liver cancer, and indications for orthotopic liver transplantation. Given its high prevalence, the absence of FDA-approved drugs for NAFLD is noticeable. In the pathogenesis of NAFLD, it is well known that mitochondrial dysfunction arises as a result of changes in ETC complexes and the membrane potential ($\Delta\psi_m$). Mitochondrial dysfunction in NAFLD is accompanied by incomplete/suboptimal fat oxidation, which leads to the accumulation of toxic lipid intermediates such as ceramides and diacylglycerol, which can cause inflammation and disrupt insulin signaling [6]. Also, hepatocyte mitochondrial respiratory chain overload in obesity promotes formation of reactive oxygen species (ROS) [6].

In normal physiological conditions, cellular ROS are found at minimal concentration as byproducts of aerobic metabolism and secondary messengers in many signaling pathways. Moreover, there is a stable balance between prooxidants and antioxidants in normal physiological conditions [4]. However, while rate of free radical formation exceeds the capacity

of antioxidant protection, oxidative stress develops, followed by serious damage to the cellular apparatus [4]. The cell has several defense and repair mechanisms counterbalancing oxidative stress. However, it has been shown that antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione (GSH), glutathione peroxidase (GSHPx), and glutathione reductase (GSHR) exhibit decreased activity in the affected brain areas during neurodegenerative diseases [5]. It is likely that in NAFLD, the defense system is also affected and cannot cope with a large number of errors in lipid metabolism, which aggravates the disease course and progression. Thus, the study aimed to study a role of hepatic *SOD1* expression in the NAFLD pathogenesis in obesity.

Materials and methods

166 obese patients were included in the study group. Of these, 28 patients (9 men, 19 women) with obesity without type 2 diabetes (T2DM) and body mass index (BMI) < 40 kg/m² (group 2) (age 42±10 years; BMI 35.2±2.9 kg/m²), 47 patients (14 men, 33 women) with obesity without T2DM and BMI > 40 kg/m² – group 3 (age 45±9 years; BMI 48.6±8.0 kg/m²). Groups 4 and 5 included obese patients with T2DM – 21 patients (8 males, 13 females) with BMI < 40 kg/m² (age 46±10 years; BMI 36.5±3.0 kg/m²) and 45 patients (12 males, 33 females) with a BMI > 40 kg/m² (age 48±8 years; BMI 50.2±8.2 kg/m²). 25 (15 males; 10 females) apparently healthy donors with normal anthropometric and biochemical parameters (age 38±9 years; BMI 22.7±3.7 kg/m²) were included in the control group (group 1). These groups were age- and sex-comparable. The diagnosis of T2DM was established based on examination at specialized hospital, guided by the diagnostic criteria for diabetes mellitus and other types of hyperglycemia of the World Health Organization (1999-2013) (IDF, 2013). Venous blood (Vacuette with a clot activator or EDTA) samples were used for biochemical and serological assays. Liver biopsies were collected during elective bariatric surgery and used to study gene expression, protein production, mitochondrial DNA (mtDNA), and histological analysis. Voluntary informed consent for the study was signed and provided by all patients. Permission to conduct the study was obtained from the local ethics committee (Protocol No. 2 of IKBFU, March 6, 2017).

The serum glucose level was determined on an automatic biochemical analyzer CA-180 (FURUNO ELECTRIC CO., LTD, Japan), using commercial kits (Dia-m, Russia). Plasma insulin levels were determined by flow fluorimetry using commercial test systems (Bio-Plex, Pro Human Diabetes 10-Plex Assay, Bio-Rad, USA) on an automated analyzer (Bio-Plex® 200 Systems, Bio-Rad, USA) and Bio-Plex Manager software (Bio-Rad, USA). Insulin values were converted from pg/ml to mIU/ml by using an online calculator (<http://www.endmemo.com/medical/unitconvert/Estradiol.php>). The insulin resistance index was calculated by using an online software (<https://www.omnicalculator.com/health/homa-ir>).

Total RNA from liver biopsies was isolated by using the ExtractRNA reagent (Evrogen, Russia). Reverse transcription of RNA samples was performed using the MMLV RT kit (Evrogen, Russia). Complementary DNA (cDNA) synthesis was performed according to the manufacturer's protocol. *SOD1* gene expression was determined by quantitative PCR using qPCRmix-HS reagents (Evrogen, Russia) on a CFX96 amplifier (Bio-Rad, USA). Protein fraction from liver biopsies was isolated by using RIPA buffer (RIPA Buffer, Thermo Scientific, USA), and concentration measurement was performed by the Bradford method (BCA Protein Assay Kit, ThermoFisher, USA). Semi-quantitative analysis of protein production in liver biopsies was performed by running immunoblotting assay to confirm results of gene expression. Semi-quantitative protein measurement was carried out by using specific monoclonal antibodies to *SOD1* (Invitrogen, USA) and *GAPDH* (Thermo Fisher, USA); and blotting systems (Mini-PROTEAN Tetra Systems, Trans-Blot Turbo, Bio-Rad, USA). The target proteins were detected on a ChemiDoc MP Imaging System (BioRad, USA). Membrane images were analyzed using the software ImageLab, Bio-Rad. The liver mtDNA copy number was estimated by Droplet Digital PCR (ddPCR) using the QX200 Droplet Digital PCR System (BioRad, USA). A detailed protocol is available in study published earlier (<https://pubmed.ncbi.nlm.nih.gov/29429383/>).

The data were analyzed for normal distribution using the Kolmogorov-Smirnov test. Differences were assessed using Student's *t*-test for normal distribution (two groups, parametric test) and Mann-Whitney test for abnormal distribution (two groups, nonparametric test). Data are presented as mean and standard deviation as well as median and 25%-75% quartiles. The analysis of relationship between two variable was carried out using the Spearman correlation method. Statistical significance was set at $p < 0.05$ level.

Results and discussion

NAFLD is a liver disease that begins with steatosis and progresses to non-alcoholic steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma [6]. The liver is central to the intermediate metabolism

of glucose, lipids, and ketones, which are needed to meet the energy requirements in peripheral tissues, heart, and brain. Triglycerides and free fatty acids accumulate in excess in the liver during obesity and insulin resistance, which leads to the formation of lipid droplets in hepatocytes. In turn, steatosis increases β -oxidation of fatty acids, induces the tricarboxylic acid cycle, and stimulates oxidative phosphorylation [6]. Overloading these processes leads to decreased ATP level, increased formation of reactive oxygen species (ROS), and excessive fat deposition [6]. Oxidative stress is one of the key mediators of liver damage and is the main contributor to the progression from steatosis to steatohepatitis [1]. In normal physiological conditions, mitochondria can efficiently remove elevated ROS levels via the antioxidant system and cell metabolic adaptation to altered conditions [1]. However, ROS production is too high so that compensatory mechanisms cannot cope with such a load during NAFLD. Superoxide dismutase-1 (*SOD1*) is a well-known intracellular antioxidant enzyme. Intracellular *SOD1* (cytosolic Cu/ZnSOD) is a homodimer (32 kDa) mainly localized in the cytosol, as well as in the intermembrane space of mitochondria [2] especially superoxide anion ($O_2^{\bullet-}$). Presumably, *SOD1* may play a role in NAFLD. Thus, monitoring changes in hepatic *SOD1* expression in NAFLD will help to understand the antioxidant system state.

Plasma glucose levels in obese patients without T2DM with BMI < 40 kg/m² exceeded those in control group ($p = 0.006$) (Table 1). Plasma glucose levels in obese patients with T2DM (with BMI < 40 kg/m² and BMI > 40 kg/m²) were significantly higher than in the control group ($p < 0.001$), obese patients without T2DM ($p = 0.007$ and $p < 0.001$, respectively) with BMI < 40 kg/m² and BMI > 40 kg/m² ($p < 0.001$) (Table 1). As expected, glucose levels went outside the reference range (3.9-6.4 mmol/l) only in obese patients with T2DM (Table 1). Plasma insulin levels in obese patients without T2DM with BMI > 40 kg/m² exceeded those in control group ($p = 0.003$) (Table 1). Plasma insulin levels in obese patients with T2DM (with BMI < 40 kg/m² and BMI > 40 kg/m²) were significantly higher than in the control group ($p < 0.001$), obese patients without T2DM ($p = 0.01$ and $p = 0.03$, respectively) with BMI < 40 kg/m² and BMI > 40 kg/m² ($p = 0.004$ and $p = 0.02$, respectively) (Table 1). Similar differences between groups were found for the HOMA-IR index (Table 1).

In this experiment, the liver histological analysis was carried out to determine the area of lipid droplets and inflammation by counting number of lymphocytes. The area of lipid droplets in the liver of obese patients without T2DM with BMI < 40 kg/m² significantly exceeded that one in control group ($p = 0.03$) (Table 1). The area of lipid droplets in the liver of obese patients with T2DM (with BMI < 40 kg/m² and BMI > 40 kg/m²) was significantly higher than that in control group ($p < 0.001$) and obese patients without

TABLE 1. STUDIED PARAMETERS IN GROUPS

Parameters	Parameters of studies groups				
	1 Control group	2 Obese patients without type 2 diabetes with BMI < 40 kg/m ²	3 Obese patients without type 2 diabetes with BMI > 40 kg/m ²	4 Obese patients with type 2 diabetes with BMI < 40 kg/m ²	5 Obese patients with type 2 diabetes with BMI > 40 kg/m ²
Insulin (pg/ml)	45.93 (39.26-61.25)	93.42 (44.41-170.50)	134.30 (67.79-237.10) p ₁ = 0.003	502.2 (255.70-1248.00) p ₁ < 0.001 p ₂ = 0.01 p ₃ = 0.004	262.2 (134.00-530.70) p ₁ < 0.001 p ₂ = 0.03 p ₃ = 0.02
glucose (mmol/l)	5.05 (5.30-5.51)	5.66 (4.87-6.48) p ₁ = 0.006	5.14 (4.71-6.08)	6.83 (5.61-9.38) p ₁ < 0.0001 p ₂ = 0.007 p ₃ = 0.0002	7.09 (5.92-9.74) p ₁ < 0.001 p ₂ < 0.001 p ₃ < 0.001
hOMA-IR (c. u.)	6.15 (4.55-8.30)	13.11 (5.99-22.68)	19.68 (11.62-36.41) p ₁ < 0.001	127.90 (48.77-292.50) p ₁ < 0.001 p ₂ = 0.006 p ₃ = 0.004	52.00 (30.22-211.00) p ₁ < 0.001 p ₂ = 0.007 p ₃ = 0.01 p ₄ = 0.03
area of lipid droplets (%)	3.37 (1.84-5.26)	5.16 (2.71-26.46) p ₁ = 0.03	4.10 (2.20-7.98)	22.24 (16.78-25.46) p ₁ < 0.001 p ₃ < 0.001	19.17 (11.37-32.24) p ₁ < 0.001 p ₂ < 0.003 p ₃ < 0.001
lymphocyte count	–	121.00 (92.50-134.50)	75.00 (50.50-94.50) p ₂ = 0.03	98.00 (71.00-137.00)	127.00 (102.00-163.50) p ₃ < 0.001
sOD gene expression (c. u.)	1.01 (0.99-1.03)	0.96 (0.88-1.06)	0.92 (0.89-0.94) p ₁ = 0.001	0.90 (0.89-1.05)	0.91 (0.88-0.96) p ₁ = 0.001
amounts of mtDNA	3253 (2097-4280)	1412 (531-3221) p ₁ = 0.002	711 (520-777) p ₁ < 0.0001	1390 (818.3-2072.0) p ₁ = 0.008 p ₃ = 0.005	1035 (745-1566) p ₁ < 0.001 p ₃ < 0.001

Note. Significance determined using the nonparametric Mann–Whitney test (Mean±SD); “p_x”, significant differences.

T2DM with BMI > 40 kg/m² (p < 0.001) (Table 1). It should be noted that the area of lipid droplets in the liver of obese patients with T2DM with a BMI < 40 kg/m² vs BMI > 40 kg/m² was significantly higher (Table 1). Healthy donors showed no signs of liver inflammation. The number of leukocytes in the liver of obese patients without T2DM with a BMI > 40 kg/m² vs BMI < 40 kg/m² was higher (p = 0.03). The number of leukocytes in the liver of obese patients with T2DM with BMI > 40 kg/m² vs BMI < 40 kg/m² was significantly higher (p < 0.001) (Table 1). The control group showed a normal liver structure without signs of inflammation. However, focal liver steatosis (small- and medium-droplet) and single drops were observed in some healthy donors. Small, medium, and large droplet steatosis was observed in obese patients without disturbed carbohydrate metabolism. Hepatocyte dystrophy and karyolysis was found in 40% and 17%, respectively.

Intracellular cholestasis and signs of inflammation (lymphocyte infiltration) were detected in 41.6% and 58% of patients, respectively. At the same time, 30% subjects had no steatosis or single drops similar to the control group. Small, medium, and large droplet steatosis was observed in obese patients with impaired carbohydrate metabolism. Hepatocyte dystrophy was observed in almost all patients (93.75%), whereas karyolysis manifestations in lower percentage (62.5%). Intracellular cholestasis and lymphocytic infiltration were detected in 81.25% of patients. Thus, steatosis and inflammation progressed with increased BMI and developing insulin resistance.

In this study, the level of *SOD1* gene expression in the liver of obese patients with and without T2DM with BMI > 40 kg/m² was significantly lower than in healthy donors (Table 1), which indicates reduced antioxidant protection along with increased BMI. Positive correlations were found between the *SOD1*

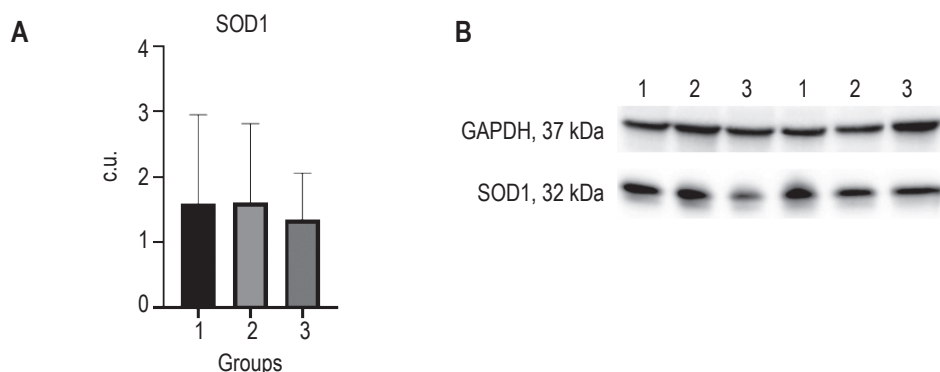


Figure 1. SOD1 protein production in the liver

Note. 1, healthy donors; 2, obese patients without T2DM; 3, obese patients with T2DM. A, production of SOD1 protein in the liver, normalized to the reference protein GAPDH in three groups. B, chemiluminescence images of SOD1 and GAPDH proteins in the liver.

expression and BMI ($r = 0.74$) as well as between the *SOD1* expression and mtDNA ($r = 0.65$) in the control group. Consequently, in normal physiological conditions in the liver, mitochondria begin to function vigorously in response to an excessive FFA intake with increased BMI. In turn, elevated β -oxidation activates the antioxidant mechanisms of the cell defense against high ROS levels, which are formed due to errors in electron transfer in the mitochondrial respiratory chain. A negative correlation between the *SOD1* expression and the index of insulin resistance HOMA-IR ($r = -0.61$) was found in obese patients without impaired carbohydrate metabolism. Thus, the likelihood of developing insulin resistance increases along with decreased antioxidant protection.

We also performed a semi-quantitative analysis of SOD1 protein level in the liver. Thus, the value of densitometry of the SOD1 protein relative to the reference GAPDH protein in the liver in healthy donors was 1.6 ± 1.3 c. u., 1.6 ± 1.2 c. u. in obese patients without T2DM and 1.5 ± 0.9 c. u. in obese patients with T2DM (Figure 1). A negative correlation between the production of SOD1 protein and the area of lipid droplets in the liver was found in obese patients without T2DM confirming the theory about decreased activity of the antioxidant system in obesity.

As previously mentioned, changes in mitochondrial structure and function are considered as a key hallmark of NAFLD [3] liver cancer, and indications for orthotopic liver transplantation. Given its high prevalence, the absence of FDA-approved drugs for NAFLD is noticeable. In the pathogenesis of NAFLD, it is well known that mitochondrial dysfunction arises as a result of changes in ETC complexes and the membrane potential ($\Delta\psi_m$). The results obtained using electron microscopy showed that mitochondria were enlarged and the mitochondrial matrix contained paracrystalline inclusions in mice with altered fatty acid oxidation and hepatic steatosis [7].

MtDNA is most susceptible to ROS effects since it is located near the site of ROS production [7]. Consequently, mtDNA will be more susceptible to ROS when the activity of the antioxidant system de-

creases. In this experiment, the amount of mtDNA in the liver in all groups was significantly lower in comparison with healthy donors ($p < 0.05$) (Table 1). The amount of mtDNA in the liver of obese patients without T2DM with BMI > 40 kg/m² was significantly lower than in obese patients with T2DM with BMI < 40 kg/m² ($p = 0.005$) and BMI > 40 kg/m² ($p < 0.001$) (Table 1). A negative correlation between the amount of mtDNA and the area of lipid droplets in the liver was found in healthy donors ($r = -0.71$) and obese patients without impaired carbohydrate metabolism ($r = -0.46$). This proves that in normal physiological conditions, the amount of mtDNA increases along with increasing steatosis to compensate for excess FFA levels. However, this process is disrupted in obesity. A negative correlation between the amount of mtDNA in the liver and the plasma insulin level ($r = -0.53$) and the insulin resistance index HOMA-IR ($r = -0.54$) was found in obese patients with T2DM. A decrease in the amount of mtDNA in the liver may lead to the development of T2DM.

Thus, a decrease in liver antioxidant defense in obese patients leads to mitochondrial vulnerability, which contributes to steatosis progression.

Conclusion

1) The expression *SOD1* and the number of mtDNA copies in the liver of obese patients with and without T2DM with BMI > 40 kg/m² is lower than in healthy donors.

2) The SOD1 protein level and the number of mtDNA copies are interrelated and negatively correlate with the area of lipid droplets in the liver of obese patients without T2DM.

3) The number of mtDNA copies is negatively associated with the plasma insulin level and HOMA-IR index in patients with T2DM.

4) A decrease in antioxidant protection in the liver leads to the vulnerability of mitochondria in obese patients, contributing to the progression of steatosis and insulin resistance.

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ВЛИЯНИЕ РЕАКТИВАЦИИ ВИРУСОВ ГЕРПЕСА ЧЕЛОВЕКА НА СИСТЕМНУЮ ПРОДУКЦИЮ ЦИТОКИНОВ У ПАЦИЕНТОВ С БОЛЕЗНЬЮ БЕХЧЕТА И УВЕИТАМИ

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Резюме. Болезнь Бехчета (ББ) — системное аутовоспалительно-аутоиммунное заболевание (хронический системный васкулит) невыясненной этиологии, увеит развивается почти у 70% пациентов. Патогенез ББ сложен, среди инфекционных триггерных факторов важную роль играют герпесвирусы человека (HHV). Известна способность HHV модулировать продукцию цитокинов и уклоняться от иммунного ответа организма. Цель работы: определить влияние *Herpes simplex virus type 1 (HSV-1)*, *Herpes simplex virus type 2 (HSV-2)*, *Cytomegalovirus (CMV)*, *Epstein-Barr virus (EBV)* на системные уровни хемокинов, про- и противовоспалительных цитокинов при ББ с симптомами увеитов и без. Сыворотки 116 пациентов с ББ, хронически инфицированных герпесвирусами, исследовали в иммуноферментном анализе на наличие IgG-антител к предданным антигенам HSV-1,2 и раннему антигену EBV (маркеров реактивации HHV). Концентрацию IL-1β, IFNγ, MCP-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12p70, IL-13, IL-18, TNFα, GM-CSF, Eotaxin, GRO-α, IP-10, MIP-1α, MIP-1β, SDF-1α, RANTES определяли в мультиплексном анализе, TGF-β1, TGF-β2 — в иммуноферментном анализе. В зависимости от наличия и активности увеита выделили 3 группы пациентов с ББ: 1-я группа — активный увеит, 2-я группа — увеит в ремиссии, 3-я группа — ББ без поражения глаз. По результатам серологического анализа в каждой группе выделили 2 подгруппы: а) пациенты с наличием антител-маркеров реактивации хотя бы одного из исследованных HHV, б) пациенты, хронически инфицированные HHV, без признаков реактивации. Средний уровень и частоту выявления цитокинов и хемокинов у пациентов с активными увеитами (1а, 1б) и в стадии ремиссии (2а, 2б) сравнивали с результатами обследования пациентов без поражения глаз (3а, 3б), сопоставляли подгруппы с хронической ВГЧ-инфекцией (подгруппа «б») и ее реактивацией (подгруппа «а»).

Достоверное повышение сывороточного содержания хемокинов MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, IP-10, SDF-1α, а также IFNγ, TGF-β1 и TGF-β2 отмечено у пациентов с увеитами (независимо от их активности) и маркерами реактивации HHV по сравнению с пациентами без увеитов.

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Полученные данные указывают на возможное влияние реактивации хронических герпесвирусных инфекций на системную продукцию цитокинов и хемокинов у пациентов с ББ и увеитами, причем наибольшие изменения касаются хемокинов.

Ключевые слова: болезнь Бехчета, вирусы герпеса человека, цитокины, хемокины, увеит, реактивация ВГЧ

EFFECT OF HUMAN HERPES VIRUS REACTIVATION ON SYSTEMIC CYTOKINE PRODUCTION IN PATIENTS WITH BEHCET'S DISEASE AND UVEITIS

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Abstract. Behcet's disease (BD) is a systemic autoinflammatory-autoimmune disease (chronic systemic vasculitis) of unknown etiology, almost 70% of patients develop uveitis. BD pathogenesis is complex, human herpesviruses (HHV) play an important role among infectious trigger factors. Ability of herpesviruses to modulate cytokine production and evade host's immune response is known. Aim of the study was to assess the effect of *Herpes simplex virus type 1*, *Herpes simplex virus type 2*, *Cytomegalovirus*, *Epstein-Barr virus* on systemic levels of chemokines, pro- and anti-inflammatory cytokines in BD with and without uveitis. Serum samples were collected from 116 BD patients chronically infected with HHV and examined in ELISA-test for markers of HHV reactivation (IgG-antibodies to immediate early HSV antigens 1, 2 and CMV, early EBV antigen). Concentration of IL-1 β , IFN γ , MCP-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12p70, IL-13, IL-18, TNF α , GM-CSF, Eotaxin, GRO- α , IP-10, MIP-1 α , MIP-1 β , SDF-1 α , RANTES detected in multiplex analysis. TGF- β 1, TGF- β 2 were measured in ELISA-test. Depending on presence and activity of uveitis 3 groups of patients with BD were identified: group 1 – active uveitis, group 2 – remission of uveitis, group 3 – BD without ocular manifestations. After serological study 2 subgroups were highlighted in each group: a) patients with antibody markers of reactivation of at least one HHV, b) patients chronically infected with HHV, without serological signs of reactivation. Mean level and detection rate of cytokines and chemokines in patients with active uveitis (1a, 1b) and in remission (2a, 2b) were compared with patients without eye damage (3a, 3b). Chronic HHV infection (subgroup “b”) was compared with reactivation (subgroup “a”). A significant increase of MCP-1/CCL2, MIP-1 α /CCL3, MIP-1 β /CCL4, RANTES/CCL5, IP-10, SDF-1 α chemokines in serum, as well as IFN γ , TGF- β 1, and TGF- β 2 was observed in patients with uveitis (regardless of their activity) and HHV reactivation compared to patients without uveitis. Our data indicate that systemic production of cytokines and chemokines in BD patients and uveitis could be affected by the activity of chronic herpesvirus infections, and the greatest changes are related to chemokines.

Keywords: Behcet's disease, human herpes viruses, cytokines, hemokines, uveitis, HHV reactivation

Introduction

Behcet's disease (BD) is a systemic autoinflammatory-autoimmune disorder (chronic systemic vasculitis) of unknown etiology characterized by recurrent inflammatory lesions in various organs [12]. Inflammation of the vascular tract of the eye (uveitis) mainly involves posterior segment and is considered to be one of the most common symptoms in BD [3].

BD pathogenesis is multilayered and largely unclear. It is believed that hyperactivation of innate

and adaptive immunity can be triggered by infectious factors, particularly herpes viruses, which then cause production of a large number of cytokines and chemokines aimed at suppressing the pathogen life cycle [4, 10, 13]. An experimental *HSV*-induced BD model was developed in mice [10].

Human herpes viruses (*Human herpesviridae*, HHV) hold a pivotal place in the structure of infectious and infection-associated eye pathology, being not only etiological agents but also factors that trigger and aggravate the inflammatory process. It is accounted

for by their wide distribution in human population, lifelong persistence in infected host and tendency to reactivation, which often leads to manifestation of a new infection or exacerbation of existing disease. Persistent nature of *HHV* infections is associated with their ability to evade host immune system by modulating systemic and local cytokine production.

The aim of this study was to assess an effect of some reactivated *HHVs*: *Herpes simplex virus type 1, HSV-1, Herpes simplex virus type 2, HSV-2, Cytomegalovirus, CMV, Epstein-Barr virus, EBV*, on systemic level of chemokines, pro- and anti-inflammatory cytokines in BD with and without uveitis.

Materials and methods

Serum samples were collected from 116 chronically *HHV*-infected BD patients. Mean age ranged from 19 to 61 (mean age $36,6 \pm 9,9$) years, in 73 males and 43 females.

BD was diagnosed at the V.A. Nasonova Research Institute of Rheumatology in accordance with the International Criteria for Behcet's Disease (ICBD, 2014) [5]. Ophthalmological examination was performed at the Helmholtz National Medical Research Center of Eye Diseases.

During initial visit to ophthalmologist blood sample from cubital vein was collected. IgM- and IgG-antibodies against *HSV-1, HSV-2, CMV, EBV* structural late antigens (test systems VectoHSV-IgM, VectoHSV-1,2-IgG; VectoCMV-IgM, VectoCMV-IgG; VectoEBV-VCA-IgM, VectoEBV-NA-IgG, AO Vector-Best, Russia) were measured by using ELISA assay. Reactivation of chronic *HHV* infection was diagnosed by detecting specific IgG antibodies to nonstructural immediate-early antigens of *HSV-1, HSV-2, CMV* (test systems BioSet-aktiv-HSV, BioSet-aktiv-CMV, Bioservice Biotechnology Company Ltd, Moscow) and early antigens of *EBV* (test system VectoEBV-EA-IgG, AO Vector-Best, Russia) in serum samples [8].

Serum levels of IL-1 β , IFN γ , MCP-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12p70, IL-13, IL-18, TNF α , GM-CSF, Eotaxin, GRO- α , IP-10, MIP-1 α , MIP-1 β , SDF-1 α , RANTES were detected using the Procarta PlexTM test system "Human Th1/Th2 & Chemokine 1 Panel 20 plex" (eBioscience, Austria) on a MAGPIX analyzer (Luminex Corp., USA) [11]. TGF- β 1, TGF- β 2 cytokines were measured in ELISA-test (Invitrogen test systems, Thermo Fisher Scientific, Austria).

Statistical analysis was performed by using the STATISTICA 12.0 software. The data were processed by using Fisher's exact test and Student's T-test. Differences were considered significant at $p \leq 0.05$.

Results and discussion

116 BD patients were divided into 3 groups depending on verified uveitis and its activity: group 1 – active uveitis ($n = 41$); group 2 – remission of uveitis ($n = 64$); group 3 – BD without ocular manifestations ($n = 11$). In each group 2 subgroups were highlighted: a-patients with serological markers of reactivated chronic *HHV* infection, b-patients without serological markers of reactivated chronic *HHV* infection.

Reactivation of at least one herpes virus was detected in 50-58% of BD patients; albeit, these data did not significantly differ from those in subjects with endogenous uveitis of other etiology ($p > 0.05$).

Mean level and detection rate of cytokines and chemokines in patients with active uveitis (1a, 1b) and in remission state (2a, 2b) were compared with patients without eye involvement (3a, 3b), and inter-subgroup comparison was performed for patients with chronic *HHV* infection (subgroup "b" vs subgroup "a") (Table 1).

Because uveitis is one of the most common symptoms of BD, group 3 contained very few patients. Therefore, the quantitative data obtained for several cytokines and chemokines are insufficient for analysis, and requires to be further investigated (Table 1).

Changes in the systemic production of cytokines and chemokines in BD patients with uveitis associated with reactivation of chronic *HHV* infection.

Differences in systemic immune mediator production were more significant in patients with uveitis having serological markers of *HHV* reactivation, both with active uveitis (group 1a) and in remission phase (group 2a), when compared to patients without eye involvement (group 3). Levels of several immune mediators, mainly chemokines, were significantly increased, and detection rate of investigated cytokines changed much less frequently (Table 2).

Chemokines ensure increased influx of cells into site of inflammation. MCP-1, MIP-1 α , MIP-1 β , RANTES (CC-chemokines) induce chemotaxis of monocytes, T-lymphocytes, NK-cells, dendritic and other cells to the site of inflammation [1, 3, 4]. Both active uveitis and uveitis in remission phase are featured with increased systemic production of CCL-chemokines (MCP-1/CCL2, MIP-1 α /CCL3, MIP-1 β /CCL4, RANTES/CCL-5) and CXCL-chemokines (IP-10, SDF-1 α), as well as IFN γ , TGF- β 1 and TGF- β 2.

Histological findings in BD are characterized by non-granulomatous occlusive vasculitis with chronic perivascular infiltration by neutrophils and T-lymphocytes [3]. CXCL12 chemokine family includes angiostatic IP-10 chemokine and SDF-1 α – a chemoattractant for B-lymphocytes. According to

TABLE 1. SERUM LEVELS OF CYTOKINES (pg/ml) IN BD PATIENTS WITH AND WITHOUT UVEITIS IN CONDITIONS OF CHRONIC HHV INFECTION AND ITS REACTIVATION

Cytokines	Serum levels (pg/ml) and detection rate (%)					
	Group 1 BD with active uveitis (n = 41)		Group 2 BD with remission of uveitis (n = 64)		Group 3 BD without uveitis (n = 11)	
	1a reactivation of chronic HHV infection (n = 24)	1b chronic HHV infection (n = 17)	2a reactivation of chronic HHV infection (n = 40)	2b chronic HHV infection (n = 24)	3a reactivation of chronic HHV infection (n = 6)	3b chronic HHV infection (n = 5)
IFN γ	5.5 $\pm$ 0.7* p ^{1a-3a} = 0.042 14.75%** p ^{1a-2a} = 0.048#	3.8 $\pm$ 0.4 9-53%	7.0 $\pm$ 0.8 p ^{2a-3a} = 0.001 24.60%	4.9 $\pm$ 1.0 18.24%	3.8 $\pm$ 0.3 3.50%	3.3 $\pm$ 0.5 2.40%
MCP-1	87.0 $\pm$ 9.8 p ^{1a-3a} = 0.002 24.100%	62.7 $\pm$ 13.7 17.100%	99.3 $\pm$ 21.4 p ^{2a-3a} = 0.016 40.100%	91.5 $\pm$ 15.1 p ^{2b-3b} = 0.001 24.100%	38.3 $\pm$ 10.5 6.100%	32.2 $\pm$ 4.6 5.100%
IL-8	5.6 $\pm$ 1.1 6.25%	6.7 $\pm$ 5.8 2.12%	9.4 $\pm$ 5.9 6.15%	31.4 $\pm$ 16.3 7.29%	0	2.42 1.20%
IL-18	7.6 $\pm$ 1.3 p ^{1a-2a} = 0.02 17.71%	12.2 $\pm$ 4.4 11.65%	16.3 $\pm$ 3.4 p ^{2a-3a} = 0.040 25.66%	13.0 $\pm$ 4.3 19.79%	7.7 $\pm$ 2.1 6.100%	4.1 $\pm$ 3.4 3.60%
IL-1 β	1.40 $\pm$ 0.13 p ^{1a-2a} = 0.037 4.16.7%	9.8 $\pm$ 6.5 2.12%	1.0 $\pm$ 0.1 8.20%	1.4 $\pm$ 0.3 7.29%	0	5.7 1.20%
IL-12p70	3.40 $\pm$ 0.15 12.50%	3.7 $\pm$ 0.4 5.29%	4.9 $\pm$ 1.6 18.45%	3.4 $\pm$ 0.2 14.58%	0	2.8 1.20%
IL-2	16.0 $\pm$ 8.8 5.21%	13.9 $\pm$ 4.3 5.29%	9.9 $\pm$ 4.7 4.10% p ^{2a-2b} = 0.023	7.7 $\pm$ 2.2 8.33%	12.5 1.17%	21 1.20%
IL-4	10.5 $\pm$ 3.5 6.25%	17.6 $\pm$ 12.0 2.12%	6.9 $\pm$ 0.3 12.30%	9.0 $\pm$ 2.4 10.41%	0	5.7 1.20%
IL-5	0 0	14.7 1.6%	0 0	0 0	0	11.8 1.20%
IL-6	9.9 $\pm$ 3.1 p ^{1a-2a} = 0.008 6.25%	24.7 $\pm$ 1.5 p ^{1b-2b} = 0.037 2.12%	5.9 $\pm$ 2.2 12.30%	9.9 $\pm$ 2.6 9.38%	12.5 1.17%	0
TNF α	2.4 $\pm$ 0.4 10.42%	4.2 $\pm$ 1.8 3.18%	2.1 $\pm$ 0.3 14.35%	3.7 $\pm$ 0.9 12.50%	0.1 1.17%	10.4 $\pm$ 9.0 2.40%
GM-CSF	24.3 $\pm$ 4.8 3.13%	20.6 $\pm$ 14.4 2.12%	14.4 $\pm$ 1.8 2.5%	22.1 $\pm$ 4.5 4.17%	0	0
Eotaxin	57.3 $\pm$ 7.6 24.100%	65.2 $\pm$ 12.6 17.100%	71.1 $\pm$ 6.2 p ^{2a-3a} = 0.016 40.100%	61.8 $\pm$ 7.9 24.100%	43.1 $\pm$ 9.3 6.100%	42.0 $\pm$ 9.7 5.100%
GRO- α	27.5 $\pm$ 14.6 12.50%	30.8 $\pm$ 21 13.77%	12.3 $\pm$ 2.5 p ^{2a-3a} = 0.029 21.53%	39.5 $\pm$ 17.4 11.46%	6.4 $\pm$ 0.4 3.50%	8.9 $\pm$ 2.9 2.40%
IP-10	38.7 $\pm$ 11.0 p ^{1a-3a} = 0.006 24.100%	70.3 $\pm$ 43.0 17.100%	35.9 $\pm$ 6.2 p ^{2a-3a} = 0.0008 40.100%	43.3 $\pm$ 14.9 p ^{2b-3b} = 0.048 24.100%	12.8 $\pm$ 1.6 6.100%	12.1 $\pm$ 2.1 5.100%
MIP-1 α	13.0 $\pm$ 3.6 p ^{1a-3a} = 0.016 15.63%	11.0 $\pm$ 2.2 14.88%	9.1 $\pm$ 1.5 p ^{2a-3a} = 0.0001 27.68%	18.7 $\pm$ 4.9 13.54%	1.7 $\pm$ 0.6 3.50%	3.86 1.20%

Cytokines	Serum levels (pg/ml) and detection rate (%)					
	Group 1 BD with active uveitis (n = 41)		Group 2 BD with remission of uveitis (n = 64)		Group 3 BD without uveitis (n = 11)	
	1a reactivation of chronic HHV infection (n = 24)	1b chronic HHV infection (n = 17)	2a reactivation of chronic HHV infection (n = 40)	2b chronic HHV infection (n = 24)	3a reactivation of chronic HHV infection (n = 6)	3b chronic HHV infection (n = 5)
MIP-1 β	98 $\pm$ 16 $p^{1a-3a} = 0.002$ 23.96%	69.0 $\pm$ 19.5 17.100%	84.2 $\pm$ 11.5 $p^{2a-3a} = 0.002$ 39.98%	116.1 $\pm$ 23.2 $p^{2b-3b} = 0.025$ 23.96%	36.5 $\pm$ 8.7 6.100%	48.9 $\pm$ 16.2 5.100%
SDF-1 α	436 $\pm$ 27 $p^{1a-3a} = 0.0009$ 24.100%	418 $\pm$ 28 $p^{1b-3b} = 0.02$ 17.100%	444.5 $\pm$ 24.2 $p^{2a-3a} = 0.0001$ 40.100%	525.5 $\pm$ 57.3 $p^{2b-3b} = 0.003$ 24.100%	302.6 $\pm$ 23.2 6.100%	300.0 $\pm$ 37.3 5.100%
IL-13	2.2 $\pm$ 0.8 11.46%	2.8 $\pm$ 1.7 4.24%	2.3 $\pm$ 1.4 15.38%	1.2 $\pm$ 0.2 10.42%	1.8 1.17%	2.3 $\pm$ 1.9 2.40%
RANTES	64.6 $\pm$ 7.4 $p^{1a-3a} = 0.0015$ 24.100%	56.5 $\pm$ 7.0 17.100%	73.0 $\pm$ 6.6 $p^{2a-3a} < 0.0001$ 40.100%	72.8 $\pm$ 8.7 24.100%	37.5 $\pm$ 2.1 6.100%	56.9 $\pm$ 14.1 5.100%
TGF- β 1	29753 $\pm$ 3919 $p^{1a-3a} = 0.002$ 21.100%	25774 $\pm$ 4095 20.100%	26993 $\pm$ 2312 $p^{2a-3a} = 0.0002$ 34.100%	22967 $\pm$ 3484 24.100%	14561 $\pm$ 2019 8.100%	29296 $\pm$ 7510 4.100%
TGF- β 2	6700 $\pm$ 1354 $p^{1a-3a} = 0.002$ 20.100%	3485 $\pm$ 630 18.100%	5568 $\pm$ 697 $p^{2a-3a} = 0.0003$ 38.95%	4525 $\pm$ 664 23.100%	1417 $\pm$ 767 2.66%	2552 $\pm$ 2480 3.75%

Note. * 5.5 $\pm$ 0.07, mean serum cytokine concentration, pg/ml (M $\pm$ m); ** 14.75%, cytokine detection rate: absolute count and detection percentage; # $p^{1a-2a} = 0.048$, statistical significance between two comparison groups P value of 0.05 or less was considered as statistically significant.

TABLE 2. DIRECTION OF CHANGES IN SERUM LEVELS OF CYTOKINES AND CHEMOKINES IN PATIENTS WITH ACTIVE UVEITIS AND IN REMISSION PHASE IN COMPARISON WITH BD PATIENTS WITHOUT EYE INVOLVEMENT IN CONDITIONS OF CHRONIC HHV INFECTION AND ITS REACTIVATION

Serum levels of cytokines	Reactivation of chronic HHV infection		Chronic HHV infection	
	1a active uveitis	2a remission of uveitis	1b active uveitis	2b remission of uveitis
Increased*	IFN γ	IFN γ	SDF- α /CXCL12	MCP-1/CCL2
	MCP-1/CCL2	MCP-1/CCL2		IP-10/CXCL10
	MIP-1 α /CCL3	MIP-1 α /CCL3		MIP-1 β /CCL4
	MIP-1 β /CCL4	MIP-1 β /CCL4		SDF-1 α /CXCL12
	RANTES/CCL5	RANTES/CCL5		
	IP-10/CXCL10	IP-10/CXCL10		
	SDF-1 α /CXCL12	SDF-1 α /CXCL12		
	TGF- β 1	TGF- β 1		
	TGF- β 2	TGF- β 2		
		GRO- α /CXCL1		
		Eotaxin/CCL11		
		IL-18		
Decreased				

Note. * $p < 0.05$

some reports, IP-10 is involved in the pathogenesis affecting nervous system in BD [6]. Thus, its role in the pathogenesis of peripheral portion of the visual analyzer – retina, cannot be ruled out.

During reactivation of chronic *HHV* infection, levels of IFN γ significantly increased in serum of patients with BD and uveitis, regardless of the activity of intraocular inflammation (groups 1a, 2a), when compared with patients without uveitis (group 3a). Increase of serum IFN γ level in patients with active uveitis associated with BD was reported by Zhu Y. et al. [14]. Authors discussed a role of infections as a factor stimulating T-lymphocytes to intensively secrete several cytokines, although they did not perform such analysis. We believe that increased levels of serum IFN γ in patients with serological markers of reactivated chronic *HHV* infection is necessary to suppress viral replication. Clinical remission of uveitis cannot completely exclude subclinical activity of intraocular inflammation, which is often supported by long-term replication of herpes viruses.

A significant increase of TGF- β 1 and TGF- β 2 serum levels in patients with active and inactive uveitis was observed only in association with *HHV* reactivation. TGF- β is a pleiotropic context-dependent cytokine which can induce development of Th17 or Treg-cells [76].

In remission of uveitis (group 2a) we detected an increased level of the two other angiogenic chemokines: Eotaxin and GRO- α , as well as immunoregulatory IL-18 cytokine, which induces IFN γ production, thus determining its important role in the host anti-infectious defense [2].

Belguendous H. et al. [2] found a significant increase of serum IL-18 in patients with active vs inactive uveitis. Corticosteroid therapy reduced IL-18 levels and decreased disease activity. It was considered that IL-18 might serve a good marker for monitoring activity of uveitis and related treatment

efficacy. However, in our study patients with active uveitis and *HHV* reactivation had significantly higher serum IL-18 level than patients with remission of uveitis. This might result from intensive corticosteroid treatment during the period of active uveitis.

Systemic production of cytokines and chemokines in BD with uveitis coupled to chronic *HHV* without reactivation differed from patients without eye involvement by showing fewer range of produced immune mediators: systemic level of SDF- α was significantly higher both in active uveitis and in remission stage, than in patients without uveitis. Patients with remission of uveitis also showed significant increase in 2 other immune mediators: IP-10 and MIP-1 β . Serum SDF- α turned out to be the only chemokine which was significantly elevated in all examined BD subgroups with uveitis than in the corresponding subgroups without eye involvement. SDF- α has been shown to be crucially involved in both physiological and pathological processes [9].

In contrast to BD without uveitis, patients with remission of uveitis produced a wider range of immune mediators than those with active uveitis, which is hard to interpret precisely but their protective role by lowering the risk of ocular inflammation relapse with underlying subclinical herpes viruses replication, cannot be ruled out.

It is noteworthy that all patients with uveitis showed increased concentration of GRO- α chemokine compared to patients without uveitis. Besides its oncogenic properties, GRO- α is a pro-angiogenic chemokine and plays an important role in inflammation and wound healing.

Thus, our data indicate that the systemic production of cytokines and chemokines in BD patients with uveitis is affected by the activity of chronic herpesvirus infections, and the most prominent changes are related to chemokines.

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РОЛЬ GSK-3 В Wnt/ β -CATENIN-СИГНАЛЬНОМ ПУТИ ПРИ ОЖИРЕНИИ

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Резюме. На механизм адипогенеза оказывают влияние многочисленное количество факторов, важными из них являются компоненты Wnt-сигнального пути. Поиск возможных маркеров развития заболеваний, связанных с ожирением, обусловил интерес к изучению GSK-3 (glycogensynthase kinase), β -катенина. GSK-3 β — внутриклеточная серин/треонинкиназа, обнаружена в цитоплазме, ядре, митохондриях, синтезируется во всех тканях организма и участвует в регуляции таких процессов, как метаболизм, клеточная пролиферация, апоптоз и другие. GSK-3 β в активном состоянии фосфорилирует и ингибирует гликогенсинтазу. Когда инсулин связывается с рецептором на клетке через инозитол-3-фосфат, то активация протеинкиназа B (Akt1) активируется и, в свою очередь, фосфорилирует и ингибирует GSK-3 β . Также GSK-3 β участвует в регуляции обмена глюкозы. Важная функция GSK-3 β — ингибирование белка β -катенина. Когда клетка GSK-3 β в комплексе с белками APC и Axin покоится, то происходит связывание и фосфорилирование транскрипционного фактора β -катенина, затем его убиквитинирование и деградация. Когда Wnt действует на клетку белков, то белок Dvl активируется, связывается с GSK-3 β , высвобождая β -катенин, что препятствует его распаду. При этом роль GSK3 α/β в воспалительной реакции адипоцитов до сих пор полностью не исследована, поэтому представляется перспективным изучение места GSK-3 в Wnt/ β -catenin-сигнальном пути при ожирении.

Целью исследования явилась оценка активности компонентов Wnt-сигнального пути у пациентов с ожирением посредством определения уровня GSK-3 и β -катенина в сыворотке крови. В исследование были включены 32 пациента, у которых было определено ожирение I–III степени с прогрессирующими формами, сахарный диабет отсутствует. Чтобы определить концентрацию GSK-3 α , GSK-3 β и β -катенина в сыворотке крови, был использован метод иммуноферментного анализа. Данные представлены в виде абсолютного и относительного (%) числа больных; среднего арифметического; медианы, 1-го и 3-го квартилей — Me ($Q_{0,25}$ – $Q_{0,75}$). В сыворотке крови пациентов, страдающих ожирением, выявлено повышение уровня GSK-3 α (785 (371–1317,5) пг/мл) в 7,5 раз по сравнению со здоровыми лицами 105 (102,5–110) пг/мл, ($p < 0,001$), также повышение уровня GSK-3 β в сыворотке крови, уровень которого у пациентов с ожирением составил 295 (190–695) пг/мл, что на 18,3% превышало аналогичные показатели, полученные у здоровых лиц 241 (218,75–287,5) пг/мл, $p = 0,111$. Была отмечена тенденция к увеличению количества GSK-3 в зависимости от степени ожирения, при этом часто наблюдается снижение β -катенина, что согласуется с исследованиями ряда авторов. Эти данные можно рассматривать в качестве прогностического критерия течения патологических процессов при ожирении.

Ключевые слова: ожирение, GSK-3 α , GSK-3 β , β -катенин, Wnt-сигнальный путь

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ROLE OF GSK-3 IN Wnt/ β -CATENIN SIGNALING PATHWAY IN OBESITY

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Abstract. The complexity of the adipogenesis mechanism results from the impact of multiple cues, among which an important place is held by the components of the Wnt signaling pathway. The search for potential markers of the development of diseases related to obesity aroused an interest in the study of GSK-3 (glycogen synthase kinase), β -catenin. GSK-3 β is an intracellular serine / threonine kinase found in the cytoplasm, nucleus, mitochondria, synthesized in all body tissues and involved in regulating metabolic processes, cell proliferation, apoptosis etc. The first of the discovered functions of GSK-3 β was the regulation of glycogen synthesis. Active GSK-3 β phosphorylates and thereby inhibits glycogen synthase. As a result of the insulin binding to the cell receptor via inositol-3-phosphate, protein kinase B (Akt1) is activated, which, in turn, phosphorylates and inhibits GSK-3 β . In addition, GSK-3 β is involved in the regulating glucose metabolism. The most important function of GSK-3 β is the inhibition of the β -catenin protein. In a resting cell, GSK-3 β in complex with the APC and Axin proteins binds and phosphorylates the β -catenin transcription factor, which leads to its ubiquitination and degradation. When Wnt proteins act on the cell, the Dvl protein is activated, which, by binding to GSK-3 β , releases β -catenin, preventing its degradation, however, the role of GSK-3 α/β in the adipocyte inflammatory response has not yet been fully investigated, therefore it seems promising to study the role of GSK-3 in the Wnt/ β -catenin signaling pathway in obesity.

The aim of the study was to assess the activity of the components of the Wnt signaling pathway in obese patients by measuring the serum level of GSK-3 and β -catenin. There were enrolled 32 patients with progressive forms of I-III degree obesity in the absence of diabetes mellitus. The concentration of serum GSK-3 α , GSK-3 β , and β -catenin was measured by enzyme-linked immunoassay. Data are presented as absolute and relative (%) number of patients; arithmetic mean; medians, 1 and 3 quartiles – Me ($Q_{0.25}$ - $Q_{0.75}$). Obese patients contained a 7.5-fold increased serum level of GSK-3 α (785 (371-1317.5) pg/ml) compared to healthy individuals 105 (102.5-110) pg/ml, ($p < 0.001$), paralleled with increased amount of GSK-3 β , which level in obese patients was 295 (190-695) pg/ml, which is by 18.3% higher than those in healthy individuals 241 (218.75-287.5) pg/ml, $p = 0.111$. Amount of GSK-3 depending on the degree of obesity tended to increase, most often coupled to decreased β -catenin level which is consistent with the literature data and can be considered as a prognostic criterion for the course of pathological processes in obesity.

Keywords: obesity, GSK-3 α , GSK-3 β , β -catenin, Wnt- signaling pathway

Introduction

“The Wnt signaling pathway plays a key role in many processes, including cell proliferation, tissue regeneration, and embryonic development. Impaired signaling is involved in the pathogenesis of various human diseases. Two signaling pathways in the WNT system exist: canonical and non-canonical. The canonical pathway involves activation of the β -catenin transcriptional coactivator. The pathways activated by WNT ligands independently of β -catenin are classified as non-canonical WNT pathways. A key element of the canonical Wnt/ β -catenin pathway is the regulation of the β -catenin protein, which acts as a transcriptional cofactor and performs dual functions as well as also participates in cell adhesion, forming a stable complex with cell adhesion molecules of the cadherin family. In the absence of WNT ligands, cytosolic β -catenin interacts with other components of the destruction

complex, including Axin 1, adenomatous polyposis coli (APC), glycogen synthase kinase-3 (GSK-3), casein kinase 1 (CK1), protein phosphatase 2A (PP2A), and protein containing β transducin repeat E3 –ubiquitin ligase (β -TrCP)” [1, 5].

“Glycogen synthase kinase 3 (GSK-3) was first described more than 30 years ago as an enzyme phosphorylating glycogen synthase after ligating insulin and thereby inhibits its activity. Later, the isoforms of this protein were discovered: α and β . The GSK-3 α isoform is highly homologous to GSK-3 β (98% identity in the kinase domain)” [13].

“The aberrant activity of GSK-3 is associated with the pathogenesis of many diseases, such as atherosclerosis, pathology of the cardiovascular system, neurological disorders, oncopathology, immune disorders, etc. It is known that the inhibitory activity of GSK-3 β leads to decreased glycogen synthesis in the

liver and muscles, as well as increased blood glucose levels, therefore GSK-3 β is presumably associated with the pathogenesis of diseases such as diabetes mellitus and obesity” [8, 10].

According to current concepts [5, 11], WNT/ β -catenin signaling inhibits adipogenesis. Studies carried out by foreign authors [6, 11] revealed the transmission of WNT signals in the regulation of adipocyte differentiation. “In particular, activation of the canonical pathway in pre-adipocytes by overexpression of Wnt1 or β -catenin mutant with a defect in GSK3 β phosphorylation has been shown to inhibit adipogenesis. Similarly, treatment with GSK3 β pharmacological inhibitors blocked adipocyte differentiation” [7].

It is known that IL-6 is not only a pro-inflammatory cytokine, but also regulates energy and glucose metabolism. A group of scientists [15] in experiments with pigs found that blocking IL-6 trans-signaling can prevent the recruitment of macrophages from adipose tissue with a high fat content, but does not induce body weight gain and improved insulin resistance. “Overexpression of the IL-6 gene induces a significantly reduced body weight, improves insulin sensitivity, and increases the mRNA level of lipolysis genes. There is a strong link between IL-6 and obesity-associated inflammation. Presumably, the mechanism of Gsk3 β expression regulated by IL-6 in LPS (lipopolysaccharide)-induced pig adipocytes is as follows: LPS increased the level of Gsk3 β phosphorylation and then inhibited the activity of Gsk3 β kinase. Inhibition of Gsk3 β attenuated LPS-induced IL-6 production in porcine adipocytes. It is believed that the infection and inflammation by their ability to increase pro-inflammatory cytokines, chemokines and adhesion molecules play a key role in the pathophysiology of insulin resistance and type 2 diabetes. Studies carried out by the group of authors [13, 15] have shown that GSK-3 plays a central role in the regulating such inflammation. In particular, it was found that inhibition of GSK3 α/β suppresses inflammation in response to various stimuli such as TNF- α , IL-1 β and LPS in vitro”. Thus, it appears that GSK-3 plays a role in many inflammatory diseases. For example, according to Lappas M. et al., GSK-3 activity increases in adipose tissue and skeletal muscles of pregnant women with gestational diabetes mellitus and regulates proinflammatory mediators caused by infections and inflammations [8, 9, 10].

Thus, according to studies by a number of authors [7, 8, 10, 13, 15], an increase in GSK3 activity leads to the development of insulin resistance; however, the role of GSK3 α/β in the inflammatory response of adipocytes has not yet been fully investigated, therefore it seems promising to study the place of GSK-3 in the Wnt/ β -catenin signaling pathway in obesity. **The aim of the study** was to assess the activity

of the Wnt signaling pathway in obese patients by determining the level of GSK-3 in the blood serum.

Materials and methods

There were enrolled 32 patients with progressive forms of I-III degree obesity in the absence of diabetes mellitus, aged 40 ± 10 years. To determine serum concentration of GSK-3 α , GSK-3 β and β -catenin, considered as physiological normal range, we examined 20 sex- and age-matched healthy individuals in the control group free of chronic diseases, lacking signs of acute infectious diseases upon examination and not registered with dispensaries.

Body mass index was calculated by using the Quetelet formula: BMI = body weight (kg)/height (m²). Serum concentrations of GSK-3 α , GSK-3 β and β -catenin were determined by enzyme-linked immunosorbent assay (ELISA) using Human GSK kits, Sunlong Biotech Co., Ltd, China; Human β -catenin, Sunlong Biotech Co., Ltd, China.

The results were statistically processed using the Microsoft Excel XP software package. Data are presented as absolute and relative (%) number of patients; arithmetic mean; medians, 1 and 3 quartiles – Me ($Q_{0.25}$ – $Q_{0.75}$). Differences between groups in quantitative parameters were calculated by using the nonparametric Mann–Whitney U-test, were considered significant at $p < 0.05$.

The exclusion criteria from the study were as follows: age of the patients under 18 and over 65 years old, concomitant exacerbated or decompensated somatic diseases, concomitant acute respiratory infections, pregnancy, sepsis, immunosuppression due to neoplasms or HIV infection, active viral hepatitis, patient refusal to participate. The study, after obtaining informed consent from each patient, was carried out in accordance with the Good Clinical Practice and the principles of the Declaration of Helsinki, approved by the Ethics Committee of FSBEI HE “Oryol State University named after I.S. Turgenev”.

Results and discussion

As a result of the examination of obese patients, it was found that 18 (56.25%) of them had degree 1 obesity, 12 patients (37.5%) – degree 2, 2 patients (6.25%) – degree 3. Analyzing the anthropometric data showed that the average height rate was 170 ± 15 cm, weight – 95 ± 15 kg, respectively.

According to the data of general clinical, laboratory and instrumental studies, it was found that accelerated ESR and eosinophilia was revealed in the general blood test in 2 (6.25%) patients, in one person (3.125%) – increased leukocyte count; 2 (6.25%) – altered general urine test containing increased leukocyte count, presence of bacteria and salts; biochemical blood assay analysis in 6 (18.75%) subjects showed increased total cholesterol, 4 patients

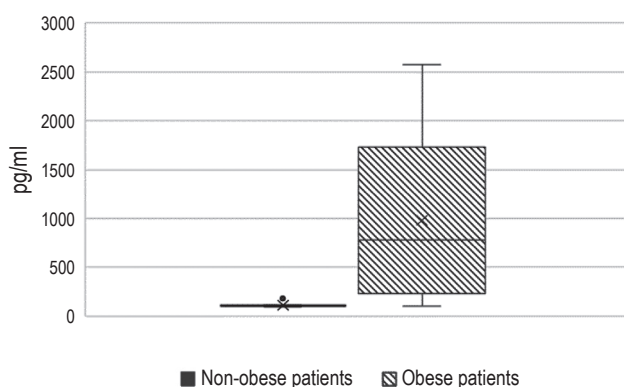


Figure 1. GSK-3 α concentration in blood serum (pg/ml)

Note. For distributions that differ from normal in Figures 1, 2, Me (median) is given; 25-75 percentiles (upper and lower quartiles $Q_{0.25}$ - $Q_{0.75}$); minimum and maximum sample values; outlier / single data point.

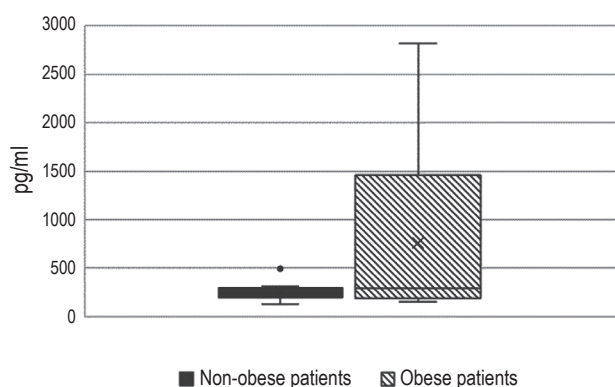


Figure 2. GSK-3 β concentration in blood serum (pg/ml)

Note. As for Figure 1.

TABLE 1. GSK-3 α AND GSK-3 β CONCENTRATION IN BLOOD SERUM DEPENDING ON THE PATIENTS DEGREE OF OBESITY (pg/ml)

Protein (m)		Study groups by degree of obesity		p
		I degree	II degree	
GSK-3 α		690.111	1159.167	0.056
GSK-3 β		663.333	726.667	0.624
β -catenin		168.78	126.25	0.004
number of patients	n	18	12	
	%	56.25	37.5	
Protein (m)		I degree	III degree	p
GSK-3 α		690.111	2567.0	0.023
GSK-3 β		663.333	1850.0	0.128
β -catenin		168.78	130.0	0.205
number of patients	n	18	2	
	%	56.25	6.25	
Protein (m)		II degree	III degree	p
GSK-3 α		1159.167	2567.0	0.043
GSK-3 β		726.667	1850.0	0.133
β -catenin		126.25	130.0	0.455
number of patients	n	12	2	
	%	37.5	6.25	

Note. p, significance of differences between indicators is calculated according to the nonparametric Mann-Whitney U test, the differences are considered reliable and statistically significant when $p < 0.05$; m, sclerostin and β -catenin, concentration mean (pg/ml).

(12.5%) – increased level of bilirubin, 3 patients (9.38%) – increased transaminases (ALT, AST) level.

In patients with complaints of epigastric pain (14 people), EGD was performed, which data confirmed chronic gastritis in 14 (43.75%) patients. At the same time, the *Helicobacter pylori* bacterium was detected in 6 (18.75%) patients (the *Helipil* test system for invasive rapid diagnosis of *Helicobacter pylori* infection by the urease activity of a biopsy sample obtained during an endoscopic examination of the gastric mucosa). According to the ultrasound of the abdominal cavity organs, fatty hepatosis was confirmed in 13 (40.625%) patients, biliary dyskinesia in 5 (15.625%) patients.

We compared the levels of GSK-3 α and GSK-3 β in obese patients (32 people) and healthy people with normal body weight (20 subjects). Serum GSK-3 α levels were measured by enzyme-linked immunosorbent assay (Figure 1), and found significant changes such as increased GSK-3 α in obese individuals (785 (371-1317.5) pg/ml) by 7.5-fold compared to healthy individuals 105 (102.5-110) pg/ml, ($p < 0.001$) (Figure 1).

The concentration of GSK-3 β was determined by the method of enzyme-linked immunosorbent assay in the blood serum of patients (Figure 2). As shown by the results of studies, obese patients had a wide variability in concentration of serum GSK-3 β , the level of which was 295 (190-695) pg/ml, this is 18.3% higher than in healthy individuals 241 (218.75-287.5) pg/ml, $p = 0.111$ (Figure 2).

As noted above, activation of the Wnt/ β -catenin signaling pathway inhibits adipogenesis. Impaired Wnt/ β -catenin signaling leads to spontaneous adipogenesis [2, 4, 6, 11]. Considering the important role of β -catenin in the regulation of adipogenesis, we were also interested in evaluating its production in obese patients. According to our previous studies [9], it was convincingly established that the mean β -catenin in the blood serum in obese people was higher (150.406 ± 6.41584) than in healthy individuals (87.25 ± 36.29348), $p < 0.001$. In overweight patients 141 (128.5-185) pg/ml, and in healthy individuals 66.0 (59.5-125), a wide variability in serum β -catenin concentrations was found, $p < 0.001$.

The table shows the concentration level of GSK-3 α , GSK-3 β and β -catenin depending on the degree of obesity (table 1). The study found that patients with grade III obesity had a significantly increased level of GSK-3 α ($p = 0.043$) and increased level of GSK-3 β ($p = 0.133$) compared to those with grade II obesity, and insignificant changes in the concentration β -catenin ($p = 0.455$). Patients with grade III obesity had significantly increased level of GSK-3 α ($p = 0.023$) and increased level of GSK-3 β ($p = 0.128$) compared to those with grade I obesity, decreased concentration of β -catenin was also noted

($p = 0.205$). In patients with grade II obesity had significantly increased level of GSK-3 α ($p = 0.056$) and slightly increased level of GSK-3 β ($p = 0.624$), simultaneously with strongly significantly decreased concentration of β -catenin ($p = 0.004$) (Table 1).

As mentioned above [8, 10, 13, 15] GSK-3 is a kinase involved in the insulin signaling pathway to control glycogen metabolism. However, GSK-3 is currently recognized as a multifunctional kinase that regulates a number of additional cellular functions. According to some studies [3, 12], small molecular weight inhibitors of GSK-3 have beneficial metabolic effects in rodents, including prevented obesity caused by overfeeding and improving glucose tolerance. Thus, in animal models it was revealed that activation of GSK-3 β stimulates adipogenesis. Therefore, it seemed to us interesting to compare the serum concentrations of GSK-3 α and GSK-3 β in obese and healthy individuals. As a result, an increased level of GSK-3 α and GSK-3 β was revealed in obese individuals compared with healthy individuals, which is consistent with other publications [3, 10, 12].

It was shown that GSK-3 inhibitors increased the expression and nuclear translocation of β -catenin. GSK-3 phosphorylates β -catenin, causing its destabilization and degradation to maintain low cytosolic / nuclear β -catenin levels. The accumulated nuclear levels of β -catenin in cells treated with GSK-3 inhibitors indicate that GSK-3 activity is inhibited. In connection with the above data, our interest was aimed at assessing serum levels of GSK-3 α , GSK-3 β , and β -catenin in patients with different degree of obesity. Our data on changes in concentration of GSK-3 α , GSK-3 β and β -catenin depending on the degree of obesity may indicate that the Wnt/ β -catenin signaling pathway involving GSK-3 plays a negative regulatory role in limiting adipocyte differentiation, i.e., interruption of Wnt/ β -catenin signaling promotes adipogenesis, which is consistent with the results of several studies [2, 6, 10, 11, 14].

Conclusion

Thus, obese patients showed a wide variability in the level of the GSK-3 α and GSK-3 β proteins, which tended to increase depending on the degree of obesity. Moreover, increased level of serum GSK-3 α and GSK-3 β occurred in parallel with decreased β -catenin, which is consistent with the literature data and can be considered as a prognostic criterion for the course of pathological processes in obesity. Since the Wnt/ β -catenin-dependent pathway inhibits adipogenesis, its activation represents an attractive target for drug development to combat obesity and associated metabolic complications.

There is no conflict of interest.

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ИММУНОЛОГИЧЕСКАЯ РЕАКТИВНОСТЬ ОРГАНИЗМА ПРИ ОПИЙНОЙ НАРКОМАНИИ

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Резюме. В связи с общей тенденцией изменения реактивности при многих экзогенных и эндогенных заболеваниях, в настоящее время все большее внимание уделяется изменению иммунологической реактивности при наркомании. Целесообразность оценки состояния иммунных механизмов при опийной наркомании определяется необходимостью прогнозирования их течения и исхода. Цель исследования — изучение иммунологической реактивности организма у больных опийной наркоманией в состоянии абстиненции. С этой целью были проведены клинко-иммунологические исследования у 80 больных, употребляющих препараты опийной группы. Длительность заболевания колебалась в пределах 0,5-19 лет. Из них давность заболевания до 3 лет — у 28 больных (I группа), а свыше 3 лет — 52 больных (II группа). Количество исследуемых в контрольной группе составляло $n = 50$. Была проведена оценка психического, наркологического, соматического, неврологического статуса. Исследованы общие клинические, биохимические и иммунологические показатели. В результате данного исследования, было установлено, что у больных I группы наблюдалась более выраженная Т-лимфоцитопения. Выявлено стойкое повышение величины сывороточного IgM как в динамике абстиненции, так и в зависимости от давности заболевания, что может говорить о напряжении гуморального звена иммунитета при опийной наркомании. Кроме того, по мере увеличения длительности хронической наркотизации отмечается тенденция к повышению относительного количества В-лимфоцитов. Таким образом, можно сделать такие выводы, что у больных опийной наркоманией в состоянии абстиненции развивается Т-лимфоцитопения; с увеличением длительности заболевания наблюдается повышение в 2-2,6 раза уровня IgM; выявленные изменения иммунологической реактивности организма предполагают необходимость включения иммунокорректирующей терапии в комплекс лечебных мероприятий при опийной наркомании.

Ключевые слова: иммунологическая реактивность, опиомания, психиатрия и наркология, реактивность организма, опийная зависимость

BODY IMMUNOLOGICAL REACTIVITY IN OPIOMANIA

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Abstract. Due to the general trend of changes in reactivity in many exogenous and endogenous diseases, more and more attention is currently being paid to changes in immunological reactivity in drug addiction. The expediency of assessing the state of immune mechanisms in opioid addiction is determined by the need

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to predict their course and outcome. The aim of the study was to study the immunological reactivity of the body in patients with opium addiction in a stage of abstinence. For this purpose, clinical and immunological studies were conducted in 80 patients who use opioid drugs. The duration of the disease ranged from 0.5-19 years. Of these, the disease duration is up to 3 years – 28 patients (group I), and over 3 years – 52 patients (group II). The number of subjects in the control group was $n = 50$. Authors carried out the assessment of the mental, narcological, somatic, and neurological status. In addition, they have studied the general clinical, biochemical and immunological parameters. As a result of this study, it was found, that patients of group I had more pronounced T-lymphocytopenia. A persistent increase in the value of serum IgM was revealed both in the dynamics of abstinence and depending on the duration of the disease, which may indicate a strain on the humoral link of immunity in opium addiction. In addition, as the duration of chronic narcotization increases, there is a tendency to increase the relative number of B-lymphocytes. Thus, at the patients with opium addiction in a state of abstinence develop T-lymphocytopenia. Moreover, with an increase in the duration of the disease, an increase in the level of IgM by 2-2.6 times. The revealed changes in the immunological reactivity of the organism suggest the need to include immunocorrective therapy in the complex of therapeutic measures for opium addiction.

Keywords: immunological reactivity, opiomania, psychiatry and narcology, organism reactivity, opiate addiction

Introduction

In connection with the general trend of changes in reactivity in many exogenous and endogenous diseases, more attention is now paid to changes in immunological reactivity during drug addiction. The feasibility of assessing the state of immune mechanisms is accounted for by the need to predict their course and outcome [2, 8].

A number of studies showed that patients with opium addiction have functional insufficiency of polymorphonuclear leukocytes and T-lymphocytes, a decreased percentage of peripheral blood T- and B-lymphocytes as well as macrophages; decreased natural killer cell activity and antibody-dependent cell-mediated cytotoxicity; autoimmune disorders are noted [2, 4, 7].

On the contrary, other studies showed increased absolute number of peripheral blood T-lymphocytes in drug addicts, and that both during the period of abstinence and period of remission IgM level and T-lymphocyte count were significantly increased [2]. In addition, recently a growing interest have been paid to detection of blood anti-morphine antibodies in opium addiction. A significant increase in the level of anti-morphine antibodies was not typical for other types of drug addiction [1]. A direct addiction between degree of absorption of complement by blood serums in the presence of morphine and severity of drug tolerance in patients with opium addiction was recorded [3].

The aim of the study was to study the immunological reactivity in patients with opiate addiction during state of abstinence.

Materials and methods

Clinical and immunological studies were carried out in 80 patients (64 males and 16 females) who use homemade opium group preparations. All 80 patients were injected with drugs intravenously, clinically diagnosed with stage 2 of the disease with

detailed clinical picture represented by a large drug addiction syndrome. The patients were hospitalized to narcological hospital. The age of the patients ranged from 15 to 40 years, the duration of the disease ranged from 0.5 to 19 years. Of these, the duration of the disease up to 3 years was observed in 28 patients (group 1), and over 3 years – in 52 patients (group 2). Assessment of mental, narcological, somatic, and neurological status was carried out. General clinical, biochemical and immunological parameters were studied.

Immunological studies were carried out dynamically: upon admission to the hospital, after 7 days and after 14 days along with ongoing withdrawal syndrome therapy. In the blood, T- and B-lymphocytes, subpopulations of T-cells, immunoglobulins of classes A, G, M were determined by a unified method. The control group consisted of 50 age- and sex-matched apparently healthy individuals.

Results and discussion

The results of examining parameters of cellular and humoral immunity in patients with opium addiction are presented in Table 1. Upon admission to the clinic, a decrease in T-lymphocytes by 24.4% was found compared to the control group ($p < 0.05$). Analysis of T-cell subpopulations in the examined patients revealed a 26.7% decrease in T-helper cells and by 37.5% in T-cytotoxic ($p < 0.05$). At the same time, there was an increase in the number of null cells by 25% ($p < 0.05$).

Assessing humoral immunity indices revealed that IgM level shifts were more informative, which increased by 2.5-fold than in the control group ($p < 0.05$), which agrees with the literature data.

According to some publications, an increased serum IgM level in opium addicts was more common than in other types of drug addiction, which, apparently, can serve as a hallmark of opium addiction [5]. Serum IgA and IgG levels showed no apparent deviation from normal values.

Despite the ongoing therapy of withdrawal symptoms on day 7 and 14, the body immunological

reactivity did not recover, while T-lymphocytopenia persisted, and decreased level of immunoregulatory T-cells was observed (Table 1).

We analyzed parameters of cellular and humoral immunity in opium addiction in a state of abstinence, depending on the duration of the disease.

The data shown in Table 2 revealed that patients in group 1 had more pronounced T-lymphocytopenia. A persistent increase in serum IgM level was found both dynamically during abstinence and depended on the duration of the disease, which may indicate the tension of the humoral immune arm in opium addiction. In addition, along with increasing duration of chronic anesthesia, there is a tendency to increase percentage of B-lymphocytes.

Taking into account the data on detected serum anti-brain antibodies in opium addiction, it can be assumed that, namely, anti-brain antibodies underlie increased level of serum IgM. It is known, that IgM exerting a high pathogenic potential reacted with polymorphonuclear leukocytes to promote development of local chronic antigenic stimulation of multilayered immune inflammation and can aggravate

destructive processes. The changes we described in some immunological parameters are consistent with the data of experimental studies showing that long-term administration of morphine to animals stimulates the formation of specific antibodies, which neutralize the pharmacological drug effects. This may indicate about importance of antibody formation in development of opiate addiction and suggest an important role of body immunological changes in development of drug addiction [6]. The latter is confirmed by studies showing that the appearance of high levels of antigen-specific cells and antibodies that bind neurotransmitters has a significant effect on the course of opium addiction. Summing up, we can say that patients with opium addiction in a state of abstinence develop T-lymphocytopenia; with increased duration of the disease, serum IgM level increased by 2-2.6-fold; changes revealed in organism immunological reactivity suggest a need to include immunocorrective therapy in the set of therapeutic measures for opium addiction.

TABLE 1. INDICATORS OF CELLULAR AND HUMORAL IMMUNITY IN PATIENTS WITH OPIUM ADDICTION IN A STATE OF ABSTINENCE (M±m)

Indicators	Control group, n = 50	Experimental group, n = 80		
		Upon admission to the hospital	7 th day	14 th day
T-lymphocytes (%)	53.1±1.5	40.17±2.86*	42.0±4.9*	34.5±6.1*
B-lymphocytes (%)	10.2±0.9	12.30±1.29	10.3±1.3	8.8±1.8
O-cells (%)	36.60±1.97	45.8±2.7*	30.9±4.4	31.5±5.7
T-helpers (%)	42.30±1.31	31.00±2.18*	23.3±4.4**	30.6±3.9*
T-cytotoxic (%)	18.70±0.62	13.60±1.35*	11.30±1.54**	10.25±2.10**
Th/Tc	2.70±0.17	2.9±0.3	2.5±0.4	1.9±0.4*
IgA (g/l)	1.76±0.18	1.71±0.16	1.79±0.16	0.8±0.2*
IgM (g/l)	0.90±0.07	2.3±0.2**	1.9±0.3*	1.01±0.50*
IgG (g/l)	8.38±0.58	9.6±1.2	8.7±1.4	10.7±2.2

Note. *, reliability of differences in relation to the control group – $p < 0.05^*$, $p < 0.001^{**}$.

TABLE 2. INDICATORS OF CELLULAR AND HUMORAL IMMUNITY IN OPIUM ADDICTION IN A STATE OF ABSTINENCE, DEPENDING ON THE DURATION OF THE DISEASE (M±m)

Indicators	Control group, n = 50	1 st group (up to 3 years), n = 28	2 nd group (over 3 years), n = 52
T-lymphocytes (%)	53.1±1.5	37.5±4.1*	46.2±2.7*
B-lymphocytes (%)	10.2±0.9	10.8±0.9	13.1±1.8
O-cells (%)	36.60±1.97	52.5±4.4*	42.6±3.2
T-helpers (%)	42.30±1.31	28.4±3.9*	31.8±2.5*
T-cytotoxic (%)	18.70±0.62	10.23±1.90**	15.7±1.6
IgA (g/l)	1.76±0.18	1.3±0.3	1.7±0.2
IgM (g/l)	0.90±0.07	1.9±0.3*	2.4±0.3**
IgG (g/l)	8.38±0.58	8.12±1.50	10.57±1.50

Note. *, reliability of differences in relation to the control group – $p < 0.05^*$, $p < 0.001^{**}$.

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ПЕНТОКСИФИЛЛИН СПОСОБСТВУЕТ ПОВЫШЕНИЮ *IN VITRO* ПРОТИВООПУХОЛЕВОЙ ЦИТОТОКСИЧНОСТИ Т-КЛЕТОК БОЛЬНЫХ РАКОМ МОЛОЧНОЙ ЖЕЛЕЗЫ

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Резюме. Транскрипционный фактор NF-κB контролирует экспрессию генов, ответственных за реализацию клеточного цикла, апоптоза и ряда других иммунорегуляторных функций. После обнаружения возможности блокирования опухолевого роста через подавление активности NF-κB был обнаружен ряд неспецифических ингибиторов NF-κB, применение которых, однако, было осложнено множественными побочными эффектами, такими как системное воспаление, вызванное чрезмерной экспрессией интерлейкина-1β, или не связанными с иммунитетом осложнениями, которые могут быть следствием ингибирования субъединицы p65 NF-κB, играющей центральную роль в органогенезе и воспалении. Ингибирование же субъединицы c-Rel приводит к ограничению роста опухоли за счет модуляции свойств Т-регуляторных клеток.

В 2017 году Grinberg-Bleyer и соавт. проверили гипотезу о том, что субъединицу c-Rel можно избирательно ингибировать пентоксифиллином, регулируя тем самым активность Treg в ходе опухолевого роста. Авторы показали, что пентоксифиллин действительно может вызывать избирательную деградацию c-Rel, не влияя на p65, и предположили, что подобное воздействие может быть эффективным для подавления опухолевого роста. В связи этим нами была поставлена цель исследовать *in vitro* влияние пентоксифиллина на функции и противоопухолевый цитотоксический потенциал Т-клеток пациентов со злокачественными опухолями.

В качестве объекта исследования использовались мононуклеарные клетки периферической венозной крови 25 пациенток с первичным РМЖ (отсутствие метастаз), 15 пациенток с прогрессирующим РМЖ (наличие метастаз), а также 25 условно здоровых женщин без диагностированной патологии молочных желез. Исследование проводилось с добровольного информированного согласия доноров и пациентов. Исследование одобрено локальным этическим комитетом НИИФКИ.

В результате исследования было показано, что использование пентоксифиллина для ингибирования транскрипционного фактора NF-κB *in vitro* усиливает проапоптотический и цитотоксический

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противоопухолевый ответ, приводя к повышению экспрессии TRAIL на Т-лимфоцитах, в основном у здоровых доноров и пациентов с метастатическим раком молочной железы, как на интактных Т-клетках, так и в ответ на клетки опухолевой линии карциномы молочной железы человека ZR-75-1. У здоровых доноров в присутствии пентоксифиллина появляется популяция высокоэкспрессирующих TRAIL CD4 и CD8 Т-клеток.

Ключевые слова: пентоксифиллин, ингибиторы NF-κB, цитотоксичность, рак молочной железы, регуляция иммунного ответа, Т-лимфоциты

PENTOXIFYLLINE ENHANCES *IN VITRO* T-CELL ANTITUMOR RESPONSE IN BREAST CANCER PATIENTS

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Abstract. The NF-κB transcription factor controls the expression of genes responsible for cell cycle, apoptosis, and other immunoregulatory functions. Some nonspecific NF-κB inhibitors were found after discovering the possibility of blocking tumor growth through suppression of NF-κB activity, but their use was complicated by multiple side effects, such as interleukin-1β-related systemic inflammation or non-immune-related complications, which may be due to inhibition of the p65 NF-κB subunit that plays a central role in organogenesis and inflammation. Inhibition of the c-Rel subunit leads to tumor growth restriction by modulating the T-regulatory cell activity.

In 2017, Grinberg-Bleyer and co-authors checked the hypothesis that selective inhibition of the c-Rel subunit can be performed using pentoxifylline and will effectively regulate Treg activity during tumor growth. The authors showed that pentoxifylline, an FDA-approved drug, could indeed induce selective degradation of c-Rel without affecting p65, and suggested that such an effect could be effective in suppressing tumor growth. In this regard, we aimed to investigate *in vitro* how pentoxifylline affect the functional activity and antitumor cytotoxic potential of T-cells in cancer patients.

The objects of the study were peripheral blood mononuclear cells from 25 patients with primary breast cancer (no metastases), 15 patients with metastatic breast cancer, and 25 healthy women without breast pathology. Informed consent was obtained from all donors and patients. The study was approved by the local ethics committee.

Here we showed that pentoxifylline treatment *in vitro* enhances the pro-apoptotic and cytotoxic antitumor response via increasing the expression of TRAIL on T-lymphocytes, mainly in healthy donors and patients with metastatic breast cancer, both on intact T-cells and in response to the cells of the tumor line of human breast carcinoma ZR-75-1. In healthy donors, in the presence of pentoxifylline, a population of highly expressing TRAIL CD4 and CD8 T-cells appears.

Keywords: pentoxifylline, NF-κB inhibitors, antitumor cytotoxicity, breast cancer, regulation of the immune response, T-lymphocytes

Introduction

The principle of the complex interplay between tumor and immune system is currently described by the theory of immunoediting, formulated at the beginning of the 21st century, according to which the immune system is directly related to the processes of malignant growth, performing not only antitumor surveillance, but also participating in the processes of maintaining tumor growth. During development, a malignant tu-

mor enables many different strategies to maintain its own growth and metastasis, prevent recognition processes and elimination by the immune system. One of the main tumor mechanisms in immune evasion is based on the interaction of immunosuppressive receptors on effector T-cells with cognate ligands expressed in the tumor microenvironment. New approaches to cancer immunotherapy are aimed at overcoming these tolerance mechanisms and promoting a productive antitumor immune response.

The blocking of immune control molecules (checkpoint molecules) using monoclonal antibodies in clinical practice and experimental studies has been shown to be effective in inhibiting tumor growth, which was rightfully confirmed by awarding the 2018 Nobel Prize to researchers who proposed inhibition of CTLA-4 and PD-1 molecules as an effective method for cancer immunotherapy. However, despite a significant breakthrough in this field, it remains obvious that a thorough study of other mechanisms of cancer immunoediting, which promote tumor evasion from the immune response, and methods of modulating these mechanisms are required. Despite the fact that the emergence of cancer immunotherapy methods based on blocking immune control molecules (immunoregulatory receptors or checkpoint molecules) has become a real breakthrough in the treatment of malignant tumors, there is a significant number of tumors whose therapy does not lend itself to such methods (Sharma and Allison, 2015). One possible explanation for this resistance is that such tumors involve other immunosuppressive mechanisms, such as the accumulation of CD4⁺FoxP3⁺T-regulatory cells (Treg) suppressing antitumor responses (Nishikawa and Sakaguchi, 2010; Tang and Bluestone, 2008).

The NF- κ B protein, discovered more than 30 years ago, is a transcription factor that controls the expression of genes responsible for executing cell cycle processes, apoptosis, and a number of other immunoregulatory functions. After discovering an opportunity for blocking tumor growth through suppression of NF- κ B activity, it was actively searched for nonspecific NF- κ B inhibitors, but their use was complicated by multiple side effects, such as systemic inflammation due to overexpressed interleukin-1 β or non-immune-related complications, which may be due to inhibition of the p65 subunit of NF- κ B, which plays a central role in organogenesis and inflammation. Inhibition of the c-Rel subunit leads to restriction of tumor growth by modulating the properties of T-regulatory cells.

A number of studies have shown that the transcription factor NF- κ B particularly the c-Rel subunit, is critical for the expression of FoxP3 and the development of T-regulatory cells in the thymus (Isomura et al., 2009). The literature detailed the role of the transcription factor NF- κ B in the initiation, proliferation, and spread of tumors. This led several groups to test whether global inhibition of NF- κ B affects tumor growth. As a result, the use of nonspecific NF- κ B inhibitors has been complicated by multiple side effects such as systemic inflammation resulting from overexpressed interleukin (IL)-1 β or non-immune complications, which may result from inhibition of the p65 NF- κ B subunit playing a central

role in organogenesis and inflammation. Inhibition of the c-Rel subunit leads to restriction of tumor growth by modulating the properties of T-regulatory cells.

In a 2017 study, a group of researchers headed by Grinberg-Bleyer tested the hypothesis that inhibition of c-Rel may be a viable method of selectively modulated Treg activity in cancer and that pentoxifylline, an FDA-approved drug, can induce selective degradation of c-Rel without affecting p65 (Grinberg-Bleyer et al., 2017). In this regard, we set out to select conditions for inhibiting the transcription factor NF- κ B by pentoxifylline and functional assessment of the cytotoxic potential of human T-cells in normal conditions and in a malignant tumor process.

Materials and methods

Object of study

Mononuclear cells (PBMCs) from peripheral venous blood of 25 patients with primary breast cancer, 15 patients with metastatic breast cancer (presence of metastases), as well as healthy women without clinically and instrumentally diagnosed breast pathology (25 people) were examined. The study was conducted with the voluntary informed consent from all healthy donors and patients. The study was approved by the local ethics committee of RIFCI.

Venous blood from patients with primary breast cancer (T 1-3 N 0-2 M 0) was collected before surgery (radical mastectomy or radical breast resection) and patients with metastases before the course of chemotherapy. Blood sampling from donors and patients was carried out in vacuum tubes added with anticoagulant EDTA (Improvacuter, China).

PBMCs were isolated under sterile conditions by a standard method in a Ficoll-Urographin gradient ($\rho = 1.077$ g/l). PBMCs of peripheral blood and tumor cells of the breast carcinoma line ZR-75-1 were cultured at 10:1 ratio. Pentoxifylline treatment was carried out by adding it to cell cultures (500 μ g/ml) for 15 minutes at 37 °C. For the final culture, a number of functional tests were performed to assess the cytotoxic activity.

Assessment of cytotoxic activity

To assess the PBMC antitumor cytotoxic activity, we used the non-radioactive cytotoxic test CytoTox96 (Promega, USA), based on measuring amount of lactate dehydrogenase (LDH). Human mammary ductal carcinoma cell line ZR-75-1 (Bank of cell cultures, Institute of Center of the Russian Academy of Sciences, Russia) were used as target cells. The procedure was carried out according to the manufacturer's protocol. The level of cytotoxicity was estimated as the ratio of the optical density in a sample with a mixture of effectors and targets to the optical density in a sample with completely lysed targets,

expressed as a percentage. The corrections for the presence of LDH in the medium and the spontaneous release of LDH from effectors and target cells were taken into account.

In addition to the non-radioactive cytotoxic test CytoTox96, the level of tumor cell apoptosis was assessed using Annexin V and the level of TRAIL expression on CD4⁺ and CD8 T-cells was analyzed. T-cells were stained with antibodies CD3-APC-Cy7 (clone HIT3a, Cat # 300318), CD4-PE-Cy7 (clone RPA-T4, Cat # 300512), CD8-APC (clone RPA-T8, Cat # 301049), TRAIL-PE (clone RIK-2, Cat # 308206) (Biolegend, USA). The analysis was performed on a BD FACS Verse flow cytometer.

Statistical methods

Statistical data processing was carried out using GraphPadPrism 6. The results are presented as median and interquartile range; for statistical processing of the data, ANOVA with post-hoc Tukey test was used.

Results and discussion

To assess the effect of pentoxifylline on the functional activity of T-lymphocytes, we investigated spontaneous and target cell-induced protein expression of the tumor necrosis factor family members TRAIL on CD4 and CD8 T-cells treated/untreated with pentoxifylline. The analysis identified such parameters as percentage of TRAIL⁺CD4 and CD8-cells, the percentage of CD4 and CD8 T-cells with high TRAIL expression (TRAIL^{high} cells) and the mean fluorescence intensity (MFI, mean fluorescence intensity) for medium and highly expressing TRAIL-cells.

The study of the spontaneous TRAIL expression by T-cells showed that pentoxifylline affects the level of TRAIL⁺CD4 and CD8 T-cells in the group of healthy donors and patients with metastatic breast cancer (Figure 1A). Moreover, in the group of healthy donors, pentoxifylline led to decreased TRAIL expression, while in the group of patients with metastatic breast cancer, on the contrary, it was increased after treatment. At the same time, analysis of TRAIL MFI on positive cells showed a significant increase in the fluorescence intensity of TRAIL⁺CD8 T-cells in the group of patients with primary breast cancer after treatment with pentoxifylline (Figure 1B).

After culture of T-lymphocytes with ZR-75-1 target cells, a significant increase in TRAIL expression on CD4 and CD8 T-cells was observed in all study groups. In this case, the appearance of cells with medium and high intensity of TRAIL expression was found. In patients with primary breast cancer, no significant effect of pentoxifylline on TRAIL expression on CD4 and CD8 T-cells was shown in the presence of ZR-75-1 cells. The percentage of TRAIL⁺CD4 and CD8 T-cells in healthy donors increased significantly after

treatment with pentoxifylline in the presence of ZR-75-1 cells (Figure 2A).

The fluorescence intensity of TRAIL⁺CD4 and CD8 T-cells, which is an indirect indicator of molecule quantity, decreased after exposure to pentoxifylline in the presence of ZR-75-1 cells in patients with metastatic breast cancer, remaining unchanged for other groups (Figure 2B). It was also shown that percentage of highly expressing TRAIL CD4 T-cells treated with pentoxifylline in the presence of ZR-75-1 cells was increased in patients with metastatic breast cancer, whereas level of highly expressing TRAIL CD8 T-cells in same patients slightly decreased (Figure 2C). The fluorescence intensity of highly expressing TRAIL CD4 T-cells under the action of pentoxifylline in the presence of ZR-75-1 cells was increased only in healthy donors and patients with metastatic breast cancer (Figure 2D).

A study of the tumor cell apoptosis level after culture with the effector T-cells (using fluorescently labeled annexin V) showed that T-cells from healthy donors and patients with primary breast cancer significantly stronger stimulate apoptosis of tumor cells in coculture compared with patients with metastatic breast cancer. After adding pentoxifylline, the level of apoptosis significantly increased in healthy donors and patients with metastatic breast cancer, while in patients with breast cancer the level of apoptosis reached that of healthy donors, which leads to no significant differences between the groups (Figure 3A).

When studying the cytotoxic activity of mononuclear cells against ZR-75-1 tumor cells, it was shown that addition of pentoxifylline leads to increased release of LDH from tumor cells in all studied groups (Figure 3B).

Thus, it was shown that the use of pentoxifylline as an inhibitor of the transcription factor NF- κ B to suppress the functions of Treg by upregulating expression of TRAIL mainly in healthy donors and patients with metastatic breast cancer both on intact T-cells and in response to ZR-75 tumor cells. In healthy donors, in the presence of pentoxifylline, a population of highly expressing TRAIL CD4 and CD8 T-cells emerged. The cytotoxic activity and the level of apoptosis induced by T-cells differ in breast cancer patients and healthy donors, but while using an inhibitor of the transcription factor NF- κ B, the antitumor activity in breast cancer patients is stimulated as high as in healthy donors. The data obtained on the stimulation of the cytotoxic response against tumor cells upon hindering activation of the transcription factor NF- κ B would allow to combine the mechanisms of blockade and activation in the most effective way while creating models for inducing antitumor response.

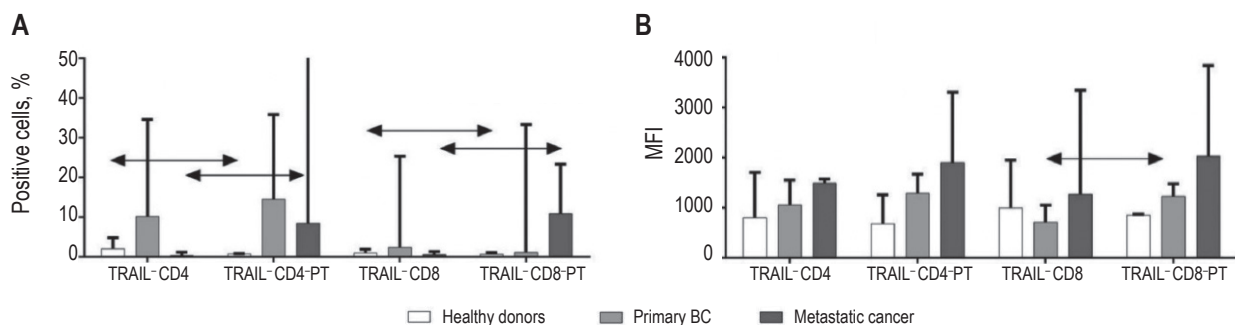


Figure 1. Influence of pentoxifylline on (A) spontaneous expression of TRAIL and on (B) the intensity of TRAIL fluorescence on CD4 and CD8 T-cells of healthy donors (n = 25), patients with primary breast cancer (n = 25) and metastatic breast cancer (n = 15)

Note. Data are presented as median and interquartile range. Arrows indicate statistically significant differences at p < 0.05.

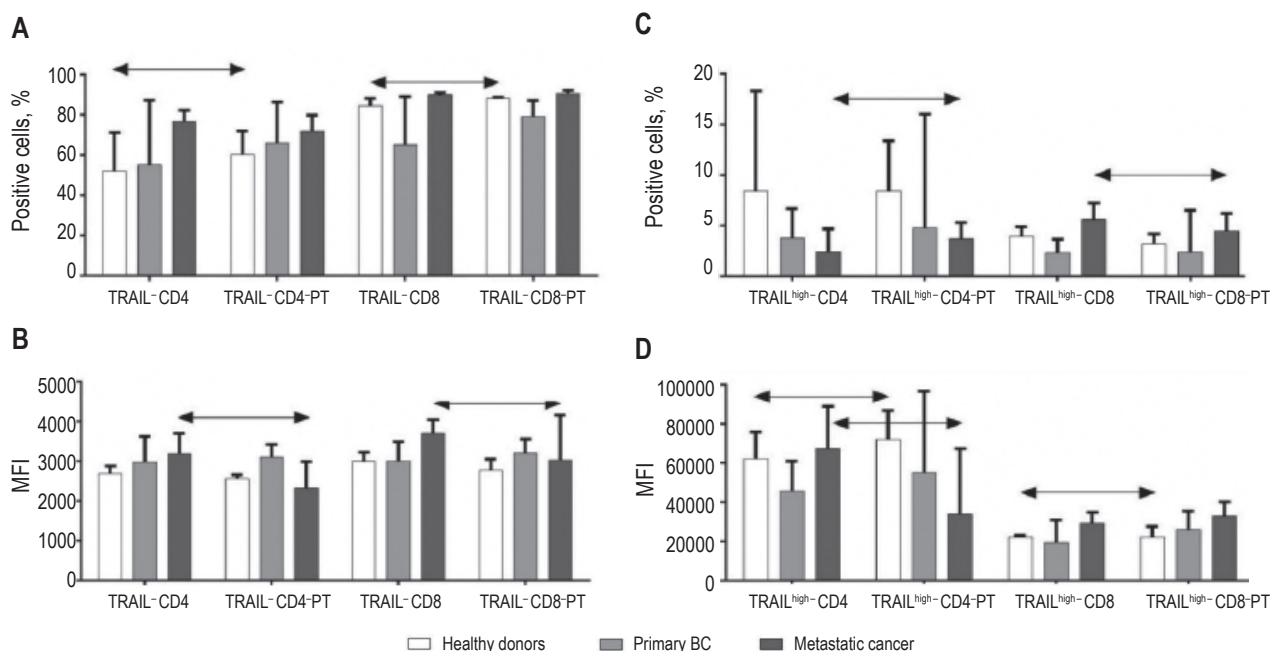


Figure 2. Influence of pentoxifylline on the surface expression of TRAIL CD4 and CD8 by T-lymphocytes of healthy donors (n = 25), patients with primary breast cancer (n = 25) and metastatic breast cancer (n = 15) when cultured with cells of the tumor line ZR-75-1

Note. As for Figure 1. A, the content of TRAIL⁺CD4 and CD8 T-cells. B, fluorescence intensity of TRAIL⁺CD4 and CD8 T-cells. C, content of TRAIL^{high} CD4 and CD8 T-cells. D, intensity of fluorescence of TRAIL^{high} CD4 and CD8 T-cells.

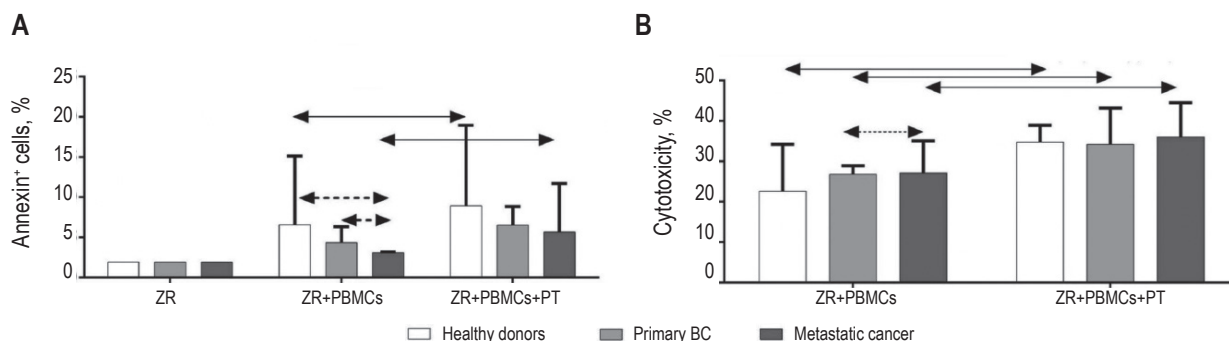


Figure 3. Study of the effect of pentoxifylline on the level of T-cell cytotoxicity using (A) cytometric analysis of the level of apoptosis of ZR-75-1 tumor cells labeled with Annexin V and (B) determination of LDH release in the CytoTox96 test in healthy donors (n = 25), patients with primary breast cancer (n = 25) and progressive breast cancer (n = 15) after cultivation with cells of the tumor line ZR-75-1

Note. As for Figure 1.

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ХЕМОКИНЫ CCL17 И CCL22 ПРИ САРКОИДОЗЕ

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Резюме. В иммунопатогенезе саркоидоза и механизмах образования гранулем принимают участие различные клетки иммунной системы и продуцируемые ими цитокины. В настоящее время активно изучается роль хемокинов, идет поиск ключевых молекул, важных для привлечения клеток иммунной системы в очаги поражения и формирования гранулем, а также влияющих на исходы процесса гранулемообразования. Целью исследования явилось определение уровней CCL17/TARC и CCL22/MDC хемокинов в плазме крови больных саркоидозом, не получавших иммуносупрессивную терапию. Что является актуальной задачей для уточнения ряда аспектов иммунопатогенеза заболевания, а также для поиска информативных клинико-лабораторных критериев оценки активности и прогноза заболевания. Были исследованы образцы плазмы крови больных саркоидозом (n = 52). У 37% (19/52) отмечалось острое, а у 63% (33/52) — хроническое течение заболевания. Контролем служили образцы периферической крови, полученные от 22 практически здоровых добровольцев. Концентрации хемокинов (пг/мл) определялись методом мультиплексного анализа по технологии xMAP (Luminex), тест-системы Milliplex MAP (Millipore, США). У обследованных больных обнаружено достоверно повышенное содержание хемокинов относительно здоровых лиц: CCL17 — 78,24 пг/мл против 26,24 пг/мл, $p < 0,001$; CCL22 — 660,60 пг/мл против 405,00 пг/мл, $p < 0,001$. Анализ клинико-лабораторной значимости уровней хемокинов в плазме крови обследованных больных саркоидозом выявил параметры их чувствительности и специфичности. У больных с острым течением саркоидоза они составили: для CCL17 — 63% и 78%, CCL22 — 63% и 91%; при хроническом: CCL17 — 58% и 83%, CCL22 — 67% и 86% соответственно. У больных с хроническим течением заболевания установлена прямая положительная связь между уровнем ангиотензин-превращающего фермента (АПФ) и концентрацией хемокинов: для CCL17 ($r = 0,530$; $p = 0,003$), для CCL22 ($r = 0,446$; $p = 0,014$). У больных саркоидозом с признаками системности заболевания был достоверно повышен уровень CCL17: 102,82 пг/мл против 32,72 пг/мл, $p = 0,011$. Уровни хемокина CCL17 были достоверно повышены у больных с признаками гепатомегалии по сравнению с больными, не имеющими изменений данного органа: 130,73 пг/мл против 51,60 пг/мл, $p = 0,022$. При наличии спленомегалии относительно больных без таких признаков отмечалось повышение концентраций хемокинов: CCL17 — 249,18 пг/мл против 46,87 пг/мл,

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$p = 0,002$; CCL22 – 1271,40 пг/мл против 660,63 пг/мл, $p = 0,003$. Таким образом, уровни хемокинов CCL17 и CCL22 при саркоидозе могут быть использованы в качестве дополнительных прогностических маркеров при хроническом течении саркоидоза с разной степенью активности клинических особенностей, в том числе, с наличием или отсутствием системных проявлений заболевания.

Ключевые слова: саркоидоз, хемокины, CCL17, CCL22, плазма крови

CHEMOKINES CCL17 AND CCL22 IN SARCOIDOSIS

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Abstract. Various immune cells as well as related cytokines are involved in immunopathogenesis of sarcoidosis and mechanisms of granuloma development. Currently, a role for chemokines in sarcoidosis has been extensively investigated, which is paralleled with a search for key molecules necessary for recruiting immune cells to intrusion site and granuloma formation as well as affecting outcome of the latter. Our study was aimed for determining level of plasma CCL17/TARC and CCL22/MDC chemokines in patients with sarcoidosis who received no immunosuppressive therapy is of high priority for clarifying some aspects in underlying immunopathogenesis as well as seeking out for secure clinical and laboratory criteria for assessing activity and disease prognosis. We studied peripheral blood plasma samples of the patients with sarcoidosis ($n = 52$). In 37% (19/52), they exhibited acute clinical manifestations, and 63% (33/52) had chronic sarcoidosis. The control group included peripheral blood samples from healthy volunteers ($n = 22$). The chemokine concentrations (pg/ml) were determined by multiplex analysis using xMAP technology (Luminex), and Milliplex MAP test system (Millipore, USA). In the patients with sarcoidosis, significantly higher levels of chemokines were shown relative to healthy volunteers: CCL17 – 78.24 pg/ml vs 26.24 pg/ml, $p < 0.001$; CCL22 – 660.60 pg/ml vs 405.00 pg/ml, $p < 0.001$. Evaluation of clinical and laboratory diagnostic characteristics for plasma chemokine levels in sarcoidosis patients allowed to assess their sensitivity and specificity. The respective values were as follows: in acute sarcoidosis: for CCL17 – 63% and 78%, CCL22 – 63% and 91%; in chronic sarcoidosis: CCL17 – 58% and 83%, CCL22 – 67% and 86%, respectively. In chronic sarcoidosis the levels of this chemokine correlated with the activity of angiotensin-converting enzyme (ACE), for CCL17 ($r = 0.530$; $p = 0.003$), for CCL22 ($r = 0.446$; $p = 0.014$). Patients with systemic lesions vs no systemic lesions (sarcoidosis of the respiratory system only) had significantly elevated CCL17 level: 102.82 pg/ml vs 32.72 pg/ml, $p = 0.011$. The concentration of chemokine CCL17 was significantly increased in patients with vs without signs of hepatomegaly: 130.73 pg/ml vs 51.60 pg/ml, $p = 0.022$. Levels of chemokines was significantly increased in patients with vs without ultrasound signs of splenomegaly comprising: for CCL17 – 249.18 pg/ml vs 46.87 pg/ml, $p = 0.002$; for CCL22 – 1271.40 pg/ml vs 660.63 pg/ml, $p = 0.003$. Thus, it should be noted that the peripheral blood plasma level of chemokines CCL17 and CCL22 may be used as additional prognostic markers in chronic sarcoidosis with varying scoring of clinical signs including with/without systemic disease manifestations.

Keywords: sarcoidosis; chemokines; CCL17; CCL22; peripheral blood plasma

Introduction

Sarcoidosis is a multisystem disease of unknown etiology, featured with heterogenous clinical manifestations and outcomes, morphologically belonging to granulomatous diseases and characterized by developing specific necrosis-free sarcoid granuloma

in diverse organs, preferentially in the lungs and bronchopulmonary lymph nodes [10, 18, 23]. Various immune cells as well as related cytokines and chemokines are involved in immunopathogenesis of sarcoidosis and mechanisms of granuloma development. Sarcoid granulomas contain macrophages able to fuse and form multinucleated giant cells as well as

helper CD4⁺T-cells (Th), whereas cytotoxic CD8⁺T-cells (Tcyt), regulatory T-cells (Tregs), fibroblasts and B-cells reside on periphery [23]. Still, it is unclear what cell types largely play a crucial pathogenetic role in sarcoidosis. In particular, granuloma formation and macrophage activation in site of inflammation were shown to be linked to type 1 Th-cells (Th1) as well as plastic subsets of type 17 Th-cells (Th17) and relevant cytokines [6, 9, 26].

A directed cell migration to the site of intrusion is mediated by surface expression of specific chemokine receptors able to ligate tissue-derived chemotactic cytokines (chemokines). Currently, a role for chemokines in sarcoidosis has been extensively investigated, which is paralleled with a search for key molecules necessary for recruiting immune cells to intrusion site and granuloma formation as well as affecting outcome of the latter [1, 2, 3, 14, 16, 17, 18, 19]. Such molecules, in turn, are related to resolution of granuloma development and disease remission, or, inversely, to triggering fibrogenesis in affected organs [12, 16, 18, 21].

Chemokines CCL17/TARC (thymus and activation-regulated chemokine) and CCL22/MDC (macrophage-derived chemokine) recruit Th-cells bearing surface chemokine receptor CCR4 from the circulation to inflammatory foci [11, 18, 24]. CCR4 receptor is expressed on Th2-cells, Tregs as well as skin-migrating T-cells. Upon formation of inflammatory focus in the connective tissue beneath skin layers, CCL17 is expressed by dermal microvascular endothelial cells, whereas CCL22 is mainly found in dermal dendritic cells. In this regard, key function for CCL17 is recruitment and penetration of lymphocytes through vascular wall in the immediate vicinity to inflammatory site, whereas CCL22 mainly accounts for directed cell migration within the connective tissue outside blood vessels [8].

The level of CCL17 chemokine is an important marker for assessing ongoing atopic dermatitis, bullous pemphigoid and other skin disorders [22]. Severe progressive sarcoidosis was associated with parallel rise in CCL17 level and peripheral blood CCR4⁺CD4⁺T-cells compared to apparently healthy subjects, as well as in biopsy histology samples from damaged skin containing sarcoid granulomas [18, 20]. Dynamic three-year monitoring of sarcoidosis patients evidences about prognostic importance of elevated CCL17 level related to more aggravated clinical course, lung injury, and transition to fibrogenesis stage in affected tissues [18].

Chemokines CCL17 and CCL22 contribute to skewing towards M2-polarized macrophage phenotype exerting pro-fibrotic properties and expressing a whole set of cytokines and chemokines, including CCL17 and CCL22 [18, 21]. The latter chemokines were examined in activating fibrogenesis both in

experimental models and clinical practice in patients with sarcoidosis, idiopathic pulmonary fibrosis and some other diseases [7, 12, 13, 18, 21].

Thus, assessing level of peripheral blood plasma CCL17 and CCL22 chemokines in patients with sarcoidosis who received no immunosuppressive therapy is of high priority for clarifying some aspects in underlying immunopathogenesis as well as seeking out for secure clinical and laboratory criteria for assessing activity and disease prognosis.

Materials and methods

Patients with sarcoidosis (n = 52) aged 20–67 years, who received no immunosuppressive therapy including systemic steroid therapy and plasmapheresis were enrolled to the study. Blood samples were collected after peripheral vein puncture into vacuum test tube added with K₃EDTA.

Sarcoidosis was verified based on histology examination in 73% (38/52) patients and clinical and chest X-ray data in 27% (14/52) subjects. All patients were subdivided into two groups: acute onset sarcoidosis (Löfgren's syndrome, n = 19) and chronic onset sarcoidosis (non-Löfgren's syndrome, n = 33). In the latter case, diagnosis was verified by histology examination in 94% cases (31/33). All sarcoidosis patients were examined in the Department of the Research Institute of Interstitial and Orphan Lung Diseases at the First St. Petersburg State I. Pavlov Medical University of the Ministry of Healthcare of Russian Federation. In control group, samples of peripheral blood were collected from 22 age- and sex-matched apparently healthy subjects. All subjects provided informed consent, studies were conducted in accordance with the WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects with 2013 Amendments, Clinical Practice Rules in the Russian Federation approved by the Order of the Ministry of Healthcare of the Russian Federation dated of 19.06.2003, No. 266, and Good Clinical Practice Rules in the Russian Federation approved by the Order of the Ministry of Healthcare of the Russian Federation dated of 01.04.2016, No. 200n.

Peripheral blood plasma cytokine level was analyzed in parallel with assessing clinical, instrumental and laboratory parameters. While examining patients, there were assessed complaints, symptoms, systemic organ injuries, elevated pulmonary artery pressure (pPA, mm Hg) according to echocardiographic examination (Echo-CG), enlarged lymph nodes, aggravated pulmonary changes, spread of injury foci, emergence and progression of fibrotic signs and other manifestations of unfavorable sarcoidosis course according to multispiral computed tomography (MSCT), as well as change in pulmonary function test by evaluating pulmonary volume. Intensity of

sarcoidosis was assessed by measuring activity of angiotensin converting enzyme (ACE = IU/ml) in colorimetric assay with peptide substrate. Positive data were considered at ACE activity > 70 IU, reference level for subjects > 18 years of age was within the range 20-70 ACE IU. Detailed description and subsequent group subdivision based on ACE activity were published earlier [15].

Concentration of plasma chemokines CCL17/TARC and CCL22/MDC (pg/ml) was measured by using xMAP® Technology-based multiplex assay (Luminex, USA), commercially available kit Milliplex MAP (Millipore, USA) with magnetic micro-beads Milliplex Mag (USA), according to the manufacturer's instruction. Data were recorded and analyzed on Luminex MAGPIX Instrument System (Luminex, USA).

Statistical processing was carried out by using software suites Statistica 8.0 (StatSoft, USA) and GraphPad Prism 5.00 for Windows (GraphPad Prism Software Inc., USA). The data on relative and absolute count of various T-cell subsets were presented as median (Me) and interquartile range ($Q_{0.25}$ - $Q_{0.75}$). A nonparametric Mann-Whitney test was used for comparing data samples, whereas correlation analysis was performed using the r-Spearman rank correlation coefficient. Informativity of parameters, comparison of two distinct parameters and selection of optimal partition point were assessed by analyzing ROC (receiver-operating-characteristic)-curve followed by calculating area under ROC-curve (AUC). In addition, parameters of diagnostic sensitivity and specificity were described.

Results and discussion

It was found that among patients with sarcoidosis (n = 52) acute and chronic onset disease was observed in 37% (19/52) and 63% (33/52) cases, respectively. All patients with sarcoidosis vs healthy volunteers had significantly elevated level both for CCL17 and CCL22 chemokines.

The level of CCL17 comprised 78.24 (35.72-167.40) pg/ml compared to volunteers showing 26.24 (13.28-49.55) pg/ml ($p < 0.001$), whereas CCL22 reached up to 660.60 (417.40-914.60) pg/ml vs 405.00 (287.50-508.00) pg/ml ($p < 0.001$).

While comparing chemokine level in patients with acute (group 1) and chronic (group 2) onset with control group it was found as follows: CCL17 was significantly elevated compared to control group comprising up to 58.51 (31.80-169.10) and 60.02 (35.99-124.70) vs 26.24 (13.28-49.55) pg/ml ($p = 0.005$ and $p = 0.001$), respectively. In contrast, no inter-group difference was found for CCL17 level ($p = 0.894$). On the other hand, level of CCL22 was also increased in group 1 and group 2 vs control group reaching up to 621.30 (348.50-840.80) and 660.60

(438.20-1073.00) vs 405.00 (287.50-508.00) pg/ml ($p = 0.007$ and $p < 0.001$). Finally, no difference in CCL22 level between patients with acute vs chronic onset sarcoidosis was observed ($p = 0.582$).

The informativity of the examined laboratory parameters was assessed using ROC-analysis in both acute and chronic onset sarcoidosis:

- acute onset sarcoidosis: sensitivity for CCL17 was 63%; specificity – 78%; AUC = 0.771; criterion > 49.01 pg/ml; $p = 0.005$; sensitivity for CCL22 – 63%; specificity – 91%; AUC = 0.750; criterion > 536.30 pg/ml; $p = 0.006$.

- chronic onset sarcoidosis: sensitivity for CCL17 – 58%; specificity – 83%; AUC = 0.783; criterion > 51.43 pg/ml; $p < 0.001$. Sensitivity for CCL22 – 67%; specificity – 86%; AUC = 0.780; criterion > 520.90 pg/ml; $p < 0.001$.

It is worth noting that in chronic onset sarcoidosis with relatively low-grade activity characterized with normal ACE magnitude (< 70 ACE IU), the level of chemokines showed no significant difference from those found in volunteers that for CCL17 reached 29.03 (25.25-51.60) pg/ml (volunteers – 26.24 (13.28-49.55)); for CCL22 – 499.59 (312.87-639.92) pg/ml (volunteers – 405.00 (287.50-508.00)).

In contrast, sarcoidosis patients with elevated ACE level (≥ 70 ACE IU) had these chemokines at significantly higher level compared to those with normal ACE concentration, and reached for CCL17 – 87.00 (37.86-118.77) vs 29.03 (25.25-51.60) pg/ml, $p = 0.039$; for CCL22 – 734.48 (505.82-1063.44) vs 499.59 (312.87-639.92) pg/ml, $p = 0.044$.

Thus, concentration of chemokines CCL17 and CCL22 in the peripheral blood of patients with sarcoidosis was significantly increased during active inflammatory process that was confirmed by correlation analysis in patients with chronic disease course: a direct positive relation found between the ACE level and chemokine concentration: for CCL17 the value Spearman's correlation coefficient was $r = 0.530$; $p = 0.003$, for CCL22 – $r = 0.446$; $p = 0.014$.

To assess a potential role of such chemokines in formation of systemic lesions during sarcoidosis, the group of patients with chronic onset sarcoidosis was divided into two subgroups: subgroup 1 – no systemic lesions (sarcoidosis of the respiratory system only), subgroup 2 – with systemic lesions.

Patients from subgroup 2 (systemic lesions) vs subgroup 1 had significantly elevated CCL17 level: 102.82 (51.60-162.42) pg/ml vs 32.72 (17.75-60.02) pg/ml, $p = 0.011$.

There were identified groups of patients with/without signs of hepatosplenomegaly to conduct higher precision analysis of systemic manifestations in sarcoidosis by comparing chemokine levels.

TABLE 1. PERIPHERAL BLOOD PLASMA LEVELS OF CHEMOKINES CCL17/TARC AND CCL22/MDC (pg/ml) IN PATIENTS WITH SARCOIDOSIS (n = 32) WITH/WITHOUT HEPATO-SPLENOMEGALY, Me (Q_{0.25}-Q_{0.75})

Chemokine	Signs of hepatomegaly		Statistically significant (p)	Signs of splenomegaly		Statistically significant (p)
	Yes (n = 9)	No (n = 23)		Yes (n = 9)	No (n = 23)	
CCL17/TARC	130.73 (102.82-249.18)	51.60 (27.58-92.35)	p = 0.022	249.18 (102.82-379.39)	46.87 (27.58-92.35)	p = 0.002
CCL22/MDC	1063.44 (734.48-1414.98)	660.63 (398.83-840.84)	p = 0.068	1271.40 (906.14-1417.06)	660.63 (347.64-835.85)	p = 0.003

It was noted that such chemokines were significantly elevated in patients with vs without hepatosplenomegaly (Table 1).

The concentration of chemokine CCL17 was significantly increased in patients with vs without signs of hepatomegaly: 130.73 pg/ml (102.82-249.18) and 51.60 pg/ml (27.58-92.35), p = 0.022. Along with that, level of CCL17 was significantly increased in patients with vs without ultrasound signs of splenomegaly comprising 249.18 pg/ml (102.82-379.39) vs 46.87 pg/ml (27.58-92.35) respectively, (p = 0.002). Moreover, similar pattern was also observed for CCL22: 1271.40 pg/ml (906.14-1417.06) vs 660.63 pg/ml (347.64-835.85) (p = 0.003).

The ROC analysis allowed to establish the peak value of the informativity for plasma CCL17 level in patients with sarcoidosis with and without splenomegaly, with sensitivity – 89%; specificity – 65%; AUC = 0.865; criterion > 78.24 pg/ml; p = 0.002.

Patients with chronic onset sarcoidosis were found to have significantly increased concentration of CCL17 and CCL22 chemokines coupled to splenomegaly signs: CCL17 was 176.57 (90.57-373.32) pg/ml and 49.23 (27.58-99.62) pg/ml, p = 0.009; CCL22: 1221.06 (714.88-1416.025) pg/ml and 650.28 (385.59-790.76) pg/ml, respectively, p = 0.011.

Previously, it was published that high amount of serum chemokine CCL17 was observed in some disorders characterized by Th2-dependent mechanisms of immune response. Among those are atopic dermatitis, eosinophilic pneumonia, bronchial asthma etc. [18, 22, 25, 27]. The data obtained over the last years provided new insights into M2 macrophage polarization triggering tissue remodeling for cytokines from Th2 T-cells expressing chemokine receptor CCR4 [13]. Such chemokines examined by us are involved in activation and recruitment of regulatory CCR4⁺T-cells profoundly contributing to immune regulation and subsequent remodeling in damaged

tissues [5]. CCL17 and CCL22 are major chemokines accounting for recruitment of CCR4⁺CD4⁺T-cells from the circulation into lesion foci [8, 11, 18, 24]. In connection with this, it is worthy that the level of serum CCL17 as well as count of peripheral blood CCR4⁺CD4⁺T-cells were increased [20].

The data of our study evidence about significantly elevated level for CCL17 and CCL22 chemokines both in acute and chronic onset sarcoidosis compared to control group. Moreover, a direct correlation between level of such chemokines and ACE concentration as a major clinical and laboratory marker of sarcoidosis activity upon chronic disease course was also noted. Such data evidence about potential use of measuring CCL17 and CCL22 level as additional parameter for sarcoidosis scoring that was also confirmed by rather high sensitivity and specificity parameters as assessed by ROC-analysis.

Nguyen CTH et al. suggest that 78% sarcoidosis patients vs volunteers had elevated serum TARC/CCL17 chemokine level. Subjects with higher amount of this marker were featured with more severe clinical symptoms as well as significantly higher level of markers of disease activity and severity: serum ACE level and soluble interleukin-2 receptor (sIL-2R).

Along with this, immunohistochemistry study of biopsy materials revealed that sarcoid granulomas of such patients had increased expression of CCR4⁺T-cells and CCL17. High concentration of CCL17 was also found in patients with lesions in the three or more organs, i.e., in those having signs of the systemic disease course [18].

The data obtained evidence about marked changes in the level of examined chemokines not only in patients with more active sarcoidosis, but also in those with manifested systemic course: concentration of the chemokine CCL17 was significantly higher in patients with systemic manifestations, i.e., in subjects with organs affected along with lungs.

Signs of hepato/splenomegaly were evaluated by abdominal ultrasound examination. A number of patients had an articular syndrome, enlarged extra-pulmonary lymph nodes (suprahrenic, periportal, retroperitoneal, inguinal, cervical and other localization), as well as eye damage (uveitis of sarcoid etiology).

In addition, significantly higher level of plasma CCL17 was noted in sarcoidosis patients with signs of hepato / splenomegaly. The data of ROC-analysis evidence about peak magnitude of sensitivity and specificity for CCL17 level in patients with sarcoidosis with and without splenomegaly.

Thus, measuring plasma CCL17 and CCL22 chemokines upon different clinical course of sarcoidosis

contributes to deeper insight into the mechanisms of disease immunopathogenesis.

Identifying a role of CCR4⁺T-helper cells recruited along the gradient of cognate chemokines from the peripheral blood of patients with sarcoidosis to the affected organs and tissues requires to be further investigated by using immunohistochemistry methods, which might allow to conduct a more detailed study of the mechanisms underlying granuloma formation and maturation as well as its outcomes.

To conclude, it should be noted that the peripheral blood plasma level of chemokines CCL17 and CCL22 may be used as additional prognostic markers in chronic sarcoidosis with varying scoring of clinical signs including with/without systemic disease manifestations.

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ФАКТОРЫ ВРОЖДЕННОГО И АДАПТИВНОГО ИММУНИТЕТА В ПАТОГЕНЕЗЕ ВНУТРИУТРОБНОЙ ГЕНЕРАЛИЗОВАННОЙ ЦИТОМЕГАЛОВИРУСНОЙ ИНФЕКЦИИ

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Резюме. Цель: роль факторов врожденного и адаптивного иммунитета в развитии внутриутробной генерализованной цитомегаловирусной инфекции.

Под наблюдением находилось 47 новорожденных детей с врожденной генерализованной цитомегаловирусной инфекцией, которые составили I группу. На основании данных клинико-лабораторного обследования все исследуемые новорожденные были разделены на две подгруппы. Подгруппу 1.1 (29 человек) составили новорожденные с тяжелой формой ЦМВИ и подгруппу 1.2 (18 человек) — новорожденные со среднетяжелым течением ЦМВИ. В контрольную группу вошли 26 новорожденных без герпесвирусной инфекции. Определение количества моноцитов, экспрессирующих Toll-рецепторы (TLR), проводили методом лазерной проточной цитометрии (Beckman Coulter) с использованием реагентов Beckman Coulter, HyCultBiotechnology: FITC-CD282⁺, CD284⁺, CD286⁺ и PE-CD14⁺. Концентрацию IFN γ , IFN α , IL-6, IL-8 в сыворотке крови новорожденных определяли методом ИФА с использованием тест-систем Bender MedSystems.

Внутриутробная генерализованная ЦМВИ с полной клинической симптоматикой у новорожденных характеризовалась снижением количества моноцитов, экспрессирующих TLR-2 и TLR-6, что было сопряжено со снижением уровня IFN α , IFN γ , повышением уровня IL-6, IL-8 и MCP-1. Подгруппа с неполной клинической симптоматикой ЦМВИ характеризовалась снижением уровня IFN α , в сочетании с повышением уровня IL-6. Выявленные иммунные нарушения приводят к редукции противовирусного иммунного ответа, определяют тяжесть заболевания у перинатально инфицированных новорожденных.

Ключевые слова: врожденный иммунитет, TLR-рецепторы, цитокины, внутриутробная инфекция, цитомегаловирус, новорожденные дети

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FACTORS OF CONGENITAL AND ADAPTIVE IMMUNITY IN THE PATHOGENESIS OF INTRAUTERINE GENERATED CYTOMEGALOVIRUS INFECTION

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Abstract. Subject: to assess a role of factors of innate and adaptive immunity in the development of intrauterine generalized cytomegalovirus infection.

The study included 47 newborns with congenital generalized cytomegalovirus infection comprising group I. Based on the data of clinical and laboratory examination, all newborns studied were divided into two subgroups. Subgroup 1.1 (29 subjects) consisted of newborns with severe CMVI and subgroup 1.2 (18 subjects) – newborns with moderate CMVI. The control group included 26 newborns without herpesvirus infection.

Determination of the number of monocytes expressing Toll receptors (TLR) was performed by laser flow cytometry (Beckman Coulter) using Beckman Coulter, HyCultBiotechnology reagents: FITC-CD282⁺, CD284⁺, CD286⁺, and PE-CD14⁺. The newborn serum concentration of IFN γ , IFN α , IL-6, IL-8 was determined by ELISA using Bender MedSystems test systems.

Intrauterine generalized CMVI with complete clinical symptoms in newborns was characterized by a decrease in the number of monocytes expressing TLR-2 and TLR-6, which was associated with a decrease in the level of IFN α , IFN γ , an increase in the level of IL-6, IL-8 and MCP-1. The subgroup with incomplete clinical symptoms CMVI was characterized by a decrease in the level of IFN α , in combination with an increase in the level of IL-6. The identified immune disorders lead to a reduction in the antiviral immune response and determine the severity of the disease in prenatally infected newborns.

Keywords: innate immunity, TLR receptors, cytokines, intrauterine infection, cytomegalovirus, newborn children

Introduction

Intrauterine infectious pathology in newborns is recorded in 11 to 45% of deaths as well as in 14-18% of stillbirths.

Herpesvirus infections hold a leading place among the causes of premature birth, infant mortality, and morbidity in newborns, being one of the most difficult problems to solve in modern medicine. It was shown that in the structure of infant mortality, 10 to 61% cases result from intrauterine infections, among which cytomegalovirus infection (CMVI) prevails [3]. In newborns, cytomegalovirus infection may be also characterized by generalization of the process and accompanied by the DIC development. Often, newborns recovered after CMVI have persistent neurological symptoms by the end of the first year of life, development of early disability being the most important medical and social problem of modern perinatology.

The development and severity of intrauterine infection in neonates is determined by the type of pathogen, its virulence, duration of infection, tropism of the pathogen to the organs and tissues of the

fetus, maternal protective reserves, fetal potential to mount immune response [6] as well as reduced T-cell functional activity underlying the pathogenesis of severe forms of cytomegalovirus infection [2].

Taking into account the activation and regulation of adaptive immune response depends on the functioning of the innate immune system, a large number of studies has been recently carried out to study Toll-like receptors (TLR), which ligation by microbial factors leads to inflammatory response mediated by the production of cytokines, which further determines the nature, modality and intensity of body adaptive immune response [7]. In the resting state, non-activated TLRs are found monomers on the cell membrane. After the TLR interacts with the ligand, certain genes are activated and expressed to ensure destruction of invading pathogens.

As a result of TLR activation, a wide range of biological reactions occurs – from induced production of proinflammatory cytokines and interferons ensuring execution of innate immunity reactions, to the expression of co-stimulating molecules which promote activation of T-lymphocytes and stimulate development of adaptive immune response [4].

In recent years, evidence has been accumulated that defects in the TLR system such as decreased expression and function due to mutations or epigenetic disorders in TLR regulation underliedevelopment of immunodeficiency states, the implementation and generalization of infection.

It is evident that characteristics of immunological disorders in neonatal CMVI characterized by suppressed immune response should be thoroughly investigated.

Objective: the role of innate and adaptive immunity factors in the development of intrauterine generalized cytomegalovirus infection.

Materials and methods

The study included 47 newborns with congenital generalized cytomegalovirus infection comprising group I. Based on the data of clinical and laboratory examination, all newborns were divided into two subgroups. Subgroup 1.1 (29 subjects) consisted of newborns with a severe CMVI and subgroup 1.2 (18 subjects) — newborns with moderate CMVI. The control group included 26 newborns without herpesvirus infection.

Determination of the number of TLR expressing monocytes was performed by flow cytometry (Beckman Coulter) by using HyCultBiotechnology reagents: FITC-CD282⁺, CD286⁺ and PE-CD14⁺. The level of serum IFN γ , IFN α , IL-6, IL-8 was determined by ELISA (Bender MedSystems).

Quantity of CMV DNA in neonatal blood and urine of newborns was assessed by real-time PCR (Corbet-Researc, Australia). The determination of specific IgG and IgM antibodies against cytomegalovirus (CMV) in the blood serum of newborns was carried out by enzyme immunoassay using reagents from the “Vector-Best” company (Novosibirsk).

Analyzing statistical significance of differences between the study groups, the Mann–Whitney and Wilcoxon test was used. The correlation coefficient was calculated according to Spearman test. The statistical significance level (p) in our study was set at 0.05.

Results and discussion

The analysis of the conducted studies showed that all children in group I were born to women with burdened obstetric and gynecological history, from high-risk pregnancies, so that we analyzed anamnestic data of mothers and paired newborns in the surveyed subgroups.

Comparative analysis of gynecological diseases revealed that the history of endocervicitis in the mothers of paired children from both subgroups of group I was revealed at significantly higher rate than in the control group (by 24.1 and 22.2%, respectively). Similar chan-

ges were found while studying the frequency of detected adnexitis (27.6 and 27.7%, respectively).

Mothers of the examined children from both subgroups of group I vs. control group had significantly higher rate of previous of the urinary system diseases (58.6 and 55.6%, respectively), diseases of the gastrointestinal tract (48.3 and 44.4%, respectively). From the anamnesis of mothers of subgroup 1.1 it was found that 17.2% of the examined previous pregnancies were completed with non-developing pregnancies, 34.5% — spontaneous miscarriage at age of 5–11 weeks, which was significantly higher than in the control. A history of premature birth was noted in 31.5% of women.

The leading place in the pathology of pregnancy and childbirth in mothers from subgroups 1.1 and 1.2 was held by threatened termination of pregnancy (48.3% and 44.4%, respectively), which occurred significantly more often than in the control, early gestosis) — 34.5 and 33.3%, respectively). Polyhydramnios was a characteristic clinical symptom in mothers from subgroup 1.1, which was more common than in the control group and subgroup 1.2; similar data were also found for the early maturation of the placenta.

When analyzing the clinical symptoms in the examined newborns with cytomegalovirus infection, it was revealed that in the subgroup with severe CMVI it had significantly higher rate of hepatitis than the subgroup with moderate disease (37.9 and 5.6%, respectively), whereas pneumonia (51.7 and 11.1%, respectively), hemorrhagic syndrome (44.8 and 11.1%, respectively); meningoencephalitis and myocarditis were diagnosed only in newborns with severe generalized CMVI (6.9 and 10.3%, respectively). All newborns with cytomegalovirus infection had perinatal CNS lesion.

Studying the mechanisms of innate immunity found that only newborns with CMVI in subgroup 1.1 had decreased number of monocytes expressing TLR-2 (CD282⁺), compared with subgroup 1.2 and the control group (45.4 $\pm$ 8.9%, 65.1 $\pm$ 6.1% and 76.2 $\pm$ 5.6%, respectively). Similar changes were shown for TLR-6 (CD14⁺CD286⁺) (44.6 $\pm$ 5.9%, 57.8 $\pm$ 2.2% and 61.1 $\pm$ 1.4%, respectively). Signaling from TLR-2 is the main factor in the activation of T- and B-lymphocytes.

Interferon production including interferon alpha and gamma in newborns with cytomegalovirus infection was significantly decreased. Owing to interferons, cells become resistant to the virus. A decrease in the level of interferon inevitably leads to significantly lower response against invading viral agent. Insufficient activation of a specific antiviral response occurs, which further may be one of the factors in developing severe form of the disease. In this regard, the study of the cytokine system as regulators of inflammation processes is of great interest.

During the study we found a direct correlation between the number of TLR-2 expressing monocytes and level of IFN γ ($r = 0.60$, $p < 0.05$).

When conducting a study of the level of interferon alpha, it was found that in both subgroups it was significantly decreased compared to the control (8.3 ± 0.9 pg/ml and 10.7 ± 1.1 pg/ml *versus* 15.9 ± 2.1 pg/ml, respectively), with the peak decrease in subgroup 1.1 (8.3 ± 0.9 pg/ml), which may be associated with lowered number of monocytes expressing TLR-2 in such newborns.

It should be noted that the level of IFN γ in group 1.1 was significantly decreased (42.1 ± 9.7 pg/ml) both compared with group 1.2 and control group. A decrease in the production of IFN α and IFN γ , which can be both a cause and a consequence of acute and chronic viral diseases, indicates a congenital or acquired deficiency in the interferon system and can be considered a marker of severe generalized cytomegalovirus infection.

Taking into account that interleukin 6 participates as a cofactor in the maturation and differentiation of B-lymphocytes, we studied its level in the newborn cohort. It was found that during severe generalized CMVI, the amount of IL-6 was significantly higher than the value of the control group (25.8 ± 5.2 pg/ml *versus* 9.0 ± 1.6 pg/ml, disease, the concentration of IL-6 was also higher than in the control group (18.3 ± 4.2 pg/ml and 9.0 ± 1.6 pg/ml, respectively).

IL-8 is a major chemokine involved in neutrophil activation, apoptosis, chemotaxis, and migration of immune cells during inflammation and antiviral response. In the peripheral blood serum of healthy newborns, the level of IL-8 averaged 26.3 ± 7.7 pg/ml, whereas in subgroup 1.1 it reached 71.3 ± 5.8 pg/ml, being higher by 2.7-fold than in control group.

MCP-1 is a potent chemoattractant for monocytes/macrophages and activated T-lymphocytes, produced by many cell types. MCP-1 production is initiated by inflammatory mediators: IL-1, TNF α , IFN γ , and lipopolysaccharides (LPS). After analyzing the data obtained, it was revealed that in subgroup II the content of MCP-1 was significantly higher than the level of the control group (1083.2 ± 258.4 pg/ml *versus* 600.5 ± 53.9 pg/ml) compared to newborns in subgroup I.2, where no change in the level of MCP-1 was observed compared with the control group.

Infectious diseases in newborns are the most important medical and social problem of modern perinatology, which is due to the severity of the course of the disease and high frequency of complications. An increase in the frequency of fetal and neonatal infections is caused by a prominent spread of infection, including cytomegalovirus, in the urogenital tract in pregnant women, which in turn results in complicated adaptation during the neonatal period.

Innate immunity plays an important role in protecting newborns from infection because in the first month of life there is no immunological memory and the ability to develop specific antibodies to pathogens is significantly reduced. Therefore, protection against infection in newborns mainly depends on innate immunity and its humoral component.

Cytomegalovirus is able to evade immune control, including impaired both the quantitative composition of immune cells and their synthetic function. Our analysis of immunological parameters showed that the formation of cytomegalovirus infection was accompanied by impaired immunity in newborns. The subgroup with severe CMVI and full clinical picture was characterized by decreased in the number of monocytes expressing TLR-2 and TLR-6, decreased level of IFN α , IFN γ , increased level of IL-6, IL-8, and MCP-1. In the subgroup with moderate CMVI, there was a decrease in the IFN α content in combination with an increase in the IL-6 level.

The consequence of decreased count of monocytes expressing TLRs may result in no proper response against invading virus, prolonged persistence and chronicity of infection, because our data showed that the number of monocytes expressing TLRs correlates with the cell activity. Such monocytes are incapable of adequate activation – sensing and signal transmission after infection; suppressed production of pro-inflammatory cytokines which affects the timely activation of antigen-specific arm in the immune system. Consequently, the observed decrease in the level of monocytes expressing TLR-2 and TLR-6 can account for the high sensitivity to CMV and is consistent with the data [5] on the participation of TLR in the formation of generalized CMVI.

Cytokine production forms the basis for the immune response and has a direct effect on the mechanisms of immunoresistance in cytomegalovirus infection.

In our study, an increased amount of IL-6 was found in subgroups with varying degrees of CMVI severity. We hypothesized that these changes can lead to a shift in the differentiation of T-helpers towards Th2 subset and lacked effective antiviral response.

An increase in the level of IL-8 and MCP-1 in newborns with CMVI can lead to elevated CMV replication due to the use of chemokine receptors, damage to the vascular endothelium, and a reduction in the antiviral interferon activity, which is consistent with the previous data [1].

Among the factors of immune defense, the interferon system, which plays an important role both in mounting antiviral immunity and regulation of the immune response in viral infection, has been currently attracting much attention. The state of the neonatal interferon system determines host immunological maturity. These data on decreased

IFN α production indicate at imbalance in this protective mechanism resulting in the onset and severe course of intrauterine cytomegalovirus infection. A low level of IFN γ demonstrates impairment in the functional activity of CD4⁺-lymphocytes in newborns, which is of decisive importance in the formation of immune dysfunction.

Thus, the development of intrauterine cytomegalovirus infection in newborns is associated with impaired cytokine balance with a tendency to the Th2-

type, impaired processes of intercellular interaction. Deficiency in the IFN-reaction of peripheral blood leukocytes particularly IFN α and IFN γ . Decrease in the number of monocytes, expressing TLR-2 and TLR-6, which indicates suppression of the early stages of the immune response, impairment of the synthesized function of immunocompetent cells. Identified immune disorders leads to uncontrolled viremia, determines the severity of the disease in prenatally infected newborns.

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ГЕРПЕСВИРУСНЫЕ ИНФЕКЦИИ И КЛИНИКО-ИММУНОЛОГИЧЕСКИЕ ВЗАИМОДЕЙСТВИЯ ПРИ БОЛЕЗНИ АЛЬЦГЕЙМЕРА С РАННИМ НАЧАЛОМ И БОЛЕЗНИ АЛЬЦГЕЙМЕРА С ПОЗДНИМ НАЧАЛОМ

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Резюме. Болезнь Альцгеймера (БА) — в настоящее время наиболее частая причина деменции. Значительная роль в патогенезе БА принадлежит активации механизмов врожденного иммунного ответа ЦНС и нейровоспалению. Существует гипотеза, что в развитии и поддержании воспалительной реакции на ранних стадиях БА могут иметь значение хронические инфекции. Целью настоящего исследования являлось изучение частоты выявления и уровня ДНК герпесвирусов, а также их возможной связи с уровнем основных цитокинов врожденного и адаптивного иммунного ответа и с клиническими показателями при БА у больных с ранним и поздним началом заболевания. В исследование вошли 30 больных БА на стадии умеренной деменции и 33 здоровых добровольца. Количественное определение ДНК герпесвирусов (CMV, EBV, HHV-6, HHV-7) в слюне и в крови проводили методом ПЦР с гибридационно-флуоресцентной детекцией в режиме «реального времени». Уровень цитокинов и растворимого антагониста IL-1β (IL-1ra) в крови определяли методом ИФА. Герпесвирусную инфекцию с повышенной вирусной нагрузкой определяли, учитывая предыдущие данные, полученные авторами, при наличии хотя бы одного из 2 критериев: 1) уровень ДНК вирусов EBV и/или HHV-6 более 10 000 копий/мл в слюне; 2) наличие ДНК хотя бы одного из вирусов EBV, HHV-6, HHV-7 в крови. Выявлено, что при раннем начале достоверно чаще обнаруживался HHV-6 в слюне и крови, а при позднем — EBV в слюне. При раннем начале в подгруппе больных с повышенной вирусной нагрузкой было достоверно более выраженное повышение уровней ряда основных цитокинов: провоспалительных цитокинов IL-8 и IL-12p40p70, цитокина Th2-звена адаптивного иммунного отве-

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та IL-4, цитокина адаптивного иммунного ответа IL-2, хотя уровень противовоспалительного белка IL-1ra был достоверно ниже, чем в контрольной группе. Учитывая отсутствие выраженного роста уровня основного цитокина Th1-звена адаптивного ответа IFN γ , эти изменения могут свидетельствовать о нарушении реакций противовирусной защиты, с активацией системного воспаления и Th2-звена иммунитета. Также при раннем начале повышенная вирусная нагрузка была связана с более низкими баллами Бостонского теста называния, отражающего сохранность номинативной функции речи. Представленные результаты свидетельствуют, что при исследованиях механизмов БА и поиске прогностических маркеров болезни важным является учет неоднородности заболевания по генетическим факторам предрасположенности, факторам риска, особенностям иммунитета и клиническим данным, что необходимо для последующей разработки персонализированных подходов к профилактике и лечению БА.

Ключевые слова: болезнь Альцгеймера, Бостонский тест называния, aMCI, EBV, HHV-6, HHV-7, IFN γ , MMSE

HERPESVIRUS INFECTIONS AND CLINICAL-IMMUNOLOGIC INTERACTIONS IN PATIENTS WITH EARLY-ONSET ALZHEIMER'S DISEASE AND LATE-ONSET ALZHEIMER'S DISEASE

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Abstract. Alzheimer's disease (AD) is currently the most common cause of dementia. A significant role in the pathogenesis of AD belongs to the activation of the mechanisms of neuroinflammation. There is a hypothesis that chronic infections may play a role in the maintenance of the inflammatory response in AD. The aim of this work was to study the detection rate and DNA level of herpesviruses, as well as their possible relationship with the level of the key cytokines and with clinical parameters of AD in patients with early and late onset. 30 patients with AD and 33 healthy volunteers were enrolled. The quantitative determination of DNA of CMV, EBV, HHV-6, HHV-7 was carried out by PCR. The level of cytokines and soluble IL-1 β antagonist (IL-1ra) in the blood was determined by ELISA. Herpesvirus infection with increased viral load was determined if at least one of the criteria was present: 1) DNA level of EBV and/or HHV-6 > 10,000 copies/ml in saliva; 2) presence of DNA of at least one of the EBV, HHV-6, HHV-7 viruses in the blood. In the subgroup of patients with early onset and increased viral load, there was a higher increase in the levels of a number of cytokines: proinflammatory IL-8 and IL-12, a Th2-cytokine IL-4, a cytokine of the adaptive immune response IL-2. However, the level of the anti-inflammatory protein IL-1ra was lower than in the controls. These changes may indicate a dysregulation of the antiviral response, with a predominance of activation of systemic inflammation and Th2-mediated reactions. Also, in early onset AD the increased viral load was associated with lower scores on Boston naming test. The results indicate that in studies of AD mechanisms and in the search for prognostic markers of the disease, it is important to take into account the heterogeneity of AD in terms of genetic predisposition factors, risk factors, immune parameters and clinical data. Such approach is necessary for the subsequent development of personalized approaches to the prevention and treatment of AD.

Keywords: Alzheimer's disease, aMCI, Boston naming test, EBV, HHV-6, HHV-7, IFN γ , MMSE

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Introduction

Alzheimer’s disease (AD) is currently the most common cause of dementia. According to the WHO, in 2017, about 47.5 million people suffered from AD. This number is expected to triple over the next 35 years, making AD one of the most serious public health problems [1]. There are two forms of AD: early-onset Alzheimer’s disease (EOAD) and late-onset AD (LOAD). EOAD accounts for less than 10% of cases of the disease; but often progresses rapidly, and has notorious social and economic importance [11]. Most cases of EOAD are genetically determined by dominant mutations in the genes of the amyloid precursor protein (APP) presenilin-1 (PS1) or presenilin-2 (PS2) [16]. With these mutations, there is an increased formation of amyloid-beta ($A\beta$) 42 in the central nervous system (CNS) and in other tissues of the body. $A\beta$ contributes to the pronounced manifestations of oxidative stress, having a toxic effect on mitochondria: it reduces the number of mitochondrial complexes I and IV, disrupting the functioning of the oxygen transport chain and oxidative phosphorylation [11]. LOAD accounts for up to 90% of cases of Alzheimer’s disease and is a multifactorial disease that is often associated with the presence of the genetic polymorphism ApoE ϵ 4, which contributes to disorders of lipid metabolism in the CNS, to changes in the metabolism of $A\beta$ and to disruption of neuroimmune regulation in the CNS [7].

A significant role in the pathogenesis of AD belongs to the activation of the mechanisms of neuroinflammation, which contributes to neuronal damage and neurodegeneration. There is a hypothesis that chronic infections may play a role in the development and maintenance of the inflammatory response in the early stages of AD. A number of neurotropic infectious agents including herpesviruses have been found in the postmortal brain of AD patients, and there is data in the literature that supports their possible role in the pathogenesis of neuroinflammation in AD [7]. Even their latent presence can activate microglial cells, which cause oxidative stress, neuroinflammation, and neuronal damage [6].

The association of the HSV1 virus with the progression of AD and the effect of active HSV infection on the risk of AD has been established several decades ago and has been extensively studied [5]. However, the relationship of other herpesviruses (Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7)) with the risk and progression of AD is much less studied. There are isolated works on the increased frequency of HHV-6 DNA detection in the postmortem brain

of AD patients [12]. In one of the studies, an increase in the frequency of detection of EBV and HHV-6 in blood leukocytes in AD was shown [3]. At the same time, there are practically no studies reporting the results of quantitative assessment of the level of EBV, HHV-6 and HHV-7 DNA in EOAD and LOAD. Also, the relationship of herpesviruses with the clinical course of AD and with immunological disorders in AD has been little studied.

The aim of this work was to study the detection rate and DNA level of herpesviruses, as well as their possible relationship between the level of the key cytokines and clinical parameters in early and late onset AD patients.

Materials and methods

30 AD patients (13 men, 17 women, mean age 69 ± 2 years) at the stage of moderate dementia and 33 age- and sex-matched healthy volunteers have been enrolled into the study. Early onset AD (up to 65 years) was observed in 15 patients (mean age 61 ± 1.56 years), late AD onset (after 65 years) was also noted in 15 patients (mean age 77 ± 1.71 years).

The patients and their relatives were familiarized with the study protocol and signed a voluntary informed consent. Inclusion criteria were as follows: the ability to sign and date the informed consent form or the presence of a legal representative who can sign and date the informed consent form; AD diagnosis according to NINCDS / ADRDA criteria and MMSE score ≤ 26 points; score on the Khachinski scale ≤ 4 ; geriatric depression score ≤ 10 .

DNA of CMV, EBV, HHV-6, HHV-7 were quantified by PCR on a Rotor-Gene Q analyzer (Qiagen, Germany) using the AmpliSens reagent kits (AmpliSens, Moscow). The level of serum cytokines and soluble IL-1 β antagonist (IL-1ra) was determined by ELISA on an iMARK immunoanalyzer (Bio-Rad, USA) using test systems manufactured by “Cytokine”, Russia (IFN γ , IL-4, IL-8, IL-1ra), BCM Diagnostics, USA (IL-12p40p70), and Bender MedSystems, Australia (IL-2).

Herpesvirus infection with escalated viral load was determined, based on our previous data, if at least one of the 2 criteria was present: 1) DNA level of EBV and/or HHV-6 $> 10,000$ copies/ml in saliva; 2) the presence of DNA of at least one of the EBV, HHV-6, HHV-7 viruses in the blood [4].

The following tests and scoring scales were used for neuropsychological examination: clinical dementia rating scale (CDR); mini-mental state examination (MMSE); frontal assessment battery; clock drawing test; Boston naming test.

Microsoft Excel software was used for statistical analysis. The distribution of the studied parameters was assessed using the Shapiro-Wilks test. The results were presented as means with standard error. While

comparing differences a significance level was set at $p < 0.05$. Student's *t* test was used to compare group means, the Chi-squared test was used to compare relative values.

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Results and discussion

It was found that CMV was detected neither in saliva nor blood in any patient. However, EBV and HHV-6 were detected in the patients with a high frequency (Table 1).

The frequency of herpesvirus detection and the viral load depending on the age of AD onset is presented in Table 1. DNA of at least one of the EBV, HHV-6 and HHV-7 viruses was detected in the blood of 8/15 patients with EOAD, in 4 out of 15 patients with LOAD, and in 3 out of 33 healthy volunteers. Increased viral load was observed in 9 out of 15 patients with EOAD (significantly more often compared with control group, $p < 0.05$), 8 out of 15 patients with LOAD, and 6 out of 33 healthy volunteers.

In EOAD, increased viral load was associated with lower Boston naming test scores, which reflects more pronouncedly impaired nominative function of speech ($p = 0.016$) (Figure 1).

Previously, we presented data on the level of the key cytokines in EOAD and LOAD by showing increased

level of proinflammatory cytokines IL-8, TNF α , and IL-1ra in patients vs controls [10]. Here, we analyzed the level of cytokines (interferon- γ (IFN γ), tumor necrosis factor- α (TNF α), interleukin-4 (IL-4), IL-8, IL-12p40p70, IL-2) and IL-1ra coupled to viral load in patients with EOAD and LOAD (Table 2). The subgroup of patients with increased viral load and EOAD revealed a significantly higher rise in the levels of certain cytokines: proinflammatory IL-8 and IL-12, a Th2-cytokine IL-4, a cytokine of the adaptive immune response IL-2. At the same time, the level of the anti-inflammatory protein IL-1ra was significantly lower than in the controls. Considering the lack of increased level of the key Th1-cytokine, IFN γ , these changes may indicate a dysregulated antiviral response, with a predominance of activated systemic inflammation and Th2-mediated reactions.

The role of herpesvirus infections in the pathogenesis of Alzheimer's disease has been long studied, however, most works are devoted to the herpes simplex virus type 1 (HHV-1) and refer to patients with late onset AD as the most common clinical form. In this pilot study, we for the first time assessed the levels of herpesvirus DNA in AD with early and late onset. An increased frequency of detected herpesvirus DNA was found for both EOAD and LOAD, but in the latter EBV was more frequent and at higher levels detected in saliva, whereas in the former the rate of HHV-6 DNA detection was increased in saliva and blood, and the levels of HHV-6 in saliva was increased.

TABLE 1. FREQUENCY OF DETECTION OF DNA OF HERPESVIRUSES IN SALIVA AND BLOOD AND THE LEVEL OF VIRAL LOAD IN EARLY-ONSET ALZHEIMER'S DISEASE (EOAD), LATE-ONSET ALZHEIMER'S DISEASE (LOAD) AND IN THE CONTROL GROUP

Disease/viruses	EBV, frequency and copy number	HHV-6, frequency and copy number	HHV-7, frequency and copy number	CMV, frequency
Control group, n = 33 (saliva)	6/33 $6.3 \times 10^2 \pm 5.6 \times 10^1$ copies/ml	17/33 $5.0 \times 10^2 \pm 3.2 \times 10^2$ copies/ml	30/33 $7.6 \times 10^5 \pm 2.9 \times 10^5$ copies/ml	0/33
Control group, n = 33 (blood)	2/33 $1.7 \times 10^1 \pm 1.4 \times 10^1$ copies/ 10^6 cells	1/33 $2.6 \times 10^1 \pm 2.6 \times 10^1$ copies/ 10^6 cells	3/33 $6.2 \times 10^1 \pm 6.2 \times 10^1$ copies/ 10^6 cells	0/33
EOAD, n = 15 (saliva)	8/15* $1.6 \times 10^3 \pm 4.2 \times 10^2$ copies/ml	15/15* $1.1 \times 10^3 \pm 2.2 \times 10^2$ copies/ml	14/15 $5.3 \times 10^4 \pm 1.1 \times 10^4$ copies/ml	0/15
EOAD, n = 15 (blood)	2/15 $2.9 \times 10^1 \pm 2.9 \times 10^1$ copies/ 10^6 cells	6/15* $1.0 \times 10^2 \pm 1.2 \times 10^1$ copies/ 10^6 cells	4/15 $3.9 \times 10^3 \pm 1.2 \times 10^3$ copies/ 10^6 cells	0/15
LOAD, n = 15 (saliva)	10/15* $5.2 \times 10^5 \pm 3.1 \times 10^5$ copies/ml	10/15 $3.8 \times 10^3 \pm 1.5 \times 10^3$ copies/ml	12/15 $3.9 \times 10^4 \pm 2.9 \times 10^4$ copies/ml	0/15
LOAD, n = 15 (blood)	2/15 $4.2 \times 10^2 \pm 4.2 \times 10^2$ copies/ 10^6 cells	2/15 $4.5 \times 10^1 \pm 4.5 \times 10^1$ copies/ 10^6 cells	0/15	0/15

Note. *, significant differences with the control group, $p < 0.05$.

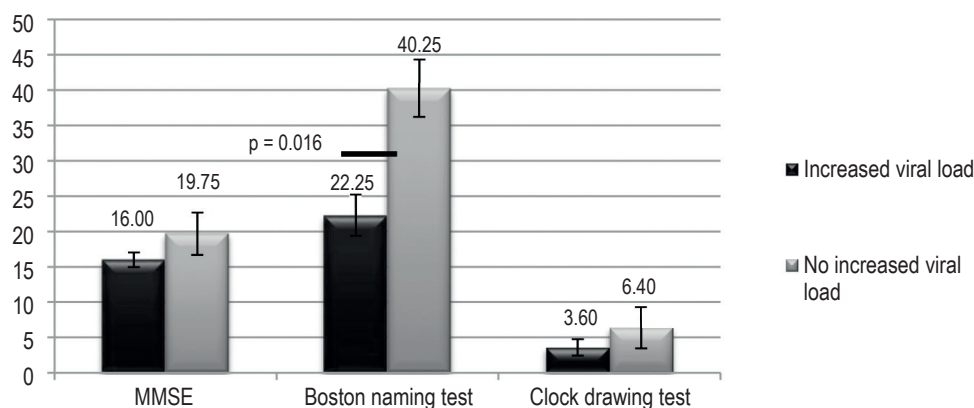


Figure 1. Results of neuropsychological examination in early-onset Alzheimer's disease in patients with and without an increased viral load (the DNA level of EBV and/or HHV-6 viruses is more than 10 000 copies/ml in saliva; or the presence of DNA at least one of EBV, HHV-6, HHV-7 viruses in the blood)

Note. Lower values indicate more pronounced neuropsychological deficits

The relationship between viral load with declined cognitive functions and immunological parameters was found in patients with early onset AD, but not in late onset AD, which might be due to the phenotypic patient heterogeneity [8]. According to the data obtained, the increased viral load in EOAD is associated with activated low-grade systemic inflammation and the Th2-arm of the immune response. According to the literature, such changes may be related to the development and progression of neurodegeneration. Thus, CNS macrophages and microglial cells acquire the M2 phenotype, disrupting the mechanisms of effective antiviral defense affected by the Th2-cytokine IL-4 [13]. Pro-inflammatory IL-8, TNF α , IL-12 stimulate macrophage activation, production of chemokines and other inflammatory factors. According to our results, the pre-dementia

AD stage and amnesic mild cognitive impairment (aMCI) were coupled to increased levels of IL-1 β , TNF α and IL-8, which correlated with a higher probability of progression to AD [8]. IL-12 is a potent stimulator of IFN γ production activating the Th1-arm of adaptive immunity; however, an impaired regulation of the immune response may be bound to dominance of its pro-inflammatory effect. The knockout of the p40 subunit of IL-12, IL-23 dimeric cytokine slows aging-associated neuronal loss and A β deposition in wild-type mice [15].

The presence of chronic systemic inflammation increases the risk of AD by 1.5-1.8 times [14]. It is also known that A β is an antimicrobial protein and an important element of the innate immune defense system. There is evidence that the reactivation of latent infections increases A β production, which contributes

TABLE 2. THE VIRAL LOAD AND THE LEVELS OF THE MAIN CYTOKINES IN THE BLOOD IN EARLY ONSET ALZHEIMER'S DISEASE (EOAD), LATE ONSET ALZHEIMER'S DISEASE (LOAD) AND IN THE CONTROL GROUP

	EOAD		LOAD		Controls
	Increased viral load	No increased viral load	Increased viral load	No increased viral load	
MMSE	16.0 $\pm$ 2.1	19.7 $\pm$ 6.0	20.8 $\pm$ 1.8	16.7 $\pm$ 4.6	30.0 $\pm$ 0.0
Boston naming test	22.3 $\pm$ 5.6*	40.2 $\pm$ 7.9	34.0 $\pm$ 11.2	30.5 $\pm$ 0.8	52.2 $\pm$ 2.2
IFN γ , pg/ml	295.5 $\pm$ 63.6	234.7 $\pm$ 48.7	225.1 $\pm$ 52.6	150.7 $\pm$ 20.6	232.5 $\pm$ 6.4
TNF α , pg/ml	6.2$\pm$0.8	9.4$\pm$2.0	4.7$\pm$0.6	5.5$\pm$0.1	3.2 $\pm$ 0.3
IL-2, pg/ml	295.8$\pm$179.1	27.9 $\pm$ 14.1	12.5 $\pm$ 0.9	12.5 $\pm$ 0.4	13.7 $\pm$ 1.1
IL-4, pg/ml	16.3$\pm$8.5	0.02 $\pm$ 0.02	0.3 $\pm$ 0.3	2.8 $\pm$ 1.0	1.42 $\pm$ 0.53
IL-8, pg/ml	190.8$\pm$90.1	60.0 $\pm$ 19.2	40.7 $\pm$ 17.3	18.3 $\pm$ 5.5	15.97 $\pm$ 2.00
IL-12p40p70, pg/ml	196.6$\pm$27.7	134.5 $\pm$ 25.2	186.1 $\pm$ 45.0	202.1 $\pm$ 41.5	142.7 $\pm$ 16.81
IL-1ra, pg/ml	30.8$\pm$16.3	845.1 $\pm$ 706.1	78.5 $\pm$ 55.6	935.4$\pm$441.7	133.1 $\pm$ 40.3

Note. *, significant differences between groups, $p < 0.05$; bold, significant differences with the control group, $p < 0.05$.

to developing neuroinflammation, and subsequent formation of senile plaques [2]. It cannot be ruled out that impaired immunity and systemic inflammation, observed upon increased EBV and HHV-6 viral load in patients with early onset AD, affect neurodegeneration, modulating the activity of neuroinflammation mechanisms through neuroimmune interactions. It is important to note that early onset AD patients with increased viral load had lower Boston naming test score, suggesting more pronouncedly impaired nominative function of speech. Taking into account our previous results and literature data, emphasizing that systemic inflammation and disorders of adaptive immunity are a risk factor for the AD progression, the results obtained indicate a possible pathogenetic role of immunological disorders associated with high viral load in early onset AD.

We have previously described immunological disorders at different stages of AD, as well as immunological heterogeneity of AD at its pre-dementia stage (aMCI), showing that the type and severity of immunological disorders are associated with the risk

of AD progression [2, 9]. According to the results of our pilot study, the relationship of infectious factors between immunological parameters and clinical characteristics in AD is uneven depending on the age of disease onset. It is known that early onset AD and late onset AD differ in genetic predisposition factors, which may affect differences in pathogenetic mechanisms. It is possible that in early onset AD, an increase in the viral load upon reactivation of chronic viral infections and the immunological disorders associated with related reactivation may contribute to neuroinflammation and more rapid progression of neurodegeneration. Further research is needed to investigate a role of infectious factors in developing Alzheimer's disease. The presented results indicate that in the studies of AD mechanisms and in the search for prognostic disease markers, it is important to take into account the heterogeneity of AD in terms of genetic predisposition factors, risk factors, immune parameters and clinical data. Such an approach is necessary for subsequent development of personalized approaches for AD prevention and treatment.

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ИЗУЧЕНИЕ ЛОКАЛЬНОЙ ЭКСПРЕССИИ мРНК ГЕНОВ МЕДИАТОРОВ ВОСПАЛЕНИЯ В МОДЕЛИ АТРОФИИ РЕТИНАЛЬНОГО ПИГМЕНТНОГО ЭПИТЕЛИЯ И ДЕГЕНЕРАЦИИ СЕТЧАТКИ, ИНДУЦИРОВАННОЙ СУБРЕТИНАЛЬНЫМ ВВЕДЕНИЕМ ФИЗИОЛОГИЧЕСКОГО РАСТВОРА В ЭКСПЕРИМЕНТЕ У КРОЛИКОВ

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Резюме. Дегенеративно-дистрофические заболевания сетчатки, в частности возрастная макулярная дегенерация (ВМД), в настоящее время считаются ведущей причиной слепоты и слабовидения в развитых странах, имеют тенденцию к неуклонному росту. В публикациях последних лет представлены доказательства участия воспалительных механизмов в развитии и прогрессировании ВМД, расшифрованных благодаря успехам в области исследований врожденного и адаптивного иммунитета. Однако иммунопатогенез атрофической формы ВМД – «географической атрофии» (ГА) остается практически неизученным. Целью работы явилось исследование локальной экспрессии мРНК воспалительных цитокинов IL-1 β , IL-18, CCL2/MCP-1 в модели атрофии РПЭ, индуцированной субретинальным введением 0,9%-ного раствора хлорида натрия в эксперименте у кроликов. Исследования выполнены в образцах тканевого комплекса сетчатки-РПЭ-хориоидеи (ТК), выделенного из глаз 23 кроликов породы новозеландских альбиносов, на которых моделировалась атрофия РПЭ путем субретинального введения 0,9%-ного раствора хлорида натрия, и 5 здоровых кроликов без глазных поражений. Животным опытной и контрольной групп за неделю до оперативного вмешательства, в раннем периоде, в сроки формирования устойчивой атрофии (РПЭ) проводились оптическая когерентная томография (ОКТ) и аутофлуоресценция глазного дна (АФ). Оценка уровней экспрессии генов провоспалительных цитокинов в ТК выполнялась методом ОТ-ПЦР. Показано, что субретинальное введение 0,01 мл 0,9%-ного раствора хлорида натрия, индуцирующее развитие атрофии РПЭ у кроликов в эксперименте, ассоциируется с разнонаправленными изменениями экспрессии mRNA

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генов IL-1 β , IL-18, MCP-1/CCL2 относительно нормы. Охарактеризованы три типа ответа в тканевом комплексе хориоидея / РПЭ / сетчатка, формируемые во время развития атрофических изменений и определяемые величиной локальной экспрессии генов цитокинов: 1) гипо/ареактивность — снижение/отсутствие экспрессии; 2) нормореактивность — умеренное повышение; 3) гиперреактивность — гиперэкспрессия. У 69,6% животных при формировании устойчивой атрофии отмечалось повышение (от умеренного до гиперответа) локальной экспрессии mRNA MCP-1/CCL2, а в трети случаев — значительное усиление экспрессии mRNA IL-1 β — факторов, повреждающих гематоретинальный барьер и способствующих нарушению иммунной привилегии заднего отрезка глаза. Полученные данные могут быть полезны в изучении различных видов атрофии РПЭ и при разработке новых стратегий лечения офтальмопатологии, в доклинических исследованиях.

Ключевые слова: ВМД, атрофия ретинального пигментного эпителия, комплекс «сетчатка — хориоидея», провоспалительные цитокины, экспрессия генов, ОТ-ПЦР

EXAMINING LOCALLY EXPRESSED mRNA OF INFLAMMATORY MEDIATOR GENES IN A MODEL OF RETINAL PIGMENT EPITHELIUM ATROPHY AND RETINAL DEGENERATION INDUCED BY SUBRETINAL SALINE INJECTION IN RABBITS

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Abstract. Degenerative-dystrophic retinal diseases, particularly age-related macular degeneration (AMD), are now considered to be the lead cause of blindness and low vision in developed countries, with a steadily increasing trend. Recent publications provide evidence for the involvement of inflammatory mechanisms in TMD development and progression unveiled due to advances in innate and adaptive immunity research. However, the immunopathogenesis of atrophic AMD form, “geographic atrophy” (GA) remains largely unstudied. Objective: to investigate local mRNA expression of inflammatory cytokines IL-1 β , IL-18, CCL2/MCP-1 in a model of RPE atrophy induced after subretinal injection of 0.9% sodium chloride solution in experimental rabbits. The investigation was carried out in tissue complex retina-RPE-choroid (TC) samples isolated from eyes of 23 albino New Zealand rabbits after modeling RPE atrophy by subretinal injection of 0.9% sodium chloride solution and 5 healthy rabbits lacking eye lesions. Animals in the experimental group (one week before surgical intervention, in the early period, and in the period of sustained RPE atrophy formation) and controls were subjected to optical coherence tomography (OCT) and ocular fundus autofluorescence (FAF). Evaluation of proinflammatory cytokine gene expression levels in TC was performed by RT-PCR. Results. Subretinal injection of 0.01 ml of 0.9% sodium chloride solution induced experimental RPE atrophy development in rabbits vs. control that was associated with multidirectional changes of IL-1 β , IL-18, MCP-1/CCL2 gene mRNA expression. Three types of response in the TC, formed during development of atrophic changes and determined by the value of local cytokine gene expression were characterized: 1) hypo/ no response — decreased/no expression; 2) normal response — moderate increase; 3) hyper response — overexpression. 69.6% of animals with persistent atrophy had a moderate to hypertrophic increase in locally expressed mRNA MCP-1/CCL2, whereas 30% cases had significantly increased IL-1 β mRNA expression — factors damaging the blood-retinal barrier and contributing to posterior segment immune privilege. It should be taken into account while developing new strategies for treatment of ophthalmic pathology, in particular the currently actively studied and tested options for RPE stem cell transplantation into subretinal space. The data obtained may be useful to investigate various types of RPE atrophy and develop new strategies of ophthalmopathology treatment in preclinical studies.

Keywords: AMD, atrophy of the retinal pigment epithelium, retina-choroid complex, proinflammatory cytokines, gene expression, RT-PCR

Introduction

Degenerative-dystrophic retinal diseases are currently considered as the lead cause of blindness and low vision in developed countries and have a steadily increasing trend. Retinal pigment epithelium damage (RPE) is the initial step in the development of degenerative changes in age-related macular degeneration, leading to photoreceptor death and irreversible loss of central vision. RPE is formed by a single layer of hexagonal polarized pigmented epithelial cells and located between the choroid and the neurosensory retina [10].

Normally, the RPE exerts many specialized functions aimed at maintaining retinal homeostasis and performing the visual cycle [4, 10]. RPE plays an important role in ocular immune privilege by forming the outer part of the haemato-retinal barrier and secreting membrane-bound and soluble immunosuppressive and apoptotic factors into the subretinal space [3, 5]. PPE cells are incapable of regeneration, and their damage is irreversible [9].

An effective treatment for GA has not been developed [11]. Despite intensive research, the obstacles in development of promising new treatments for AMD are primarily due to incomplete knowledge of the disease pathogenesis.

Recent publications show that the process of RPE and central retinal photoactive zone damage is associated with the activation of identified inflammatory mechanisms owing to advances in innate and adaptive immunity research [13].

Thus, simulating unfavorable conditions such as hypoxia, hyperglycemia, oxidative and hyperosmolar stress were shown to initiate inflammatory signaling pathways in RPE cells and neuroglia, closely related to activation of the transcription factor NF- κ B, assembly of the NLRP3-inflammatory complex and processing of IL-1 β and pro IL-18 to mature forms via secreted multiple inflammatory mediators and chemoattractant molecules [6, 7].

With the accumulation of IL-1 β and IL-18 in the intercellular space — acting in paracrine and autocrine manner can reduce the viability of RPE cells [1, 12], promote development of a new round of inflammation by stimulating intracellular proinflammatory signaling pathways, production of chemokines (IL-8, MCP-1/CCL-2) [2] and recruitment of immunocompetent cells to the lesion.

However, numerous experimental studies are currently carried out mainly on isolated RPE cells or with varying quantitative and qualitative RPE composition in combination with glial, myeloid and lymphoid cell elements *in vitro*.

It is important to consider that all closely interacting retinal cellular communities, the supplying blood vessels, the choroids, as well as leukocytes migrating to the inflammation focus are involved in the process of fundus lesions in AMD along with RPEs.

Studying the patterns of local gene expression of proinflammatory cytokines in the TC in experimentally simulated animal PPE atrophy is an important issue because it may allow determining a role and place of proinflammatory mediators in the pathological process and help developing new treatment strategies for severe degenerative retinal diseases in humans.

Purpose: to investigate the local mRNA expression for the proinflammatory cytokines IL-1 β , IL-18, MCP-1/CCL2 in a model of RPE atrophy induced after a single subretinal saline injection in rabbit experiment.

Materials and methods

Modeling RPE atrophy and retinal degeneration

The international principles of the Helsinki Declaration on the Humane Treatment of Animals, the principles of humanity set out in the European Community directive (86/609/EC) “Regulations on the Handling of Test Animals” were complied in the experimental studies. 23 male New Zealand albino rabbits (age 2.5-3.0 months, weighted 2.0-2.5 kg) were injected once with 0.01 ml of 0.9% sodium chloride solution into the subretinal space below the retina 1-1.5 mm downwards from the optic nerve head to generate a subretinal bladder. To reduce a risk of exudative-hemorrhagic intra- and postoperative complications, animals were injected intramuscularly with 0.3 ml of Zoletil 50 and 0.55 ml of 2% xylazine, and 0.3 ml of 0.4% dexamethasone, 0.5 ml of diclonine before surgery. The control group consisted of 5 somatically healthy rabbits without ocular pathology.

Specialized ophthalmological examinations

Optical coherence tomography (OCT), autofluorescence (FAF) study using TMSD-OCT (Heidelberg Engineering, Germany) were performed one week before modeling RPE atrophy, in early period (from 2 days to 1 week) and late period (from 2-4 weeks and later) after surgery by sacrificing anaesthetized animals via air embolism method (Order of the USSR Ministry of Education and Science No. 724 of 13.11.184).

RT-PCR method for determination of cytokine expression levels in eye tissues of experimental animals. Molecular biological studies were performed in 53 TC samples isolated from enucleated eyes of experimental and control rabbits according to standard protocols. Biomaterial was placed in cryovials and stored at -70°C until the study. Using the NCBI GenBank electronic database and Primer-BLAST, OligoCalc: Using the Oligonucleotide Property Calculator and BLAST Standard Nucleotide Software Package, the oligonucleotide sequences for the genes presented below were selected. IL-1 β : For 5-aatctgtacctgtcctgcgtg-3 ; Rev 5-ggttgggtctacactctcc-3 ; IL-18: For 5-accagaagag-gcttgcatca-3 ; Rev 5-tccaggttctcatcgttttcagt-3 ; MCP1/CCL2: For 5-atgaaggtctctgcaacgct-3 ; Rev

5-cccttgccagtttggtcat-3 ; GAPDH: For 5-gattgtcagcaacgcacatcctg-3 ; Rev 5-ctccacaatgccgaagtgt-3 .

TC samples were homogenized for 90 s at 45,000 rpm (homogenizer for operation in microvolumes Silent Crusher S Heidolph, Germany).

Isolation of mRNA from tissue samples was carried out using a sorbent-column method (RNeasy Mini Kit, Qiagen, Germany) added with 1 µl RNase inhibitor (RNase Inhibitor, Qiagen, Germany) and DNase treatment (DNase Max Kit, Qiagen, Germany) according to the manufacturer's instructions. The amount of obtained mRNA was monitored using a spectrophotometer at a 260 nm wavelength (Cytation 5 imaging reader (Biotek)).

cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Bio-Rad, USA): 9 µl of Nuclease-free water, 4 µl 5 iScript Reaction Mix, 1 µl of iScript Reverse Transcriptase, 5 µl mRNA and 1 µl were mixed RNase Inhibitor (Qiagen). The reverse transcription reaction was performed according to the manufacturer's protocol.

The resulting cDNA fragment was amplified by RT-PCR in real time mode on a CFX96 Touch thermal cycler (Bio-Rad, USA).

The composition of the reaction mixture: 9.4 µl dist. water, 1.5 µl buffer, 0.5 µl dNTP, 1 µl cDNA, 0.3 µl SYBR GreenI [1: 25000] (Evrogen, Russia), 0.3 µl Taq polymerase (0.5 U/µl, Biosan, Latvia), 1 µl forward primer (F1), 1 µl reverse primer (R1). Concentrations of primers of the studied and reference genes: F1_IL-1β 1 µM (Mw 63.68 g/mol); R1_IL-1β 1 µM (Mw 60.81 g/mol); F1_IL-18 1 µM (Mw 61.16 g/mol); R1_IL-18 1 µM (Mw 69.50 g/mol); F1_CCL-2 1 µM (Mw 60.98 g/mol); R1_CCL-2 1 µM (Mw 60.56 g/mol); F1_GAPDH 1 µM (Mw 63.86 g/mol); R1_GAPDH 1 µM (Mw 60.83 g/mol).

The normalized $\Delta\Delta C_t$ expression method was used to determine the relative sample cDNA amount. The results were expressed as relative units (relative units): the ratio of the threshold cycle of amplification of the studied gene to the value of the threshold cycle of amplification of the reference gene GAPDH: $\Delta\Delta C_t = (\Delta C_t \text{ sample}) / (\Delta C_t \text{ GAPDH})$.

Statistical data processing

The data were analyzed by using the Statistica 6.0 software package (StatSoft Inc., USA). The normality distribution was estimated by the Kolmogorov-Smirnov method. In case the distribution of parameters differed from normal, nonparametric methods of analysis were used. Mann-Whitney U-test was used to determine the significant differences (p) in two independent samples. The critical level of significance for statistical hypothesis testing was taken as $p < 0.05$.

Results and discussion

Between 2 and 7 days after surgery, changes in the rabbit fundus were determined as neurosensory retinal detachment with hyporeflexive content underneath, retinal tear with elevated edges according to

the cannula injection site, disorganization of retinal layers, irregular hyperfluorescence was determined on FAF indicating RPE lesion.

Reactive changes in the fundus (from 2 days to 1 week) were accompanied by significantly increased TC IL-18 mRNA expression in experimental vs. control group lacking eye damage: the mean value was 23.35 ± 5.43 rel. units (Figure 1).

Analysis of the early tissue response revealed no changes in IL-1β mRNA expression that was comparable to that in the control group.

It is known that IL-1β, in addition to enabling specific protective immunological mechanisms, acts as one of the main mediators of rapid response in development of nonspecific defense such as mounting local inflammatory response to damaging and pathogenic agents.

it might seem paradoxically, that the lack of changes in IL-1β gene mRNA expression in MCs in all animals with early reactive response noted in our study is consistent with the data obtained by N.M. Luyeshi et al. thoroughly investigating kinetics of the proinflammatory response in an mouse ischaemia/reperfusion brain injury model showing that IL-1α mRNA, but not IL-1β, was expressed by microglial cells in the early postischemic period: increased expression of the latter was observed at later stages [8].

The early post-operative period was also trended to increased local expression of MCP-1/CCL-1 gene mRNA: special attention was paid to its wide range of upward shifts relative to normal: from a slight/moderate increase (0.05 rel. units) to a hyperreactive response – a 4000-fold increase in expression compared to healthy eye tissue (Figure 1).

2-4 weeks after surgery, specialized ophthalmic examinations identified a circular light focus on the infra-red image; in this area, thinning of the outer retinal layers, hyperreflectivity of the choroid due to increased laser beam penetration beneath the retina, occurring in the absence of RPE with its high reflectivity was noted on the OCT. Thinning of the RPE and the choroid was identified in the lesion area. Autofluorescence examination identified an area of hypofluorescence corresponding to the atrophy zone, with irregular dissections of hyperfluorescent foci in the periphery.

In the late periods after induction of atrophy (from 2-4 weeks to 2 months), the parameters of the relative mRNA expression for IL-1β, IL-18, and MCP/CCL2 in MC exceeded the upper normal limit; at the same time, IL-1β mRNA was significantly increased (up to hyper-response) compared to that found in the early (reactive) postoperative period, while maintaining an enhanced CCL2 response and slightly lowered IL-18 mRNA expression (Figure 1).

A feature of the tissue response in the late stages after surgery – with established atrophy was described as multidirectional changes in the expression of all

cytokine mRNA genes studied in MC in a wide range relative to the norm.

An individual analysis of the IL-1 β and IL-18 genes mRNA showed that overexpression was observed in approximately the same frequency of cases: in 29.4% (on average, up to 2624 rel. units) and 23.5% (on average, 59 rel. units); changes in expression within the normal range were found in 35.3% of animals (on average, 1.29 rel. units – IL-1 β and 1.59 rel. units – IL-18); hypo-response and lack of expression were detected in 35.3% and 41.2% of cases, respectively. It should be noted that at later stages with established stable atrophy (from 2-4 weeks and later, up to 2 months), the level of local MCP-1/CCL2 mRNA expression also varied: hyper-response was noted in 17.6% of cases (in on average, up to 597.2 rel. units); moderately increased expression was determined in more than half of the animals in the experimental group; normal reaction and lack of expression (from 0.0 to 0.014 rel. units) were detected in 29.4% of cases.

Conclusion

1. A single subretinal injection of 0.01 ml of 0.9% sodium chloride solution, which induced RPE atrophy in experimental rabbits was found to be associated with multidirectional changes in IL-1 β , IL-18, MCP-1/CCL2 mRNA gene expression relative to normal.

2. Three types of response in the TC of the choroid / RPE / retina, formed during the development of atrophic changes and determined by the magnitude of local cytokine expression were identified:

- lack of expression – lack of response/hypo-reactivity;
- normal/moderately increased response;
- overexpression – hyperreactivity.

It is possible that certain types of responses, characterized by the magnitude of the proinflammatory cytokine expression are genetically determined being associated with polymorphisms in the IL-1 β , IL-18, MCP-1/CCL2 genes; however, this assumption requires further target research.

3. No relationship was found between the strength of the response (the magnitude of local

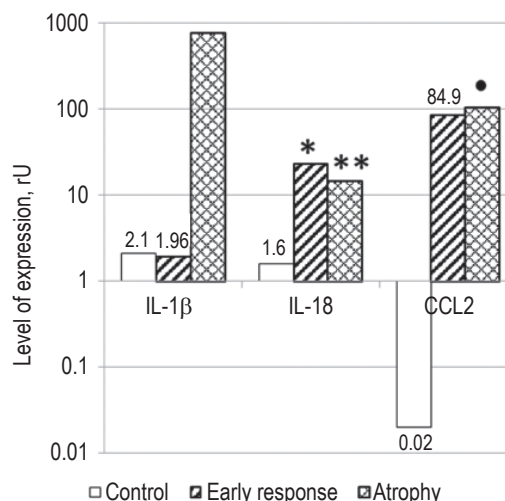


Figure 1. Level of mRNA expression of the genes IL-1 β , IL-18, CCL2 / MCP-1 (using a logarithmic scale on the ordinate) in MCs of healthy rabbits (control), at early (7 days) and distant (from 14 days and later) terms after the single subretinal injection of physiological sodium chloride solution in the experiment)

Note. *, the significance of the difference in relative expression in the early response (with reactive changes in the fundus) compared to the control ($p < 0.05$); **, the significance of the difference in relative expression in the RPE atrophy group compared to the group of early (reactive) changes ($p < 0.05$); • – a tendency to differ in the group of early (reactive) changes compared to the control ($p < 0.06$).

mRNA expression) to atrophy-inducing stimulus and the size of atrophic retinal lesion.

The circumstances noted above as well as the data that RPE induction of atrophy in one-third of animals was accompanied by significantly increased IL-1 β mRNA expression and, in 69.6% increased local MCP-1/CCL2 mRNA expression, the factors damaging haematoretinal barrier and contributing to the immune privilege disorder in the eye posterior segment, should be taken into consideration while developing new strategies of ophthalmic pathology treatment, particularly extensively studied and tested options for RPE stem cell transplantation into subretinal space.

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ЭКСПЕРИМЕНТАЛЬНАЯ ПЕРЕОРИЕНТАЦИЯ *IN VITRO* ФЕНОТИПА 4 СУБПОПУЛЯЦИЙ НЕЙТРОФИЛЬНЫХ ГРАНУЛОЦИТОВ ИЗ ПРОВОСПАЛИТЕЛЬНОГО К ПРОТИВОСПАЛИТЕЛЬНОМУ У ДЕТЕЙ С ХИРУРГИЧЕСКОЙ ГНОЙНОЙ ИНФЕКЦИЕЙ МЯГКИХ ТКАНЕЙ

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Резюме. Лечение детей раннего возраста с атипичными или рецидивирующими гнойными инфекциями мягких тканей (ГЗМТ), которые не демонстрируют хорошего ответа на хирургическое лечение и антибактериальные препараты, является наиболее сложным. ГЗМТ возникают на фоне нарушений функционирования иммунной системы и в первую очередь системы нейтрофильных гранулоцитов (НГ). Векторный эффект иммуномодулирующей терапии на конкретную субпопуляцию NG может позволить корректировать дисфункции NG без ущерба для защиты хозяина, включая стратегии усиления, подавления или восстановления их функций. Цель исследования: оценить в системе *in vitro* модулирующие эффекты влияния аргинил-альфа-аспартил-лизил-валил-тирозил-аргинин (ГП) на трансформированный фенотип 4 субпопуляций НГ, а также на функциональную активность НГ детей с гнойно-воспалительными заболеваниями мягких тканей.

Исследованы образцы периферической крови (ПК) детей раннего возраста 2–4 лет: 17 детей с нетипично протекающими острыми ГЗМТ и 10 условно здоровых детей. На I этапе проведена сравнительная оценка содержания и фенотипа 4 субпопуляций НГ CD16⁺CD62L⁺CD63⁺, CD16⁺CD62L⁺CD63⁺, CD64⁺CD16⁺CD32⁺CD11b⁺, CD64⁺CD16⁺CD32⁺CD11b⁺, фагоцитарной и микробицидной функции НГ. На II этапе в системе *in vitro* определены эффекты ГП на НГ детей с ГЗМТ по изучаемым параметрам. Методом проточной цитометрии (FC500, Beckman Coulter, (США), конъюгаты МкАТ Beckman Coulter International S.A. (Франция)), определялось относительное количество НГ исследуемых субпопуляций и плотность экспрессии рецепторов (MFI). Для оценки фагоцитарной функции НГ использован микробиологический метод с оценкой завершенности фагоцитоза со *S. aureus* (штамм 209). Активность NADPH-оксидазы НГ исследовали в НСТ спонтанном тесте (НСТсп.) и в нагрузочном в системе *in vitro* (НСТст.).

Сравнительное изучение образцов ПК условно здоровых детей и детей с ГЗМТ позволило выявить различные варианты трансформации фенотипа изучаемых субпопуляций НГ, сопряженных с дефектами их функциональной активности. В системе *in vitro* были продемонстрированы эффекты ГП,

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проявляющиеся снижением количества CD16⁺CD62L⁺CD63⁺НГ и повышением CD16⁺CD62L⁺CD63⁻НГ, модуляцией негативно измененного фенотипа субпопуляций CD64⁺CD32⁺CD16⁺CD11b⁺НГ и CD64⁺CD32⁺CD16⁺CD11b⁻НГ, направленные на восстановление фагоцитарной функции и поддержания напряженности NADPH-оксидаз.

В результате исследования установлено иммуномодулирующее действие ГП, которое проявляется в переориентации НГ с провоспалительного фенотипа на противовоспалительный, что может быть использовано в будущем при создании персонализированной таргетной иммунотерапии. Направлена на коррекцию неполноценного функционирования НГ у детей раннего возраста, страдающих ГЗМТ.

Ключевые слова: нейтрофильные гранулоциты, субпопуляции, эксперимент *in vitro*, гексапептид аргинил-альфа-аспартил-лизил-валил-тирозил-аргинин, гнойные заболевания мягких тканей, дети

IN VITRO EXPERIMENTAL REWIRING OF 4 NEUTROPHILIC GRANULOCYTE SUBSETS FROM THE PRO-INFLAMMATORY TO THE ANTI-INFLAMMATORY PHENOTYPE IN CHILDREN WITH SURGICAL PURULENT INFECTION OF SOFT TISSUE

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Abstract. Treatment of young children with atypical or recurrent purulent soft tissue infections (PSTD) that do not respond well to surgery and antibiotics is most challenging. PSTD occurs against the background of impaired functioning of the immune system and, first of all, the system of neutrophilic granulocytes (NG). The vector effect of immunotropic therapy on a specific NG subsets may allow the correction of NG dysfunctions without compromising host protection, including strategies to enhance, inhibit or restore their functions. The aim of study: to evaluate *in vitro* the modulating effects of arginyl-alpha-aspartyl-lysyl-valyl-tyrosyl-arginine (HP) on the transformed phenotype of 4 NG subsets, as well as on the functional activity of NG in children with purulent-inflammatory soft tissue diseases.

We studied samples of peripheral blood (PB) from young children 2-4 years old: 17 children with atypical acute PSTD and 10 apparently healthy children. At stage I, a comparative assessment of the content and phenotype of 4 NG subsets CD16⁺CD62L⁺CD63⁻, CD16⁺CD62L⁺CD63⁺, CD64⁺CD16⁺CD32⁺CD11b⁺, CD64⁺CD16⁺CD32⁺CD11b⁻, phagocytic and microbicidal functions of NG was carried out. At stage II, the *in vitro* system determined the effects of HP on NG in children with PSTD according to the studied parameters. By the method of flow cytometry (FC500 "Beckman Coulter" (USA), conjugates of MkAT "Beckman Coulter International S.A." (France)), the relative number of NGs of the studied subsets and the density of receptor expression (MFI) were determined. To assess the phagocytic function of NG a microbiological method was used to assess the completeness of phagocytosis with *S. aureus* (strain 209). The activity of NG NADPH oxidase was investigated in the NBT-spontaneous test (NBTsp.) and in the *in vitro* NBT-induced test (NBTind.). A comparative study of PB samples from conventionally healthy children and children with PSTD made it possible to identify various variants of transformation of the phenotype of the studied NG subsets, associated with defects in their functional activity. In the *in vitro* system the effects of HP were demonstrated, manifested by a decrease in the amount of CD16⁺CD62L⁺CD63⁺NG and an increase in CD16⁺CD62L⁺CD63⁻NG, modulation of the negatively altered phenotype of subsets CD64⁺CD32⁺CD16⁺CD11b⁺NG and CD64⁺CD32⁺CD16⁺CD11b⁻NG, aimed at restoring phagocytic function and maintaining the tension of NADPH oxidases.

As a result of the study it was found the immunomodulatory effects of HP, which is manifested in the reorientation of NG from the pro-inflammatory phenotype to the anti-inflammatory one, which can be used in the future when creating personalized targeted immunotherapy aimed at correcting defective functioning NG in early children, suffering from PSTD.

Keywords: neutrophilic granulocytes, subset, experiment *in vitro*, hexapeptide arginyl-alpha-aspartyl-lysyl-valyl-tyrosyl-arginine, purulent soft tissue diseases, children

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Introduction

Infections of the skin and soft tissues in children are common in the pediatric practice and often require surgical intervention. Localized purulent bacterial soft tissue lesions are treated with surgical aids (incision and drainage) and treatment with systemic antibiotics [3, 8].

The most difficult is the treatment of young children with atypical or recurrent purulent soft tissue infections that demonstrating no good response to surgical treatment and antibacterial drugs. It is shown that such purulent infections, as a rule, occur in parallel with impaired immune system functioning [10].

Neutrophil granulocytes (NGs) play an important role in the direct combating against purulent bacterial infection and at the same time coordinate and regulate the general, immune and inflammatory responses. It is established that NG exert various specialized functions on destruction of bacterial agents, one of which is not only phagocytic activity, but also the extrusion of their genomic DNA in the form of extracellular neutrophil traps (NET) [5].

Numerous studies indicate a significant phenotypic and functional NG heterogeneity [2, 4, 7, 9] as well as existence of diverse subsets. NGs are incredibly plastic cells able to reshape their phenotype under microenvironmental influence and change their properties. It accounts for NG potential undergo to transformational change. At the same time, it is shown that altered normal functioning of neutrophil granulocytes, both quantitatively and qualitatively, exerts negative effect on the course of purulent bacterial infection, and contributes to occurrence of atypical disease course. The vector effect of immunotropic therapy on a specific NG subset may allow to correct NG dysfunctions without compromising host protection, including strategies to enhance, inhibit, or restore their functions [6, 7].

NG is a unique and attractive target for therapeutic intervention. Correction of NG dysfunctions in atypically emerging purulent inflammatory diseases via modulating NG subset phenotype is one of the main urgent tasks in immunology [6, 12].

In this regard, it is of interest to study *in vitro* possibilities for remodeling negatively transformed phenotype of CD64⁺CD16⁺CD32⁺CD11b⁺, CD64⁺CD16⁺CD32⁺CD11b⁺, CD16⁺CD62L⁺CD63⁺, CD16⁺CD62L⁺CD63⁺NG subsets in children with purulent surgical infection of deep skin and soft tissues. It should be emphasized that these NG subsets are functionally important because the surface membrane molecules that shape their phenotypes – CD64, CD32, and CD16 are receptors for the three Fcγ fragments of IgG (FcγRs), respectively – FcγRI, FcγRII, and FcγRIII, whereas the CD11b molecule is a receptor for the C3b complement component. CD64 (FcγRI) is able to bind all IgG subclasses practically not being expressed on non-activated

peripheral blood (PB) NG in healthy subjects. In acute bacterial infection, CD64-expressing NG emerge in the PB. CD64 can activate the ROS formation to enhance phagocytosis and trigger NG ADCC via an oxygen-mediated mechanism. The CD32 receptor (FcγRIIA) in response to NG contact with pathogens enhances the processes of endocytosis, increases the ROS production, NG secretory activity, activates their cytotoxic mechanisms and immunomodulatory functions. The CD16 receptor (FcγRIIIB), binding to IgG, as well as to FcγRIIA with FcγRIIIB, enables cytotoxicity, including ADCC, enhances phagocytic function, degranulation, and oxidative burst [1, 7, 12]. High expression of CD16 molecules on the NG membrane is associated with their increased functional activity, while low expression suggest about NG immaturity or “reverse differentiation”. When the CD16 receptor interacts with CD11b/CD18 molecules, FcγRII-mediated internalization is enhanced. The CD11b receptor is the signaling partner for all Fcγ receptors, and its blockade leads to compromised activation of Fcγ receptors and impaired NG phagocytic function. CD11b is a marker of NG activation: with an adequate response to various microbial antigens, its expression level elevates. CD11b regulates chemotaxis, migration, adhesion, phagocytosis, respiratory burst, and NG degranulation [11, 12].

CD62L molecules are expressed on the membrane surface of circulating NG. The CD62L – L-selectin receptor [LAM-1], is a cellular adhesion molecule and is involved in the NG “rolling” of NG on the surface of the endothelium during inflammatory process. When the expression level of CD62L receptors is reduced, NG undergo apoptosis. The CD63 receptor, a marker of activated NG, is a glycoprotein-3 (LAMP-3) being found in the granules of non-activated NG, and is expressed on the surface of active NG. An increased level of CD63 expression is associated with the processes of NG phagocytosis and degranulation [1].

Of particular interest is the *in vitro* study of the immunomodulatory effects of the hexapeptide (HP) – arginyl-alpha-aspartyl-lysyl-valyl-tyrosyl-arginine on functionally significant NG subsets. HP is an immunotropic substance bearing an active center of the thymic hormone thymopoietin and exerts immunoregulatory and detoxifying properties, inactivates free radical and peroxide compounds, restoring the balance of redox reactions in infectious and inflammatory diseases.

The aim of study: to evaluate *in vitro* modulatory effects of arginyl-alpha-aspartyl-lysyl-valyl-tyrosyl-arginine (HP) on the transformed phenotype of 4 NG subsets, as well as on NG functional activity in children with purulent-inflammatory soft tissue diseases (PSTD).

Materials and methods

The single-center, prospective, non-randomized study was conducted. Peripheral blood (PB) samples of young children aged 2-4 years were examined.

The study group 1 consisted of 17 young children aged 2-4 years (9 boys and 8 girls) with atypical acute purulent-inflammatory soft tissue diseases (abscesses, phlegmons)-PSTD and comparison group contained 10 apparently healthy young children aged 2-4 years (5 girls and 5 boys). At the first stage, a comparative assessment of the number and phenotype of 4 functionally relevant NG subsets $CD16^+CD62L^+CD63^-$, $CD16^+CD62L^+CD63^+$, $CD64^+CD16^+CD32^+CD11b^+$, $CD64^+CD16^+CD32^+CD11b^-$ was carried out in the samples of the both groups: the study group 1 and comparison group. NG phagocytic and microbicidal functions were tested. At the second stage, the HP effects influence on NG subsets and functions were studied *in vitro* in children with PSTD. PB samples were incubated for 60 min at T 37 °C with HP (at a concentration of 10^{-6} g/l). After that, 4 functionally relevant subsets, phagocytic and microbicidal functions were also tested. The flow cytometry was used (FC500 "Beckman Coulter" (USA) for immunophenotyping of NG subsets with conjugated McAT "Beckman Coulter International S.A." (France)), percentage of NG subsets and the density of receptor expression identifying the various phenotypes via mean fluorescence intensity index (MFI) were determined. To assess the NG phagocytic function, PB samples were incubated with *S. aureus* (strain 209), for 120 min, at T-37. The level of active-phagocytic NG (%PHAN); phagocytic number (PN), phagocytic index (PHI) were tested. The percentage of digestion (%PD), the index of digestion (ID) were determined to assess the killing activity. The NG NADPH-oxidase activity was studied in the NBT-spontaneous test (NBTsp.) and NBT-induced test (NBTind.), by assessing opportunity to exert cytotoxic and cytolytic potential. The average cytochemical index and percentage of formazan-positive cells were determined (MCIsP, %FPCsp and MCInd., %FPCind), whereas the coefficient of mobilization (CM) – %FPCind/%FPCsp was calculated.

Statistical data processing was carried out by using computer-based software Microsoft Excel 2016 and StatPlus 2010. The results were presented as a median (upper and lower quartile) – Me ($Q_{0.25}$ – $Q_{0.75}$). The nonparametric Mann–Whitney U test was used to assess the significance level for differences. The significance level was set at $p < 0.05$.

We are grateful to NPP Bionox, Ltd for providing for our study pharmacological substance Hexapeptide – arginyl-alpha-aspartyl-lysyl-valyl-tyrosyl-arginine.

Results and discussion

A comparative study of PB samples from children with PSTD revealed lowered number of NG subsets $CD64^+CD16^+CD32^+CD11b^+$ comprising 86.5 (80.5–96.1) % vs 98.0 (96.9–98.7) % found in the comparison group ($p < 0.05$). A change in the NG phenotype for this subset was also found: a 1.6-fold decrease in CD16 expression density to 81.5 (64.9–97.3),

increased CD11b MFI ($p < 0.05$), but no changes in CD32 expression level compared to those in the group of apparently healthy children ($p > 0.05$) (Table 1).

At the same time, a significantly higher number of NG subsets $CD64^+CD16^+CD32^+CD11b^+$ NG was found – 14.5 (5.6–15.7) % vs 0.3 (0.2–0.4) % in the comparison group ($p < 0.05$). The $CD64^+CD16^+CD32^+CD11b^+$ NG subset expressed similar level of CD16, CD32, CD11b receptor expression density (MFI index) as the subset, $CD64^+CD16^+CD32^+CD11b^+$ NG in PSTD ($p_1 > 0.05$, $p_2 > 0.05$, $p_3 > 0.05$) (Table 1), but the expression level for examined receptors differed from those observed in the group of healthy children. Thus, a 2.4-fold decrease in MFI CD64 down to 3.4 (2.2–4.7) vs 7.9 (5.3–12.2) in the comparison group, a 7.6-fold increase in MFI CD16 to 71.2 (60.2–98.2) vs 9.4 (5.1–19.6) in the comparison group, and a 1.6-fold increase in MFI CD11b to 24.6 (17.7–37.1) vs 15.4 (4.9–15.9) in the comparison group ($p_1 < 0.05$, $p_2 < 0.05$, $p_3 < 0.05$) were found, whereas the CD32 level did not differ from its density in the comparison group ($p > 0.05$) (Table 1).

A significant 8-fold increase in the number of activated NG subset $CD16^+CD62L^+CD63^+$ NG – 64.0 (11.3–74.1) % was observed in children with PSTD compared to t healthy children – 7.6 (0.3–16.5) %, due to a decrease in $CD16^+CD62L^+CD63^-$ NG to 28.8 (6.6–32.8) %, which in apparently healthy children was 89.5 (81.5–96.8) % ($p < 0.05$). At the same time, there was a decrease in the MFI of CD16 ($p < 0.05$), and the level of expressed CD62L and CD63 surface receptors determined in both subsets did not change nor significantly differed from those in the comparison group (Table 1). The revealed transitions in phenotype and percentage of the studied functionally relevant NG subsets in children with PSTD are associated with the revealed defects in NG functional activity (Table 1).

The NG phagocytic function in children with PSTD was studied. The data obtained demonstrated a quantitative shortage of actively phagocytic NG (%PHAN 49.0 (39.3–53.0) vs 55.2 (54.0–57.0) in the comparison group, $p < 0.05$). At the same time, parameters of NG absorption capacity were increased: PHN – 5.2 (3.9–5.6) vs 4.1 (3.7–5.7) ($p > 0.05$) and PHI-2.7 (1.8–3.6) vs 2,5 (1.8–3.2) in the comparison group ($p > 0.05$). The decrease in degradation processes was detected: %D-53,1 (42.5–57.7) vs 61.6 (57.9–62.9) %, ID -1,3 (0,7–1,3) vs 1.6 (1.4–1.9) in the comparison group ($p_1 < 0.05$, $p_2 < 0.05$). At the same time, group with PSTD showed significantly increased spontaneous and induced activity of the NADPH-oxidases ($p < 0.05$) without preserving protective microbicidal potential, as evidenced by low KM ($p < 0.05$).

The effect of HP on the NG subset phenotype from children with PSTD was revealed in 1.5-fold decrease in CD16 MFI ($p < 0.05$) and 1.6-fold decrease in CD11b MFI in the $CD64^+CD16^+CD32^+CD11b^+$ NG subset ($p < 0.05$), that prominently tended to dec-

TABLE 1. EFFECT OF HEXAPEPTIDE ON THE PHENOTYPE OF FUNCTIONALLY SIGNIFICANT SUBSETS OF NEUTROPHIL GRANULOCYTES IN CHILDREN WITH PURULENT-INFLAMMATORY SOFT TISSUE DISEASES, Me ($Q_{0.25}$ - $Q_{0.75}$)

%CD64 ⁺ CD16 ⁺ CD32 ⁺ CD11b ⁺		MFI CD64	MFI CD16	MFI CD32	MFI CD11b
Healthy children	98.0 (96.9-98.7)		129.5 (115.8-131.8)	5.7 (4.8-6.1)	16.4 (9.7-17.7)
PSTD	86.5 (80.5-96.4)		81.5* (64.9-97.3)	5.3 (4.0-5.9)	19.4* (18.9-28.9)
PSTD + HP	94.7 (83.8-97.0)		73.2* (57.5-79.1)	5.4 (4.2-7.2)	10.6 (7.8-14.5)
%CD64 ⁺ CD16 ⁺ CD32 ⁺ CD11b		MFI CD64	MFI CD16	MFI CD32	MFI CD11b
Healthy children	0.3 (0.2-0.4)	7.9 (5.3-12.2)	9.4 (5.1-9.6)	5.5 (5.2-6.3)	15.4 (4.9-15.9)
PSTD	14.5* (5.6-15.7)	3.4* (2.19-4.72)	71.2* (60.2-98.2)	6.1 (5.02-7.56)	24.6* (17.7-37.1)
PSTD + HP	6.1* (5.5-12.7)	4.04 (2.6-7.2)	48.1* (35.1-75.3)	6.93 (6.1-9.2)	15.1 (11.9-17.5)

%CD16 ⁺ CD62L ⁺ CD63 ⁺		MFI CD16	MFI CD62	MFI CD63
Healthy children	89.5 (81.48-96.8)	128.9 (115.8-130.1)	7.08 (5.4-11.3)	
PSTD	28.8* (6.6-32.8)	83.5* (65.0-91.3)	5.7 (4.9-8.7)	
PSTD + HP	35.4 (27.0-73.5)	78.4* (67.5-80.1)	5.0 (4.6-5.6)	
%CD16 ⁺ CD62L ⁺ CD63 ⁺		MFI CD16	MFI CD62	MFI CD63
Healthy children	7.6 (0.3-16.5)	128.9 (115.8-130.1)	4.6 (3.5-8.9)	2.2 (1.5-3.2)
PSTD	64.0* (11.3-74.1)	83.5* (65.0-91.3)	5.6 (4.4-6.8)	2.2 (1.8-2.6)
PSTD + HP	52.0* (24.5-75.3)	78.4* (67.5-80.1)	4.7 (4.2-5.2)	2.0 (1.6-2.8)

Note. *, significant differences from the indicators of conditionally healthy children of the comparison group $p < 0.05$.

rease in MFI CD16 and MFI CD11b in the CD64⁺CD16⁺CD32⁺CD11b⁺NG subset paralleled with unchanged CD32 membrane receptor expression in both subsets (Table 1.).

HP affected CD16⁺CD62L⁺CD63⁺NG and CD16⁺CD62L⁺CD63⁺NG subsets demonstrated in children suffering from PSTD revealed by redistribution of both subsets: decreased percentage (%) of CD16⁺CD62L⁺CD63⁺NG and increased proportion (%) of CD16⁺CD62L⁺CD63⁺NG along with unchanged CD16, CD62L and CD63 expression density assessed by MFI index (Table 1.).

The study examining HP effect on NG phagocytic and microbicidal NADPH-dependent function in children with PSTD revealed the effects associated with the modulation of the receptor apparatus aimed at restoring the % of PHAN, the functions of degradation and maintaining the tension of NADPH oxidases necessary for mounting response to infectious process. The NG phagocytic activity tended to recover, but the indicators of all parameters

remained below the magnitude in apparently healthy children: %PHAN ($p < 0.05$), PHN ($p < 0.05$), PHI ($p < 0.05$), ID ($p < 0.05$). At the same time, the reserve NADPH-oxidase activity increased by 2-fold in CM compared to the baseline level, which was higher than in comparison groups ($p < 0.05$).

Conclusion

During the experimental stage of the *in vitro* study, the different modulating HP effect on the number and the phenotype of 4 NG subsets and functions were shown, as well as an opportunity of reprogramming NG subset phenotype that was changed during PSTD.

Thus, the study resulted in describing the immunomodulatory HP effects manifested in NG rewiring from the pro-inflammatory to the anti-inflammatory phenotype, which can be used in the future while creating personalized target immunotherapy aimed at correcting defective NG functioning in early children, suffering from PSTD.

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КЛЕТочный ИММУНИТЕТ и ЕГО РОЛЬ в ПАТОГЕНЕЗЕ УШИБА ГОЛОВНОГО МОЗГА

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Резюме. Черепно-мозговая травма (ЧМТ) является одной из наиболее распространенных патологий центральной нервной системы в мире, а применение методов структурной нейровизуализации — компьютерной томографии (КТ) и магнитно-резонансной томографии (МРТ) — зачастую не позволяет оценить тяжесть, полученной при травме головного мозга. Это предопределяет необходимость поиска новых методов дифференциальной диагностики степени тяжести и прогнозирования риска последствий. Одно из таких перспективных направлений является изучение иммунного статуса, так как черепно-мозговая травма характеризуется высокой частотой осложнений.

Помимо этого известно, что тяжесть при ЧМТ в значительной степени определяется вовлеченностью иммунокомпетентных клеток. Реакции со стороны иммунной системы, которые развиваются после травмы мозга и, возможно, направлены против собственных антигенов, в раннем периоде заболевания имеют отношение к повреждению нервной ткани. Однако, по последним имеющимся данным, впоследствии способны стимулировать процессы репарации и регенерации в ткани головного мозга. При повреждении нервной ткани в ответ на эндогенные молекулы, которые образуются при разрушении клеток и внеклеточного матрикса, будут активироваться клетки иммунной системы.

Современные данные указывают, что Т-клетки иммунной системы играют роль как в формировании вторичных повреждений, так и в механизмах восстановления. Они способны защищать нейроны посредством продукции нейротрофических факторов, таких как нейротрофический фактор мозга (BDNF), который стимулирует рост нейронов и формирование синапсов. С помощью многоцветного

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цитофлуометрического анализа было проведено исследование по определению количества основных субпопуляций лимфоцитов среди CD45RA-negative CD3⁺CD4⁺-клеток. Относительное количество Th17 (CXCR5⁺CXCR3⁻CCR6⁺CCR4⁻) и Th17/Th22 (CXCR5⁺CXCR3⁻CCR6⁺CCR4⁺), Th1/Th17 (CXCR5⁻CXCR3⁺CCR6⁺CCR4⁻) среди общего количества CD45RA-negative CD3⁺CD4⁺-клеток у пациентов с ушибом головного мозга достоверно повышено в сравнении со значениями в контрольной группе в свою очередь, а относительное количество Th1 (CXCR5⁻CXCR3⁺CCR6⁻CCR4⁻) среди общего количества CD45RA-negative CD3⁺CD4⁺-клеток достоверно снижено в сравнении со значениями в контрольной группе. Полученные к настоящему времени результаты позволяют рассматривать иммунные ответы среди ключевых звеньев патогенеза ушиба головного мозга. И, возможно, комплексное иммунологическое обследование пострадавших в первые сутки после травмы позволит определить параметры, которые помогут прогнозировать характер возможных осложнений у пациентов с ушибом головного мозга.

Ключевые слова: черепно-мозговая травма, проточная цитометрия, ушиб головного мозга, воспаление, клеточное звено системы иммунитета, субпопуляции CD3⁺CD4⁺-лимфоцитов

ROLE OF THE CELLULAR IMMUNITY IN THE PATHOGENESIS OF BRAIN CONTUSION

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Abstract. Traumatic brain injury (TBI) is one of the most common pathologies of the central nervous system in the world, and the use of structural neuroimaging methods – computed tomography (CT) and magnetic resonance imaging (MRI) – often doesn't allow assessment of the severity of the brain injury that has occurred. This situation predetermines the need to search for new methods of differential diagnosis of the severity of TBI and predicting the risk of consequences.

One of these promising areas is the study of the immune status, since traumatic brain injury is characterized by a high rate of complications.

One of these promising areas is the study of the immune status in patients with TBI in the acute period. It is now known that in response to brain damage, a response from the immune system is triggered.

The reactions from the immune system, which develop after brain injury and directed against its own antigens, in the early period of the disease are related to damage to the nervous tissue. However, according to the latest available data, they are subsequently able to stimulate the processes of repair and regeneration in the brain tissue. In the course of damage to the nervous tissue, in response to endogenous molecules formed during the destruction of cells and the extracellular matrix, the cells of the immune system are activated.

Current evidence indicates that T-cells play a role in both the formation of secondary damage and repair mechanisms. They are able to protect neurons through the production of neurotrophic factors such as brain neurotrophic factor (BDNF), which stimulates the growth of neurons, the formation of synapses.

Using multicolor cytometric analysis within the framework of this work, a study was carried out to determine the number of the main subpopulations of CD3⁺CD4⁺-lymphocytes. The relative number of Th17 (CXCR5⁺CXCR3⁻CCR6⁺CCR4⁻) and Th17/Th22 (CXCR5⁺CXCR3⁻CCR6⁺CCR4⁺), Th1/Th17 (CXCR5⁻CXCR3⁺CCR6⁺CCR4⁻) among total CD45RA-negative CD3⁺CD4⁺-cells population is significantly increased in comparison with the values in the control group, in turn, the Th1 (CXCR5⁻CXCR3⁺CCR6⁻CCR4⁻) subpopulations among total CD45RA-negative CD3⁺CD4⁺-cells are significantly decreased with the values in the control group. The results obtained so far make it possible to consider immune responses among the key

links in the pathogenesis of brain contusion TBI. And, perhaps, a comprehensive immunological examination of the victims in the first day after the injury will determine the parameters that will help predict the nature of possible complications in patients with brain contusion.

Keywords: traumatic brain injury, flow cytometry, contusion, inflammatory, cellular immune status, subpopulations of CD3⁺CD4⁺-lymphocytes

Introduction

Traumatic brain injury (TBI) is one of the leading causes of morbidity as well as mortality in the world. With regard to molecular and cellular mechanisms of TBI in the past 20 years, but immune status doesn't know. It is now known that cells of immunity play an important role in brain function, including the organization of cognition and neurogenesis [10]. The basis for this is that there is an interconnection between the main immune cells in the periphery and the brain, and this connection is strengthened in response to the immune response [5]. The data of the conducted studies indicate that signals from the immune system from the periphery cause an inflammatory response in the brain, while it increases with age, which, in turn, reduces the plasticity processes necessary for cognitive functions [3]. In this case, the triggering of the immune response occurs through Toll-like receptors (TLR), which are expressed on the cells of the macrophage system located in the circumventricular organs and the vascular plexus, reacting to circulating pathogen-associated molecular patterns, which further become to the formation of pro-inflammatory cytokines, or the flow of cytokines from the systemic circulation to the brain through the BBB, it is also possible that an inflammatory response is triggered through receptors for interleukin-1 (IL-1) expressed on perivascular macrophages and endothelial cells of cerebral venules [3, 8, 12].

Materials and methods

Within the framework of this study were explored 52 apparently healthy people aged 18–65 years without TBI, as well as any pathology that could lead to a change in the immunological status, and 22 patients with a diagnosis of brain contusion. Of these, 25 are men and 27 are women aged 20 to 46 years. All examination was conducted on the day of the patient's appointment. The examination included the collection of complaints, medical history, assessment of somatic and neurological status, neuropsychological testing. The number of subpopulations Th in percentage within CD45RA-negative CD3⁺CD4⁺-lymphocytes are demonstrated in quartile and medians ranges — Me (Q_{0.25}–Q_{0.75}), which were evaluated using multicolored cytometric analysis. The object of the study was venous blood from apparently healthy donors, obtained by puncture of a peripheral

vein and collected in test tubes. Definition the severity of TBI was avowed in accordance with the established criteria. Moreover, the presence of concomitant severe damage to other organs and somatic pathology, as well as concomitant intoxication, are an exclusion criterion for recruitment into the group. Immunophenotyping of the main subpopulations of T-helpers of peripheral blood was leaded on the day of blood sampling. Preparation of peripheral blood and setting up the flow cytometer in accordance with the recommendations outlined by S.V. Khaidukov et al. [7].

To identify the main subpopulations of T-helpers, 200 µl of whole EDTA-stabilized peripheral blood was stained for surface antigens using the following combination of monoclonal antibodies conjugated to fluorochromes. Antibodies against CD3 (clone UCHT1) and CD4 (clone 13B8.2) were used to identify T-helpers — phenotype CD3⁺CD4⁺T-cells in peripheral blood. To separate the total pool of Th-cells at the main stages of maturation, antibodies against surface CD45RA (clone 2H4LDH11LDB9 (2H4)) and CD62L (clone DREG56) were used.

We used antibodies against CD3, CD4, CD45RA, and CD62L conjugated with APC-Alexa Fluor 750, Pacific Blue, FITC, and PE, respectively (Beckman Coulter, USA), and antibodies against CCR4, CCR6, CXCR3, and CXCR5 were conjugated with Brilliant Violet 510™, PE/Cy7, APC and PerCP/Cy5.5, respectively (Biolegend, USA). The destruction of erythrocytes was carried out using a VersaLyse lysis solution (Beckman Coulter, USA) — to 975 µl *ex tempore* was added 25 µl of IOTest 3 Fixative Solution (Beckman Coulter, USA), which was incubated at room temperature in a dark site for 15 minutes. After the destruction of erythrocytes, the samples were washed once with sterile saline (330 g for 7 minutes), after which the resulting cell pellet was resuspended in physiological solution with pH 7.2–7.4 containing 2% paraformaldehyde (Sigma-Aldrich, USA). For each of the samples, at least 40,000 CD3⁺CD4⁺-lymphocytes were analyzed. Coexpression of the chemokine receptors CCR4, CCR6, CXCR3, and CXCR5 was assessed using the “gating” tactic based on the construction of hierarchical dendrograms for CD45RA-negative Th memory [9]. The statistical comparisons of data between TBI patients and healthy controls were performed using the Mann–Whitney U-test. Differences were considered significant when

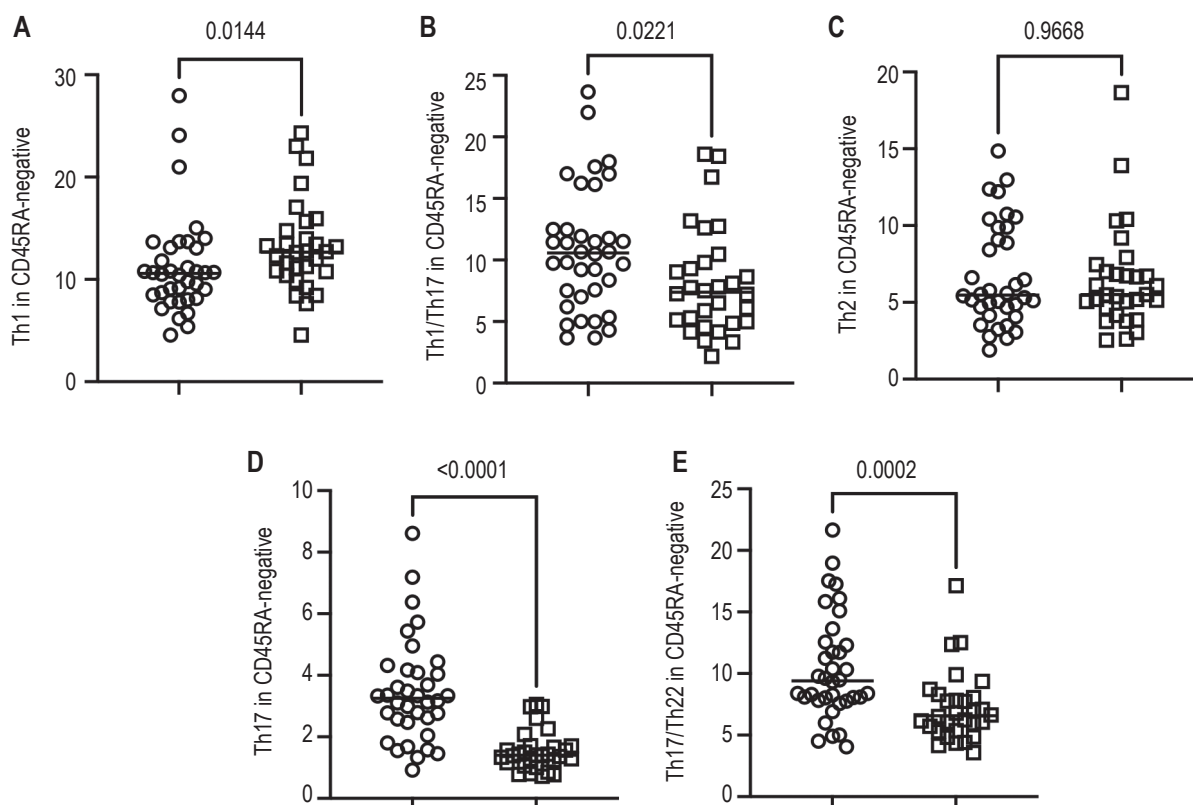


Figure 1. Comparative analysis of main subpopulation Th in brain contusion (n = 35, white circles) and healthy control (n = 52, white square)

Note. Dispersion charts A, B, C, D and E showing the percentages Th1 (CXCR5-CXCR3+CCR6-CCR4-), Th1/Th17 (CXCR5-CXCR3+CCR6-CCR4-), Th2 (CXCR5-CXCR3-CCR6-CCR4+), Th17 (CXCR5-CXCR3vCCR6+CCR4+) and Th17/Th22 (CXCR5-CXCR3-CCR6+CCR4+), accordingly in the peripheral blood of patients in brain contusion and healthy control group. The number indicates the percentage of the specified Th subpopulations amid general CD45RA-negative CD3+CD4+ cell population. Each dot represents either patient, and the horizontal bars show medians and quartile ranges – Me (Q_{0.25}-Q_{0.75}).

p values were $p < 0.05$. All of the statistical analysis of data was carried out with GraphPad Prism 6 (GraphPad Software, USA).

Results and discussion

In the analysis of the CD45RA-negative population of CD3+CD4+-cells in patients with brain contusion immediately after admission compared with a control group based on the expression of chemokine receptors (CXCR5, CXCR3, CCR6, CCR4). This allowed us to define the phenotype of Th1/Th17 (CXCR5-CXCR3+CCR6-CCR4-) and Th17 (CXCR5-CXCR3-CCR6+CCR4+), Th1 (CXCR5-CXCR3+CCR6-CCR4-) and Th2 (CXCR5-CXCR3-CCR6-CCR4+), Th17/Th22 (CXCR5-CXCR3-CCR6+CCR4+)-cells. The results demonstrate in Figure 1. The clinical significance of T-helpers (Th) with the CD3+CD4+ phenotype is shown in a very wide range of diseases, when their content can be a significant marker for determining the severity of pathological conditions

and assessing the effectiveness of the therapy used. Changes in the subpopulation composition of Th among CD3+CD4+-cells in patients with brain contusion were identified, which makes it possible to raise the question of the need to determine these cells subpopulations in clinical practice.

The comparison of these different Th subsets between patients and control group indicated significantly lower Th1 ($p < 0.05$) and significantly higher Th17 ($p < 0.0001$), Th1/Th17 ($p < 0.05$), Th17/Th22 ($p < 0.001$) with patients of brain contusion if compared with healthy controls. Also, we didn't observe significant changes in the number of Th2-cells with patients of MS-TBI if compared with healthy controls.

Among the cells of adaptive immunity, T-helpers play a leading role in the development of inflammation in response to traumatic brain injury. The revealed changes in the CD45RA-negative CD3+CD4+-cell population in patients with TBI allowed to raise the

question of the significance of determining these cells subpopulations, which may be a predictor of the course of TBI in the acute period. T-cells play a leading role in the neuroplasticity of the brain. It was shown for the first time that in patients with TBI in the peripheral blood against the background of a decrease in Th1 (CXCR5⁺CXCR3⁺CCR6⁺CCR4⁻), there is an increase in the relative amount of Th17 (CXCR5⁺CXCR3⁺CCR6⁺CCR4⁺), Th1/Th17 (CXCR5⁺CXCR3⁺CCR6⁺CCR4⁻), Th17/Th22 (CXCR5⁺CXCR3⁺CCR6⁺CCR4⁺). It is known that type Th1 play a role in learning and memory processes by limiting the activation of meningeal myeloid cells and promoting the expression of neutrophilic factor [6]. As for T-helper 17 (Th17) – are capable of secreting a broad span of cytokines such as interleukin-17A (IL-17A), IL-21, IL-22, IL-25, IL-26, TNF α and chemokines. It should be noted that it is under the influence of IL-17 and IL-22, the receptors for which are present on the endothelial cells that make up the blood-brain barrier, that the structure of tight junctions is disrupted. In addition, most of the cytokines secreted by Th17 have pro-inflammatory activity. Their role in the pathogenesis of various autoimmune diseases has been noted. Given that myelination of nerve fibers plays an important role in ensuring motor activity and neuroplasticity, damage to myelin can negatively affect recovery after TBI, causing the development

of cognitive and other disorders, contributing to the formation of cerebral atrophy. A number of authors are inclined to believe that Th17-type cells are not so much pro-inflammatory as act as modulators of the immune response [1]. The role of Th17 in human organ transplantation and autoimmune disease [2]. The revealed increase in the relative amount of Th1 among CD3⁺CD4⁺-cells consistent with the severity of the injury, since Th1 are responsible for chronic inflammation, thereby providing assistance to tissue macrophages and cytotoxic T-cells, and synthesizing a number of cytokines (IFN γ , IL-2, TNF α , TNF β), having a pro-inflammatory nature [11]. Head trauma affects cellular immunity, thereby leading to a decrease or increase in certain subpopulations of T-cells among the CD45RA-negative population of CD3⁺CD4⁺-cells. It is known that with a brain injury, the activation of cells that are involved in inflammatory processes occurs, together with cells that are involved in reparative processes. Possibly these variations can be a prognostic factor for the formation of the clinical picture of post-traumatic neurodegeneration in the long-term period, which requires an improvement in treatment algorithms with the possible inclusion of an assessment of the immune status and rehabilitation of patients with brain contusion to improve management tactics.

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СТИМУЛИРУЮЩЕЕ ВЛИЯНИЕ ВЫСОКИХ ДОЗ ГЕПАРИНА НА МИГРАЦИОННУЮ АКТИВНОСТЬ И СОХРАНЕНИЕ СТВОЛОВОСТИ МСК В ПРИСУТСТВИИ ОСТЕОЗАМЕЩАЮЩИХ МАТЕРИАЛОВ

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Резюме. Искусственные материалы, применяемые в регенеративной медицине, при имплантации индуцируют развитие воспалительной реакции, необходимой для эффективной регенерации поврежденной костной ткани. Контакт имплантата с тканями сопровождается осаждением белков крови и интерстициальной жидкости на его поверхности, что способствует активации системы комплемента, компонентов врожденного иммунитета и инициирует коагуляционный гемостаз, приводящий к образованию фибринового сгустка. На поверхности имплантата образуется внеклеточный матрикс на основе фибрина, коллагена и эластина, что обеспечивает основу для формирования тканевой структуры посредством адгезии стволовых клеток на формирующуюся костную мозоль до образования костного регенерата. Для предотвращения развития постоперационных патологических состояний, вызванных гиперкоагуляционным синдромом, используют терапевтические стратегии с применением антикоагулянтов (гепарин, варфарин). Однако их использование ограничивает нормальное образование сгустка фибрина *in vivo*. Это может привести к замедлению миграции мезенхимных стволовых клеток (МСК) и нарушить формирование костной мозоли, что ингибирует процессы остеоинтеграции имплантата и заживление кости. Целью исследования явилось изучение влияния гепарина в градиенте низких и высоких концентраций на миграционную активность и стволовость МСК человека в условиях культивирования *in vitro*. По результатам проточной цитометрии было выявлено, что высокие концентрации гепарина (130, 260 IU/ml) в 2D-модели культивирования способствуют увеличению количества клеток, экспрессирующих поверхностные маркеры CD73 и CD90, что свидетельствует о сохранении МСК высокого клоногенного потенциала. 3D-модель культивирования *in vitro* с добавлением гепарина и остеозамещающих имплантатов, несущих КФ покрытие с индексом шероховатости

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$R_a = 2,6-4,9$ мкм, способствовала сохранению «стволовости» МСК через экспрессию поверхностных маркеров CD73 и CD90. Согласно результатам, полученным с помощью системы xCELLigence, гепарин в поздние сроки (с 20–40 ч) повышает инвазию МСК через микропоры, имитирующие состояние стенки кровеносных сосудов. Однако в присутствии наночастиц ГАП, имитирующих процессы ремоделирования минерального костного матрикса и/или резорбции костного цемента, эффект гепарина был выражен в меньшей степени. Результаты могут быть использованы в области регенеративной медицины, связанной с введением МСК. Данные могут служить предпосылкой для разработки новых терапевтических стратегий для пациентов хирургического профиля с высоким риском развития послеоперационных тромбозов после проведения остеосинтеза.

Ключевые слова: мезенхимальные стволовые клетки, гепарин, миграция, стволовость, имплантат, RTCA, *in vitro*

STIMULATING EFFECT OF HIGH DOSE HEPARIN ON MIGRATION ACTIVITY AND MSC STEMNESS PRESERVATION IN THE PRESENCE OF BONE-SUBSTITUTING MATERIALS

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Abstract. Synthetic materials used in regenerative medicine, upon implantation, induce the development of an inflammatory reaction necessary for the effective regeneration of damaged bone tissue. Implant contact with tissues is accompanied by the deposition of blood proteins and interstitial fluid on its surface, contributing to the activation of the complement system, components of innate immunity, initiating coagulation hemostasis, leading to the formation of a fibrin clot. An extracellular matrix based on fibrin, collagen and elastin forms on the implant's surface, which provides the basis for the formation of tissue structure through the adhesion of stem cells to the forming bone callus before the formation of bone regenerate. To prevent the development of postoperative pathological conditions caused by hypercoagulable syndrome, therapeutic strategies are used to use anticoagulants (heparin, warfarin). However, their use limits the normal formation of a fibrin clot *in vivo*. This can slow down the migration of mesenchymal stem cells (MSC) and disrupt the formation of callus, inhibiting the processes of osseointegration of the implant and bone healing. The study's goal was to study the effect of heparin in a gradient of low and high concentrations on the migration activity and stem capacity of human MSCs under *in vitro* cultivation conditions. According to the results of flow cytometry, it was revealed that high concentrations of heparin (130, 260 IU/ml) in a 2D cultivation model contribute to an increase in the number of cells expressing surface markers CD73 and CD90, which indicates that MSCs retain high clonogenic potential. A 3D model of *in vitro* cultivation with the addition of heparin and osteosubstituting implants bearing a CF coating with a roughness index of $R_a = 2.6-4.9$ μm contributed to preserving the “stemness” character of MSCs through the expression of surface markers CD73 and CD90. According to the results obtained using the xCELLigence system, heparin at a later time (from 20–40 hours) increases the invasion of MSCs through micropores that simulate the state of the blood vessel walls. However, in the presence of HAP nanoparticles that mimic the remodeling processes of the mineral bone matrix and/or resorption of bone cement, the effect of heparin was less pronounced. The results can be used in the field of regenerative medicine associated with the introduction of MSCs. The data can serve as a prerequisite for developing new therapeutic strategies for surgical patients with a high risk of postoperative thrombosis after osteosynthesis.

Keywords: mesenchymal stem cells, heparin, migration, stemness, implant, RTCA, *in vitro*

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Introduction

Implantation of synthetic materials for regenerative medicine induces development of inflammatory reactions upon contact with body tissues after osteosynthesis. The development of local inflammation is necessary for the effective regeneration of damaged bone tissue [6].

The implanted material's contact with the recipient tissues is accompanied by the deposition of blood proteins and interstitial fluid on the implant interface. The layer of adsorbed proteins promotes the activation of the complement system, components of innate immunity and initiates coagulation hemostasis, which leads to the fibrin clot formation [5].

The protein framework is embedded in a negatively charged matrix of sulfated glycosaminoglycans (GAGs), such as heparan sulfate and others, which interact with platelet growth factors, vascular endothelial growth factor (VEGF) and transforming growth factor- β (TGF- β). It reduces the sensitivity of growth factors to enzymatic cleavage and develops a unique cellular and molecular environment regulating tissue regeneration [3].

The structure of the extracellular matrix (or bone callus) formed on the implant interface, based on fibrin, collagen, and elastin, provides the platform for tissue structure formation through the adhesion of stem cells on the callus to the formation of bone regenerate. Thus, a stable connection is formed between the recipient bone tissue bed and the implant interface with further osseointegration [3].

There are pathological conditions characterized by impaired hemostasis and hypercoagulation, often observed in the elderly and patients with chronic pathologies. To prevent the development of postoperative pathological conditions caused by hypercoagulation syndrome (for example, thromboembolism of arteries, heart attack, stroke), therapeutic strategies use antithrombotic drugs, including antiplatelet agents (e.g., aspirin, clopidogrel) or anticoagulants (heparin, warfarin) [6].

However, direct anticoagulants, particularly heparin, limit the normal fibrin clot formation *in vivo*, thereby slowing down the migration of mesenchymal stem cells (MSC) to the callus, which disrupts the processes of osseointegration of implants and osteoreparation.

Thus, this study was aimed to examine heparin effect in a gradient of low and high concentrations on the human MSC migration activity and stemness under *in vitro* culture conditions.

Materials and methods

MSCs were isolated from human lipoaspirate (Resolution No. 7 of 12/09/2015 of the local ethics committee of the Innovation Park of the I. Kant IKBFU), meeting the minimum MSC criteria: adhesion to the surface of culture plastic, expression of CD105, CD73 and CD90 combined with lacked CD45/CD34 as well as potential to differentiate in three orthodox directions – osteo-, chondro- and adipogenic lineage cells [3]. A two-dimensional (2D) plastic-based culture model served as a control to assess the morphofunctional state of cells cultured in the presence of heparin. A three-dimensional culture model with implants on plastic served as and/or a three-dimensional (3D) matrix simulating the regenerating bone tissue's relief.

For a three-dimensional cell culture model (3D), model implants were used with a size of $10 \times 10 \times 1 \text{ mm}^3$ with double-sided calcium phosphate (CP) coating, with metal backing made from commercially pure VT1-0 titanium (the content of the constituent elements in weight per cent: 99.58 Ti, 0.12 O, 0.18 Fe, 0.07 C, 0.04 N, 0.01 H) by the microarc oxidation method on a Micro arc-3.0 installation (at the Institute of Strength Physics and Materials Science, Siberian Branch of the Russian Academy of Sciences, Tomsk) in the anode mode. CP coating had an average surface roughness index (R_a) = 2.6–4.9 μm , which corresponded to the biologically active range of the relief of CP-coatings for osteogenic MSC differentiation *in vivo* [4].

To assess the reaction of cells in the presence of developed matrices and/or heparin (Belmedpreparaty, Belarus), MMSCs were cultured in 12-well sterile plastic culture flat-bottomed plates (Orange Scientific, Belgium). We examined the four experimental groups: a control group of cells cultured without heparin and implants; cells added with heparin; cells cultured with an implant; cells added with heparin and an implant. To study the anticoagulant effect, we used various concentrations of heparin: 1.3 IU/ml and 13 IU/ml (low), 130 IU/ml and 260 IU/ml (high). The implant was clipped to the edge of the hole. MSCs (1×10^5 cells/ml) were cultured in 2 ml of complete nutrient medium (CNM) (90% DMEM/F12 (1:1) (Gibco Life Technologies, USA), 10% FBS (Sigma Aldrich, USA)), 50 mg/L gentamicin (Invitrogen, UK), 280 mg/L L-glutamine (Sigma Aldrich, USA) and cultured for 14 days at 37 °C, 100% humidity in an atmosphere of 5% CO₂, by replacing cell medium every 3–4 days.

MSC antigenic determinants were identified by using the MSC Phenotyping Kit human – 130-095-198 (Miltenyi Biotec, USA) according to the manufacturer's protocol, followed by cell analysis on a MACS Quant flow cytometer (Miltenyi Biotec, Ger-

many). The data were processed using the KALUZA Analysis Software (Beckman Coulter, USA).

MSC migration potential was assessed using an electrode xCELLigence® RTCA DP (ACEA Biosciences Inc., USA) system for continuous observation (real-time cell analysis). The 16-well CIM plate is a 2 cavities membrane-separated system. Due to the small volume of the lower chamber (no more than 162 µl), the RTCA system does not allow to study bulk samples. In this regard, a nanodispersion of hydroxyapatite (HA) with a particle 10–30 nm diameter and a 1 mg/ml concentration in CNM was placed in the lower chamber. The nanopowder was prepared by the mechanochemical method at the Institute of Solid State Chemistry and Mechanochemistry of the Siberian Branch of the Russian Academy of Sciences (Novosibirsk). The concentration of the HA solution per well for the CIM-plate was 0.15 mg/150 µl. Live adhering MSCs do not directly contact HA nanoparticles deposited on the bottom of the lower microwells.

MSCs (2×10^4 L/ml) with/without heparin were placed in the plate upper microwells (maximum volume 180 µl). There were subdivided 4 groups of observation: the control group of cells without heparin and HA; cells with heparin; cells with HA in the lower chamber; cells with heparin in the upper section and HA in the lower section. The heparin was added at concentrations as described above. The reverse side of the membrane separating the microwells is 80% covered with gold electrode cells. The impedance obtained from these electrodes shows the area occupied by cells at each moment, which directly depends on their migration rate (invasion) through 8 µm micropores helping to track their migration at diverse timepoints, and expressed as a Cell Index (CI). The signals for CI determination using the RTCA Software were recorded every 15 min for 72 hrs.

Before testing the biological activity, the samples prepared and HA nanopowder were sterilized in a dry heat oven (Sanyo, Japan) at 160 °C for 1 h.

Statistical data processing was carried out by using statistical description methods, as well as methods for statistical hypothesis testing. We assessed the normality distribution by using the Kolmogorov–Smirnov test. The data had no normal distribution; therefore, for descriptive statistics, the median (M), 25% ($Q_{0.25}$) and 75% ($Q_{0.75}$) quartiles were determined. To assess the statistical significance of the differences, the nonparametric Wilcoxon T-test for dependent samples and the nonparametric Mann–Whitney test for independent samples were used.

Differences were considered statistically significant at a significance level of $p < 0.05$. Statistical analysis of the results was performed by using the GraphPad Prism version 8.0.1 software package (GraphPad Software Inc., San Diego, CA, USA).

Results and discussion

According to the flow cytometry results, the number of cells carrying the surface marker CD90 increased compared to the control values in MSC cultures supplemented with heparin at a concentration of 13 IU/ml – 260 IU/ml ($p \leq 0.05$) in a 2D culture model (Table 1). There was also a statistically significant increase in the number of CD73⁺-cells in the groups with heparin concentrations within the range of 1.3 IU/ml – 260 IU/ml (relative to the control group). A group of scientists tried to determine the authenticity of stem markers in a recent study, which included CD73, CD90 and CD105 [1]. In this study, increased number of cells expressing markers CD90 and CD73 indicated at poorly differentiated state of the cell culture [4]. A study by Moraes D.A. (2016) showed that decreased level of CD90 expression enhanced MSC osteogenic and adipogenic differentiation. These data suggest that CD90 controls the differentiation of MSCs interfering with differentiation, and therefore preserves the “stem pattern” of this cell culture [9]. The data obtained in the experiment may indicate about preserved stem nature of the MSC culture during their 2D co-culture with heparin.

We analyzed groups in a 3D model of MSC culture added with heparin at multiple concentrations together with bone-replacing implants carrying a CF coating with a roughness index $Ra = 2.6\text{--}4.9$ µm. According to the data obtained (Table 1), cell culture supplemented with heparin at a concentration of 1.3 IU/ml showed decreased number of cells expressing the surface marker CD90 ($p \leq 0.05$). This may indicate that CP-induced differentiation towards osteoblasts. Exposure to heparin at concentration of 1.3 IU/ml, 130 IU/ml, and 260 IU/ml decreased CD73 expression, which may also indicate a loss of MSC stemness under the influence of implants. Furthermore, heparin at doses of 1.3–13 IU/ml increased hematopoietic cells' yield in 3D culture by 2–3-fold (Table 1). Perhaps, bone-replacing implants in the presence of heparin at low concentrations (1.3–13 IU/ml) activate some MSC pool *in vitro* to differentiate into osteoblasts, which is accompanied by decreased expression of MSC markers. The emergence of osteoblastic niches can contribute to the accumulation of hematopoietic stem cells, which are present in small quantity in the primary culture of human adipose tissue MSCs [11].

At the same time, high doses of heparin (130–260 IU/ml) significantly reduced the proportion of single-positive CD73⁺ MSCs. However, the yield of hematopoietic cells did not change or even decreased compared to the 3D control (Table 1).

The results of the MSC migration activity under the influence of heparin gradient concentrations and/or suspension of HAP nanoparticles are presented in Figures 1, 2.

TABLE 1. EXPRESSION OF SURFACE MARKERS OF STEMNESS ON MSCs AFTER 14 DAYS OF CULTIVATION WITH HEPARIN AND/OR BONE-REPLACING IMPLANTS, Me ($Q_{0.25}$ - $Q_{0.75}$)

2D culture			
Study groups	CD90	CD73	CD14, CD20, CD34, CD45
Control	78.97 (77.56-81.39)	78.22 (74.03-78.97)	1.02 (0.99-5.86)
mSC + heparin 1,3 IU/ml	79.79 (75.15-85.02)	84.59 (84.24-95.72) $p_0 < 0.05$	5.09 (1.71-10.62)
mSC + heparin 13 IU/ml	87.91 (81.57-88.21) $p_0 < 0.05$	87.26 (84.60-88.21) $p_0 < 0.05$	5.78 (5.07-10.10)
mSC + heparin 130 IU/ml	90.73 (82.62-93.18) $p_0 < 0.05$	90.44 (83.89-91.90) $p_0 < 0.05$	5.85 (4.26-7.18)
mSC + heparin 260 IU/ml	93.84 (93.5-96.7) $p_0 < 0.05$	92.37 (89.90-94.86) $p_0 < 0.05$	3.27 (2.37-3.30)
3D culture			
Control + implant	89.69 (86.06-93.18)	90.58 (87.07-94.25)	2.3 (1.94-4.37)
mSC + heparin 1,3 IU/ml + implant	79.76 (79.75-85.39) $p_1 < 0.05$	77.85 (72.67-78.14) $p_1 < 0.05$	5.17 (3.00-5.22)
mSC + heparin 13 IU/ml + implant	88.24 (87.82-91.87)	87.65 (84.55-87.99)	7.06 (5.10-8.02) $p_1 < 0.05$
mSC + heparin 130 IU/ml + implant	92.76 (88.28-93.22)	76.63 (67.20-78.38) $p_1 < 0.05$	2.22 (1.97-4.09)
mSC + heparin 260 IU/ml + implant	84.18 (83.28-90.19)	83.81 (81.78-89.71) $p_1 < 0.05$	1.2 (1.15-1.50) $p_1 < 0.05$

Note. p_0 , statistical differences relative to the control group; P_1 , statistical differences relative to the control group in the presence of an implant.

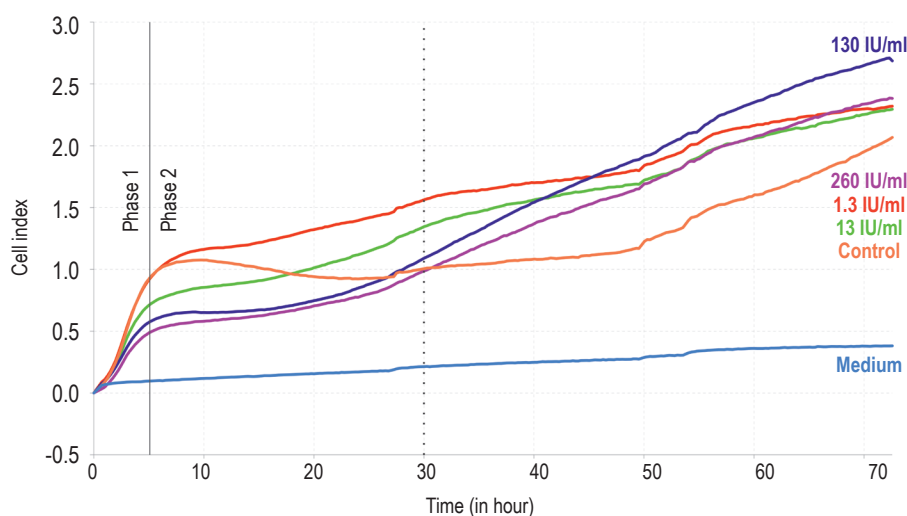


Figure 1. Curves of MSC invasion under the influence of various heparin concentrations (1.3-260 IU/ml) in the RTCA system (72-h monitoring)

Note. Phase 1, linear growth phase; Phase 2, CI logarithmic growth phase.

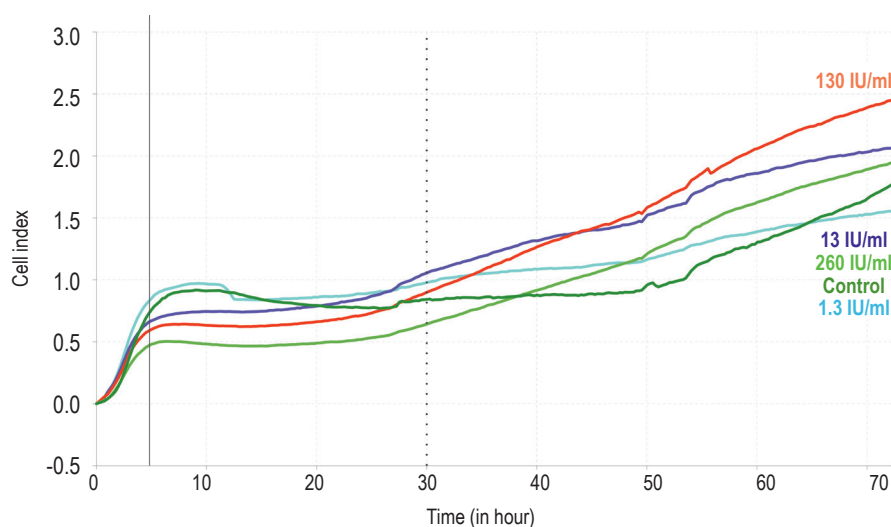


Figure 2. Curves of MSC invasion under the influence of various heparin concentrations (1.3-260 IU/ml) in the presence of a suspension of HA nanoparticles in the RTCA system (72-h monitoring)

Note. As for Figure 1.

The curves depicting dynamic migration (invasion) of cells through the porous membrane were built up by using the xCELLigence system and can be divided into 2 sections: Phase 1 – (0-5 hours) phase of linear growth; Phase 2 – (5-75 hours) phase of logarithmic growth with gradual saturation of the CI cell migration index.

The data in Figure 1 show an initial sharp increase in CI in all study groups due to rapid invasion/migration of cells within the first 5 hours. 5-7 hours after the onset of experiment, the lower chamber cellularity enters the phase of logarithmic growth associated with slowed (for control – a decline in the period of 10-25 hrs) rate of increase in the CI index of invasion. Within the first 25-30 hrs of the experiment, under the influence of heparin at high concentrations (130-260 IU/ml), it reduced MSC migration activity compared to control group ($p < 0.0001$) (Figure 1). We also noted similar dynamics up to 18 hours of observation by using heparin at dose of 13 IU/ml. Previously, it was demonstrated that heparin blocked the intercellular interaction of platelets and cancer cells [2], which may underlie heparin-related early effect.

Finally, the MSC heparin-coated migration activity was found to be significantly (according to the Wilcoxon T-test) higher than in control. The results obtained are consistent with the literature data that heparin and heparan sulfates can stimulate cell migration while interacting with heparin-binding cytokines and chemokines. Talsma D.T. demonstrated similar results on lymphocyte culture [12]. In addition, Liang Y. and Kiick K.L. showed increased cell proliferation and migration under PLGA nanoparticle action functionalized with heparin and combined with fibrin gels to form composite structures [7].

Figure 2 shows the data demonstrating the effect of heparin within the studied concentration range on MSC invasion added with suspension of HA nanoparticles to the CIM-plate lower well. In our earlier studies, we showed that the HA particles do not significantly affect the operation of the xCELLigence system [8]. The curves of the dynamic migration activity demonstrate that during the first 5 hrs, the cells are at the stage of active invasion/migration and enter the phase of logarithmic growth. Groups added with heparin at high concentrations of 13, 130 or 260 IU/ml inhibited migration activity within the first 20-40 hours similar to groups without HA ($p < 0.0001$) (Figure 1, 2). On the contrary, the CI index increases faster than the control value in the remaining time, especially when heparin is added at concentration of 130 IU/ml ($p < 0.0001$), which indicates a good migration potential of cells through the pores in the CIM-plate membrane. Simultaneously, the maximum concentration of heparin (260 IU/ml) had a less pronounced effect in both RTCA systems (Figure 1, 2) than at concentration of 130 IU/ml, which indicates its potential cytotoxicity.

Heparin at the lowest concentration (1.3 IU/ml) stimulated MSC migration activity only for up to 65 h of observation (Figure 2); the growth rate of the CI index turned out to be lower than in the system without HA nanoparticles (Figure 1, 2).

Thus, the data obtained indicate that heparin at later periods (from 20-40 hours) increases the invasion of MSCs through micropores that simulate the blood vessel wall state. However, in the presence of HA nanoparticles that mimic the processes of mineral bone matrix remodeling and/or resorption of bone cement, heparin effect is less pronounced. High concentrations of heparin (130, 260 IU/ml)

also contribute to an increase (in a 2D system) or preservation (in a 3D model) of the MSC stemness (by expression of surface markers CD73, CD90 with decreased expression of hematopoietic cell antigens).

On the one hand, the results are intriguing in terms of regenerative medicine technologies associated with the systemic administration of MSCs to correct chronic diseases. Moreover, they should be taken into account in patients' postoperative management with arthroplasty of major joints. The data may serve as a

prerequisite for developing new therapeutic strategies for surgical patients with a high risk of postoperative thrombosis after osteosynthesis.

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ДИНАМИКА БИОМАРКЕРОВ КАРДИОВАСКУЛЯРНОГО РИСКА И ЦИТОКИНОВ У БОЛЬНЫХ С ДЕКОМПЕНСИРОВАННОЙ СЕРДЕЧНОЙ НЕДОСТАТОЧНОСТЬЮ С СИСТОЛИЧЕСКОЙ ДИСФУНКЦИЕЙ ИШЕМИЧЕСКОГО ГЕНЕЗА ПРИ НАЛИЧИИ И ОТСУТСТВИИ МИОКАРДИТА

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Резюме. Развитие и прогрессирование сердечной недостаточности связано с различными патофизиологическими механизмами, особый интерес представляет изучение воспалительной реакции, как фундаментального звена в патогенезе ХСН и ее основного компонента — декомпенсации. Проведено открытое, нерандомизированное, проспективное исследование по изучению клинико-морфологических особенностей субклинического воспаления у больных с острой декомпенсацией ишемической хронической сердечной недостаточностью со сниженной фракцией выброса. В исследование были включены 25 больных с декомпенсацией ишемической ХСН с ФВ левого желудочка < 40% в возрасте от 35 до 75 лет (60,12±9,3) с подписанным информированным согласием. В данном исследовании изучалась динамика содержания в сыворотке крови С-реактивного белка (СРБ), N-концевого фрагмента белка-предшественника мозгового натрийуретического пептида (NT-proBNP), растворимого ST2 (sST2), рецептора инсулиноподобного фактора роста 1 (IGF-1R), интерлейкина-6 (IL-6), интерлейкина-10 (IL-10), фактора некроза опухоли-α (TNFα) на Multiplex Instrument FLEXMAP 3D Luminex Corporation. Все обследованные пациенты были разделены на две группы в зависимости от диагностированного миокардита: пациенты без признаков миокардита и пациенты с миокардитом. Установлено, что в группе пациентов с диагностированным миокардитом наблюдалось повышенное содержание СРБ, IGF-1R, IL-6 и IL-10, TNFα по сравнению с группой пациентов без миокардита. Средние концентрации NT-proBNP и sST2 в обеих группах не различались. При последующем визите через год наблюдалось снижение содержания СРБ, NT-proBNP, IL-6 в обеих группах. В группе больных миокардитом наблюдалось повышение содержания sST2, IGF-1R, IL-10. Таким образом, проведенное в динамике исследование выявило достоверные различия в изменении сывороточной активности про- и противовоспалительных цитокинов и биомаркеров сердечно-сосудистого риска у пациентов с декомпенсированной сердечной недостаточностью с систолической дисфункцией при диагностированном миокардите и при его отсутствии.

Ключевые слова: воспаление, цитокины, биомаркеры, сердечная недостаточность, миокардит

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DYNAMIC CHANGES IN CARDIOVASCULAR RISK BIOMARKERS AND CYTOKINES OF MYOCARDITIS-FREE PATIENTS WITH DECOMPENSATED HEART FAILURE AND ISCHEMIC SYSTOLIC DYSFUNCTION

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Abstract. The development and progression of heart failure is associated with a variety of pathophysiological mechanisms, of particular interest is the study of the inflammatory response as a fundamental link in the pathogenesis of CHF and its main component – decompensation. An open, non-randomized, prospective study was carried out to evaluate the clinical and morphological features of subclinical inflammation in patients with acute decompensation of ischemic chronic heart failure with a reduced ejection fraction. The study included 25 patients with decompensated ischemic CHF with left ventricular ejection fraction < 40% aged 35 to 75 years (60.12 ± 9.3 y. o.). In this study the dynamics of the serum content of C-reactive protein (CRP), N-terminal fragment of the brain natriuretic peptide precursor protein (NT-proBNP), soluble ST2 (sST2), insulin-like growth factor-1 receptor (IGF-1R), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor- α (TNF α) was performed by multiplex immunoassay using the FLEXMAP 3D. All studied patients were divided into two groups depending on the diagnosed myocarditis: patients with no signs of myocarditis and patients with myocarditis. It was found that in the group of patients with diagnosed myocarditis there was an increased content of CRP, IGF-1R, IL-6 and IL-10, TNF α compared to the group of patients without myocarditis. The median concentrations of the NT-proBNP and sST2 in both groups did not differ. At the follow-up visit a year later, there was a decrease in the content of CRP, NT-proBNP, IL-6 in both groups. In the group of patients with myocarditis, an increase in the content of sST2, IGF-1R, IL-10 was observed. Thus, the study carried out in dynamics revealed significant differences in the degree of changes in the serum activity of pro- and anti-inflammatory cytokines and biomarkers of cardiovascular risk in patients with decompensated heart failure with systolic dysfunction with diagnosed myocarditis and in its absence.

Keywords: inflammation, cytokines, biomarkers, heart failure, myocarditis

Introduction

Decompensation of CHF is a serious clinical and public health problem with a high level of morbidity, mortality and rate of hospitalizations [6]. The development and progression of heart failure are associated with a variety of pathophysiological mechanisms, of particular interest is examining an inflammatory response as a fundamental link in the CHF pathogenesis and its main component – decompensation [7]. An inflammatory concept has been formulated, which is based on the persistent inflammation present at the early stages of CHF, as well as at later stages, when CHF is verified [6]. Recently, the study of inflammation markers and their role in cardioprotective mechanisms, as well as growth factors and cytokines as prognostically relevant biomarkers of developing heart failure deserved particular interest [4, 5]. It is known that imbalance of pro- and anti-inflammatory cytokines significantly contributes to development and progression of CHF, but the results of experimental and exploratory clinical studies have not led to the emergence of a

single effective anti-inflammatory strategy in CHF syndrome [7].

Purpose of the study: to study the dynamics and relationship of serum cardiovascular risk biomarkers and cytokines in patients with decompensated heart failure with ischemic systolic dysfunction with/without myocarditis using xMAP technology.

Materials and methods

An open, non-randomized, prospective study was carried out to evaluate the clinical and morphological features of subclinical inflammation in patients with acute decompensation of ischemic chronic heart failure with reduced ejection fraction. This study is registered at the ClinicalTrials.gov, ID: NCT02649517. The study included 25 patients with decompensated ischemic CHF with left ventricular ejection fraction < 40% aged 35 to 75 years (60.12 ± 9.3 y.o.), who signed informed consent, 6 months after successfully performed angioplasty and/or coronary artery bypass grafting. The therapy performed in patients at the time of enrolling to the study corresponded to the

current recommendations such as: beta-blockers, diuretics, mineralocorticoid receptor antagonists, cardiac glycosides. Exclusion criteria were: acute coronary syndrome less than 6 months before hospitalization; hemodynamically significant valvular heart disease; severe concomitant diseases that could affect the course of the underlying disease and the study results. All patients underwent invasive coronary angioplasty to exclude the progression of coronary artery atherosclerosis, as well as endomyocardial biopsy with immunohistological analysis to verify myocarditis. This article analyzes the dynamics of biomarkers in such patients by using Milliplex kit (Merck KGaA, Darmshadt) ($n = 10$). The diagnosis of myocarditis was established in 5 patients according to immunohistochemical criteria: the presence of at least 14 leukocytes/mm², with the presence of CD3 positive T-lymphocytes ≥ 7 cells/mm². Fasting blood sampling for biomarkers from the cubital vein was carried out during hospitalization of patients and one year later at the checkpoint visit. The evaluation serum of high-sensitive C-reactive protein (hCRP) concentration was carried out in blood serum by ELISA (Biomedica); level of N-terminal fragment of brain natriuretic peptide precursor protein (NT-proBNP) pg/ml was determined using Human Cardiovascular Disease Panel 1. Levels of soluble ST2 (sST2) ng/ml, insulin-like growth factor-1 receptor (IGF-1R) pg/ml were investigated by using Human Cardiovascular Disease Panel 5. The level of interleukin-6 (IL-6) pg/ml, interleukin-10 (IL-10) pg/ml, tumor necrosis factor- α (TNF α) pg/ml was measured by using Human Cytokines/Chemokines-38 kit. All studies were performed using Multiplex Instrument FLEXMAP 3D Luminex Corporation and MILLIPLEX Analyst 5.1 software (Merck KGaA, Milliplex; Darmshadt), the Core Facility "Medical genomics", Tomsk NRMC.

Results and discussion

All patients were divided into two groups depending on the diagnosed myocarditis: patients with no signs of myocarditis were included in group 1 and patients with myocarditis were included in group 2. Clinical characteristics are presented in Table 1. Examining serum biomarkers from patients during hospitalization showed that in the group of patients with diagnosed myocarditis, there was an increased content of C-reactive protein, insulin-like growth factor receptor-1, interleukins-6 and 10, tumor necrosis factor- α compared to the group of patients without myocarditis. The median NT-proBNP concentration in both groups was comparable and exceeded the pathologically significant level of 125 pg/ml. The sST2 level in both groups was also comparable on admission, but did not exceed the pathologically significant level

of 30 ng/ml. The data are presented in Table 2. A decreased level of C-reactive protein was noted in both groups, at the follow-up visit one year later in patients without myocarditis, the observed decrease was by 58%, while in the group with diagnosed myocarditis it was decreased only by 28%. The NT-proBNP content in both groups decreased by 40% one year later, but its concentration also remained above the pathologically significant level. The level of sST2 one year later in the group of patients without myocarditis remained unchanged; in the group of patients with myocarditis, it was increased by 76%. In the group of patients with myocarditis, the IGF-1R content increased by 5-fold, while the median IGF-1R concentration in the blood serum of patients without myocarditis remained at the level comparable to that observed at the time of hospitalization. The content of interleukin-6 in both groups of patients decreased by 48 and 43%, respectively. The content of interleukin-10 in the group of patients with myocarditis increased by 18% compared to the baseline level; in the group of patients without myocarditis, the level of IL-10 remained unchanged. In both groups, the content of TNF α decreased by 12 and 20%, respectively. Thus, the study of the dynamics of biomarkers in both groups revealed decreased concentrations of inflammation markers such as CRP, IL-6, and NT-proBNP a marker of the risk of heart failure after 12 months of observation. Moreover, in the group of patients with myocarditis, there was increased content of sST2, IGF-1R, IL-10 after 12 months of observation. Correlation analysis in the group of patients without myocarditis revealed a negative relationship between the content of NT-proBNP and IGF-1R ($r = -0.70$, $p < 0.05$). In both groups of patients, a positive relationship with high correlation coefficient was found between serum level of sST2 and IGF-1R ($r = 0.90$) $p < 0.05$ on admission and at the follow-up visit one year later.

Cytokines are a key element of the immune system in the development of acute and chronic inflammation. The majority of chronic inflammatory diseases, regardless of their organ location, are based on imbalanced production between pro- and anti-inflammatory mediators [4]. Therefore, the basis for the treatment of any inflammatory process is attempted to normalize it by any means. It has now been established that not only lymphocytes and macrophages, but also other cells are capable of synthesizing cytokines, and in this respect the heart is a unique source of their production [9]. The concentration of hCRP is considered as the most sensitive and specific laboratory marker of inflammation and tissue damage [8] and, as we demonstrated in our study, it correlates with the synthesis of IL-6 both with/without myocarditis, which, in turn, plays an important role in the developing inflammation. It is known that cardiomyocytes in patients with heart failure produce

TABLE 1. BASELINE CHARACTERISTICS OF PATIENTS WHO WERE INCLUDED IN THE STUDY, Me (Q_{0.25}-Q_{0.75})

Parameters	Group 1 (n = 5)	Group 2 (n = 5)
Age, years	58.0 (51.0-61.0)	61.0 (56.0-67.0)
Body Mass Index, kg/m ²	31.6 (30.3-34.7)	28.0 (25.30-31.07)
Duration of chronic heart failure, month	24.0 (12.7-77.0)	12.0 (8.0-48.0)
Time between the last myocardial infarction before the development of chronic heart failure, month	26.0 (6.0-114.0)	80.0 (13.0-120.0)
Functional class of CHF by (NYHA) before hospitalization:		
II	4	2
III	–	3
IV	1	–
Systolic pressure, mmHg	130.0 (100.0-138.0)	120.0 (100.0-130.0)
Diastolic pressure, mmHg	80.0 (60.0-80.0)	70.0 (60.0-80.0)
Heart rate, beats/min.	68.0 (64.0-82.0)	72.0 (68.0-90.0)
Respiratory rate, breath/min.	18.0 (17.0-20.0)	18.0 (16.0-20.0)
Medical treatment during hospitalization:		
A2 receptor Blockers/ ACE inhibitors	–	1
Beta-blockers	3	–
Cardiac glycosides	–	2
Diuretics	4	4
Acyclovir	–	2
Viferon	1	4

TABLE 2. CYTOKINE LEVEL, Me (Q_{0.25}-Q_{0.75})

Parameter	Baseline		At 12 mounts	
	Group 1 (n = 5)	Group 2 (n = 5)	Group 1 (n = 5)	Group 2 (n = 5)
C-RP(h) mg/l	6.59** (1.03-9.66)	8.26 (1.34-10.20)	2.77 (1.99-4.32) *p = 0.06	5.93 (1.69-10.46)
Nt-proBNP pg/ml	579.70 (93.13-683.71) **p = 0.06	525.31 (212.34-532.80) **p = 0.06	362.12 (163.79-656.05)	323.40 (268.72-625.72)
sST2 ng/ml	2.22 (1.83-4.38)	2.14** (1.35-3.36)	2.10* (1.83-6.54)	8.58 (5.90-10.98)
IGF-1R pg/ml	6639.96 (4213.32-7839.19)	8245.50** (7275.64-8819.10)	7758.29* (5552.64-15202.21)	39808.16 (23040.85-95407.03)
IL-6 pg/ml	6.79 (4.69-7.05) **p = 0.06	8.11 (5.33-9.80) **p = 0.06	3.50 (3.48-6.87)	4.60 (4.02-8.84)
IL-10 pg/ml	1.70 (1.28-2.41)	2.57 (0.92-3.60)	1.55* (1.23-2.10)	3.16 (3.09-6.25)
TNF α pg/ml	7.62* (4.98-12.65)	20.46 (19.53-23.90)	6.71* (6.42-11.60)	16.53 (11.60-17.81)

Note. *, statically significant within the group 1 vs 2; **, statically significant within the group Baseline vs At 12 mounts; Group 1, there is no myocarditis; Group 2, there is myocarditis.

TNF α , even without manifested inflammatory process. In addition, TNF α induces the process of cardiomyocyte programmed death, which loses its compensatory character under the pathological conditions posing TNF α as having fundamental importance in myocardial remodeling. [8]. In our study, an increased content of serum TNF α was noted in the group of patients with myocarditis; after 12 months of observation, its level remained unchanged, whereas content of interleukin 10 tended to increase. It is known that the anti-inflammatory cytokine IL-10 can inhibit TNF α production and counterbalance its negative effects in heart failure [2]. However, the results of clinically examined IL-10 in heart failure and its progression are controversial. On the one hand, there is an evidence of decreased IL-10 levels in HF and left ventricular remodeling; on the other hand, there are reports showing its increased levels and elevated mortality in HF patients with a simultaneous increased IL-10 and TNF α concentrations [1]. IL-10 can function as a component of the feedback mechanism: elevated levels of TNF α in HF stimulate the secretion of IL-10, and IL-10, on the contrary, suppresses excessive activity of pro-inflammatory cytokines; however, a role of IL-10 regardless of TNF α , cannot be ruled out as the production of IL-10 is induced together with pro-inflammatory cytokines, TNF α in particular in inflammatory processes and in HF [3]. It was supported by showing positive correlations between the content of TNF α and IL-10 ($r = 0.70$) $p < 0.05$ in the group of patients with myocarditis. Among diverse existing modern biochemical markers, natriuretic peptides have been introduced into routine clinical practice, proving to act as markers of myocardial stress, myocardial dysfunction and heart failure [9]. Currently, not a single biomarker can account for all aspects of CHF syndrome and development of its decompensation [9]. According to the numerous modern studies, sST2 has recently been included in the European and American guidelines for CHF treatment. The data obtained in the group of patients with diagnosed myocarditis after 12 months of observation demonstrate not only increased serum sST2 content, but also a positive relationship with proinflammatory cytokines. It is possible that the level of sST2 expression not only confirms the inflammatory mechanism of CHF development, but also reflects the activity of systemic inflammatory response upon disease progression. In recent years, the association of IGF-1 with cardiovascular diseases as an independent risk factor has been discussed, but the results of these studies are rather contradictory. IGF-1 under the

influence of growth hormone is secreted in the liver as well as also produced in cardiomyocytes, smooth muscle cells and fibroblasts; it is capable of exerting an insulin-like metabolic effect that exerts a significant role in regulating the structure and function of the myocardium and blood vessels [1]. There is evidence that decreased synthesis of IGF-1 promotes enhanced apoptosis of cardiomyocytes and progression of myocardial fibrosis that appears to be a predictor of decompensated chronic heart failure [3,9]. IGF-1 exerts its action through ubiquitously expressed receptors (IGF-1R) including cardiomyocytes, which determines their role as modulators of myocardial structure and function. Insulin-like growth factors in the bloodstream are associated with proteins that play a key role in the bioavailability of ligands, as they compete with IGF-1R for IGF-I. By binding to IGF-1, IGFBPs thereby inhibit proliferation signals and, according to the feedback loop, IGF-1R becomes elevated if IGF-1R is over-activated. There is evidence that proinflammatory cytokines can be involved in blocking IGF-1 specific receptor binding due to the phosphorylation of serine residues in IRS [9]. It is possible that the significant increase in the IGF-1R content obtained in our study in the group of patients with myocarditis underlies hormonal and neurohumoral processes and serves as a biochemical marker of altered metabolic processes, whereas established positive relationships with inflammation markers seem to be a predictor of aggravated chronic heart failure decompensation, indicating an unfavorable prognosis. Thus, a study in the dynamics of biomarkers in patients with diagnosed myocarditis demonstrated increased level of IGF-1R, soluble ST2, IL-10, a decrease in NL-6, NT-proBNP and C-reactive protein and their close inter-relationship. In the group of patients without myocarditis, there was a decrease in IL-6, NT-proBNP and C-reactive protein, the levels of other biomarkers remained unchanged. The follow-up study revealed significant differences in magnitude of changes in serum level of pro- and anti-inflammatory cytokines and biomarkers of cardiovascular risk in patients with decompensated heart failure with systolic dysfunction with/without diagnosed myocarditis. The established relationships allow us to assume that the presence of myocarditis in patients with decompensated heart failure with ischemic systolic dysfunction leads to formation of more unfavorable relationships in the feedback loop of cytokine secretion, leading to evenly greater shift in the cytokine balance towards quantitative predominance of pro-inflammatory cytokines.

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ИММУНОЛОГИЧЕСКИЕ И ПАТОМОРФОЛОГИЧЕСКИЕ АСПЕКТЫ РАННЕЙ И ПОЗДНЕЙ ПРЕЭКЛАМПСИИ

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Резюме. Одно из самых распространенных осложнений беременности — преэклампсия (РЕ) может возникать начиная с 20 недель гестации и завершается только с полным удалением последа. Традиционно РЕ принято подразделять на раннюю, возникающую до 34 недель беременности включительно (ЕОРЕ), и позднюю — после 34 недель гестации (ЛОРЕ). Клинические проявления в том и другом случае одинаковы, однако факторы риска и степень тяжести ПЭ различны. Установлено, что ЕОРЕ определяется нарушением инвазии трофобласта и трансформации спиральных артерий матки в ранние сроки беременности, а позднее начало РЕ связывают со оксидативным стрессом синцитиотрофобласта, возникающим вторично, при ограниченном газообмене и недостаточном поступлении питательных веществ. Многочисленные исследования отмечали значительный вклад иммунных реакций в патогенез преэклампсии, однако состояние В-лимфоцитов при ЕОРЕ и ЛОРЕ не исследовалось. Проводилась комплексная оценка состояния женщин при раннем (до 34 недель беременности включительно) и позднем (после 34 недель) развитии преэклампсии с учетом клинических и анамнестических характеристик, особенности формирования структурных компонентов плаценты, а также определением характера дифференцировки и функциональной активности В-лимфоцитов. В периферической венозной крови исследовали содержание CD19⁺, CD20⁺, CD19⁺CD27⁺IgD[±], CD19⁺CD20⁺CD38⁺, CD20⁺CD5⁺-клеток и сывороточный уровень IL-5, IL-9, IL-13. Морфологическое исследование включало макроскопическое описание, органомерию, обзорную гистологию и трансмиссионную электронную микроскопию. В группе женщин с ранней преэклампсией в анамнезе чаще имелись перинатальные потери, преждевременные роды и медицинские аборт, а в текущей беременности внутриутробное инфицирование, маловодие, плацентарная недостаточность и задержка роста плода. При поздней преэклампсии чаще отмечался метаболический синдром, анемия, а в анамнезе артериальная гипертензия. В периферической крови всех женщин с преэклампсией отмечалось повышение содержания CD20⁺CD5⁺-клеток по сравнению с показателями при неосложненной беременности, более выраженное при позднем начале преэклампсии. Только у женщин с ранней преэклампсией в крови повышалось содержание CD19⁺CD20⁺CD38⁺ и CD19⁺CD27⁺IgD[±]-клеток, IL-5,

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IL-9 и IL-13. Исследования последа при ранней преэклампсии свидетельствовали о нарушении имплантации и патологической плацентации с развитием первичной плацентарной недостаточности, переходящей в хроническую форму. При поздней преэклампсии развитие фетоплацентарной недостаточности определяли хронические расстройства материнской и плодовой гемоциркуляции с повышенным отложением фибрина и фибриноида в базальной пластинке и в зонах некроза эпителия ворсин. Проведенное исследование показало, что сроки манифестации преэклампсии определяются действием факторов клинического анамнеза, структурной перестройкой в плаценте и иммунными реакциями В-лимфоцитов тесно взаимосвязанными между собой.

Ключевые слова: ранняя преэклампсия, поздняя преэклампсия, плацента, периферическая кровь, В-лимфоциты

IMMUNOLOGICAL AND PATHOMORPHOLOGICAL ASPECTS OF EARLY AND LATE PREECLAMPSIA

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Abstract. Preeclampsia (PE) is one of the most common complications of pregnancy, and it can be after 20 weeks of gestation. It ends only with a complete dissection of afterbirth. Traditionally, PE is subdivided into the early one, taking place through 34 weeks of pregnancy (EOPE) and the late one, which is after 34 weeks of gestation (LOPE). Clinical manifestations are similar in both cases however, risk factors and the severity of PE are different. It has been established that EOPE is determined by impaired trophoblast invasion and transformation of the spiral arteries of the uterus in early pregnancy, and late onset of PE is associated with oxidative stress of syncytiotrophoblast, which occurs secondarily, with limited gas exchange and insufficient intake of nutrients. Numerous studies have noted a significant contribution of immune responses to the pathogenesis of preeclampsia, however, the state of B-lymphocytes in EOPE and LOPE has not been studied. A comprehensive assessment of the condition of women with early (up to 34 weeks of pregnancy inclusive) and late (after 34 weeks) development of preeclampsia was carried out, taking into account clinical and anamnestic characteristics, the peculiarities of the formation of the structural components of the placenta, as well as determining the nature of differentiation and functional activity of B-lymphocytes. In peripheral venous blood, the content of CD19⁺, CD20⁺, CD19⁺CD27⁺IgD[±], CD19⁺CD20⁺CD38⁺, CD20⁺CD5⁺-cells and serum levels of IL-5, IL-9, IL-13 were examined. Morphological examination included gross description, organometry, survey histology, and transmission electron microscopy. In the group of women with early preeclampsia in history, there were more often perinatal losses, premature births and medical abortions, and in the current pregnancy, intrauterine infection, oligohydramnios, placental insufficiency and fetal growth retardation. With late preeclampsia, metabolic syndrome, anemia, and a history of arterial hypertension were more often observed. In the peripheral blood of all women with preeclampsia, there was an increase in the content of CD20⁺CD5⁺-cells in comparison with those in uncomplicated pregnancy, more pronounced in the late onset of preeclampsia. Only in women with early preeclampsia blood levels of CD19⁺CD20⁺CD38⁺ and CD19⁺CD27⁺IgD[±]-cells, IL-5, IL-9 and IL-13 increased. Studies of the placenta in early preeclampsia indicated impaired implantation and pathological placentation with the development of primary placental insufficiency, which becomes chronic. In late preeclampsia, the development of placental insufficiency was determined by chronic disorders of maternal and fetal hemocirculation with increased deposition of fibrin and fibrinoid in the basal lamina and in the zones of villous epithelium necrosis. The study showed that the timing of the manifestation of preeclampsia is determined by the action of factors of the clinical history, structural rearrangements in the placenta and immune responses of B-lymphocytes are closely interrelated.

Keywords: early and late preeclampsia, placenta, peripheral blood, B-lymphocytes

Introduction

Being a unique physiological state, pregnancy causes changes in the functioning of multiple host systems. Continuously exchanging antigen material connects the two body systems triggering a cascade of immune reactions giving the most favorable development conditions for the fetus. Nevertheless, there are instances where the balance of the immune regulatory processes is disturbed under the influence of various factors such as genetic, contagious, hormonal, etc., and the reserve capacity of the immune system becomes insufficient in response to adverse reactions. Most of the gestational process complications result from such conditions, but the timeframe for clinical symptoms varies considerably. Preeclampsia (PE) is one of the most common complications of pregnancy occurring after 20 weeks of gestation that disappears only after birth. Traditionally, PE is subdivided into the early PE occurring through 34 weeks of pregnancy (EOPE) and the late PE observed after 34 weeks of gestation (LOPE). Clinical manifestations are similar in both cases, but PE risk factors [8] and the severity of differ [13]. The cause of PE is characterized by syncytiotrophoblast stress, systemic inflammatory reaction and maternal endothelial dysfunction [9]. However, detailed analytical reviews have demonstrated that EOPE is diagnosed by trophoblast invasion dysplasia and the transformation of the uterine spiral artery in early pregnancy [5, 9]. This leads to the development of adverse reactions due to perfusion changes and increased placental stress [9]. Late-onset PE is associated with syncytiotrophoblast oxidative stress, which emerges secondarily in case of limited gas exchange and inadequate nutritional intake [9]. On the one hand, LOPE is associated with boosted apoptotic damage to the placenta, a massive inflow of syncytiotrophoblast microparticles (STBM) into the maternal bloodstream. As STBM ligands, they activate immune system cells. This leads to the release of inflammatory cytokines, coagulation factors and superoxide radicals, contributing to development of systemic inflammation associated with preeclampsia [9]. On the other hand, this condition may be determined by maternal hypersensitivity to factors of placental origin due to genetic predisposition, somatic problems associated with endothelial dysfunction, cardiovascular disease [5]. Numerous studies, including those performed in our laboratory, have noted a significant contribution of immune reactions to preeclampsia-related pathogenic mechanism. Some features of developing inflammatory reactions and immune regulatory processes were revealed both at the systems level and in the placenta under various PE forms. At the same time,

the information on B-lymphocyte state transition is sparse, paying virtually no attention to role during EOPE and LOPE.

In our study, we decided to conduct a comprehensive assessment of women with EOPE and LOPE, taking into account their clinical and anamnestic characteristics, features of placenta structural component formation, and determining the nature of B-lymphocyte differentiation and functional activity.

Materials and methods

The anamnesis and clinical course of preeclampsia were analyzed in the groups of women with early-onset preeclampsia (218 women within 24–34 weeks of gestation) and late-onset preeclampsia (92 women with more than 34 weeks of gestation). Immunoassay was performed in 38 women with PE during 24–34 weeks of pregnancy and 44 women with PE during 34 up to 40 weeks of pregnancy. The control group consisted of women with uncomplicated pregnancy, including 30 women during 24–34 weeks of gestation and 24 women during 34–40 weeks of gestation. Laboratory tests were performed on a FACSCantoII flow cytometer with the FACSDiva software (Becton Dickinson) by analyzing percentage of CD19⁺- and CD20⁺-lymphocytes in peripheral venous blood, CD27⁺IgD[±], CD20-CD38⁺ cells in the CD19⁺ population, CD5⁺-cells in the CD20⁺ population were estimated. We used Beckman Coulter monoclonal antibodies CD19, CD20, CD38, CD27, IgD; eBioscience CD5, CD20. Serum IL-5, IL-9, IL-13 from bio-plex 27 kits (Bio-Rad) were examined on a Luminex 200 analyzer (Luminex Corporation). Serum levels of immunoglobulins G, A, M were measured on a microplate reader in Immunoscreen test systems (VECTOR-BEST JSC). Morphological examination of 35 placentas in early PE and 40 placentas in late PE was performed, including macroscopic examination, organometry, observational histology, and transmission electron microscopy. The results were statistically processed using a package of licensed software packages: Microsoft Office 2010, Statistica for Windows 13.0. Values were quantified and presented as median with the 25th and 75th percentiles (Me (Q_{0.25}-Q_{0.75}) %). The significance of differences between variables was assessed by the nonparametric criteria of the Mann–Whitney U test.

Results and discussion

The analysis revealed that the mean age of the patients did not depend on the onset of PE and was 32.0 (26.0–35.0) years in the subgroup with early-onset PE (EOPE) and 30.0 (26.0–35.0) years ($p > 0.05$) in the subgroup with late PE (LOPE). The

incidence of bronchial asthma was higher in pregnant women with early-onset PE found in 2.75% of cases, while it was not recorded in another subgroup ($p = 0.01$). Metabolic syndrome manifested as obesity and arterial hypertension was significantly more frequently diagnosed in the subgroup with late PE – in 45.12% of cases compared to the another subgroup (23.03%, $p = 0.04$). At the same time, in hypertensive patients with the early PE development, the duration of this disease lasted for 8.0 (4.0-12.0) years, which was significantly longer than in late-onset PE – 6.0 (3.0-9.0) years ($p = 0.03$). Analysis of obstetric and gynecological status showed that subjects examined in the subgroup with early PE were significantly more likely to have perinatal loss (5.96%), premature delivery (16.51%), and medical abortions (7.80%) in the history compared to another subgroup (1.2%, 7.61%, and 1.09% respectively; $p = 0.02$ in all cases). During pregnancy, early-onset PE was more often associated with intrauterine infection from the trimester 1 (6.88% vs 2.17%; $p = 0.04$), placental insufficiency and VTE developed in the trimester 2 (74.31% and 57.80% vs 53.26% and 32.61%, respectively; $p = 0.01$ for all cases), and small-viscosity (13.76% and 4.35%; $p = 0.01$) compared to the subgroup with late-onset PE. The subgroup with late-onset PE had a higher incidence of anemia in pregnant women reaching 40.22%, compared to another subgroup (22.48%, $p = 0.01$). Patients with early PE on admission had higher mean BP than those

with late-onset: 118.3 (113.3-126.3) mm Hg vs 116.7 (110.0-120.0) mm Hg ($p = 0.01$); they had proteinuria of 3 g/l per urine sample significantly more frequently (26.8% vs 16.2%). ($p = 0.01$); proteinuria higher than three g/l in a urine sample was found at significantly higher rate (26.8% vs 16.2%, $p = 0.04$); they required ICU treatment significantly more often ($p = 0.01$). In this subgroup, labor was occurred at 33.0 (30.1-34.2) weeks, which was significantly earlier than in the late-onset PE being at 37.0 (35.9-37.5) weeks ($p = 0.00$). Accordingly, the number of premature deliveries in this category of patients was higher than in another subgroup – 92.2% vs 46.7% ($p = 0.00$).

Laboratory assays showed no differences in percentage of CD19⁺- and CD20⁺-lymphocytes between the control groups matched gestational age and the groups with PE (control 24-34 weeks pregnancy CD19⁺ 4.75% (3.38-6.90), CD20⁺ 5.90% (4.20-8.20), EOPE CD19⁺ 4.70% (3.18-7.25), CD20⁺ 4.60 (2.68-7.60); control more after 34 weeks of pregnancy CD19⁺ 4.70% (3.55-5.75), CD20⁺ 4.00% (2.50-5.60), LOPE CD19⁺ 4.05 (2.75-6.98), CD20⁺ 4.85% (2.78-7.10); $p > 0.05$ in all cases). Irrespective of the gestational age of PE clinical manifestations, increased CD5⁺-cell frequency in the CD20⁺B-lymphocyte population was observed in such patient peripheral blood samples (Table 1) compared to those in the control group of the corresponding gestational age. Furthermore, in LOPE vs. EOPE the level of CD20⁺CD5⁺-lymphocytes was higher. Only women

TABLE 1. CONTENT OF B-LYMPHOCYTE POPULATIONS IN THE PERIPHERAL BLOOD AND THE SERUM LEVEL OF CYTOKINES IN UNCOMPLICATED PREGNANCY, EARLY AND LATE PREECLAMPSIA, Me ($Q_{0.25}$ - $Q_{0.75}$)

Parameter	24-34 weeks pregnancy		More when 34 weeks pregnant	
	Control	Early preeclampsia	Control	Late preeclampsia
CD27 ⁺ IgD ⁺ in CD19 ⁺ , %	25.10 (17.45-30.15)	39.00 (26.30-43.90) $p = 0.003$	27.70 (23.90-39.40)	33.95 (22.95-37.95)
CD20 ⁺ CD38 ⁺ in CD19 ⁺ , %	0.55 (0.33-1.35)	3.70 (1.73-8.20) $p = 0.000$	2.60 (0.90-3.60)	3.60 (1.80-8.60)
CD5 ⁺ in CD20 ⁺ , %	11.35 (9.48-13.23)	13.85 (10.10-22.03) $p = 0.045$	8.85 (6.98-12.08)	21.20 (14.95-24.18) $p = 0.000$
IL-9, pg/ml	50.32 (41.28-92.77)	102.11 (66.56-126.08) $p = 0.03$	54.09 (48.99-94.84)	74.67 (50.31-89.08)
IL-13, pg/ml	0.07 (0.00-0.37)	1.76 (0.68-6.63) $p = 0.004$	0 (0-10.52)	2.27 (0.68-4.74)
IL-15, pg/ml	70.90 (58.18-87.33)	45.82 (9.11-69.38) $p = 0.006$	81.26 (61.61-86.98)	38.61 (6.69-56.38) $p = 0.015$

Note. p, difference from the control.

with EOPE had CD19⁺-cell population characterized by increased percentage of CD20-CD38⁺ plasmacytes and increased frequency of CD27⁺IgD[±] B-memory cells. Analysis of cytokines regulating B-cell maturation and differentiation showed that the level of IL-5, IL-9 and IL-13 increased only with EOPE. Various clinical manifestations of PE groups differed among those with lower serum IL-9 level compared to women with LOPE.

Morphological examination of the placenta showed that all PE types had implantation disorders such as marginal umbilical cord insertion and placenta hypoplasia. However, the detection and manifestation rate depended on the age of clinical manifestations for pregnancy complication. Thus, marginal cord insertion in EOPE and LOPE was diagnosed in 32.3% and 19.4% ($p = 0.04$) cases, respectively. Placenta hypoplasia, defined as grade 3, was more frequently diagnosed in EOPE (68% in EOPE, 6.3% in LOPE, $p = 0.04$) and accompanied by fetal growth restriction (FGR) in 74% of cases. Placentas of women with LOPE were twice as often diagnosed with placental villous infarcts compared with placentas with EOPE (EOPE – 31.3%, LOPE – 67.4%, $p < 0.005$) combined with obliterating angiopathy of the stem villous arteries. Premature detachment of typically situated placenta PDNSP (19.4%, $p = 0.04$) and intradecidual hemorrhages (9.68%, $p = 0.04$) were characteristic to EOPE placentas, inducing development of acute placental deficiency, while in LOPE, these disorders were not identified. Disorders of differentiation of the vascular-stromal component in villi were more frequently diagnosed in placentas with EOPE (EOPE-12.9%, LOPE-2.3%, $p = 0.04$). At EOPE, enlarged stromal canals, the peripheral orientation of vessels in the intermediate villi and hypoplasia of the muscular layer with deformed stem villi lumen were defined. In EOPE, placentas with increased deposition of fibrin and fibrinoid in areas of necrosis of the villous epithelium, in the basal lamina stroma, and intercellular areas peripheral cytotrophoblast (CTB) predominated. At the ultrastructural level, a twofold decrease in the number of mitochondria in the perinuclear zones and between the cisterns of the granular endoplasmic reticulum was observed in the cells of peripheral CTB of LOPE placentas compared with the group of EOPE placentas. However, the mean mitochondrial area of peripheral CTB was two times higher than that of EOPE ($p < 0.000$), reaching 3.91 ± 1.82 mcIU². Mitochondria of the peripheral STB in placentas at EOPE were not in contact with the granular endoplasmic reticulum (GER), and there was an increase in the cytoplasm not only in the number of loose lysosomes but also in the number of necrotized mitochondria in contact with amorphous structures.

The effect of pathological processes in the placenta is closely related to the severity of compensatory and adaptive reactions. Hyperplasia of acroteric villi (35.5%), capillaries in terminal villi (41.9%), and syncytial capillary membranes were significantly more frequent ($p = 0.04$) in LOPE placentas. While comparing the placental pathomorphological changes with the intensity of compensation and adaptation processes, the chronic placental deficiency was diagnosed in 83.6% of cases with EOPE and 73.12% with LOPE.

Our study data are broadly consistent with the generally accepted characteristics of EOPE and LOPE [5, 9]. Thus, clinical data confirm the greater severity of the pathological process in EOPE, which is combined with more pronounced changes in B-lymphocyte differentiation and cytokine status in women with EOPE. Because this group had an increased rate of medical abortions, premature delivery, perinatal miscarriages and signs of intrauterine infection in the current pregnancy, it seems most likely that trophoblast invasion disorders, spiral artery remodeling, and development of initial placental deficiency in EOPE are due to initial hypoplasia of endometrium. Pathomorphological studies of the placenta indicate implantation disorders and pathological placentation with developing initial placental deficiency. Premature detachment of the normally located placenta and intradecidual hemorrhage are indicative of acute KD in EOPE at the implantation stage. Subsequently, this process turns into a chronic form, as evidenced by disorders of maturation of the villous chorion, trophoblast mitochondrial dysfunction, syncytiotrophoblast dystrophic and necrotic changes. Such events may contribute to the enhanced release of different sized syncytiotrophoblast microparticles (STBM) into the maternal bloodstream compared to uncomplicated pregnancy [4, 10]. B-lymphocytes are known to be able to bind and phagocytize STBM [11]. This early and prolonged activation of B-lymphocytes in EOPE could determine their enhanced differentiation into memory B-cells (CD19⁺CD27⁺IgD[±], B-mems). IL-9 could play an essential role in this process, and its level was elevated in EOPE and IL-5 and IL-13. In this combination, it should be noted that increased production of IL-9, IL-5, IL-13 by the Th17-cell population is noted in some pathological conditions [1]. Excessive Th17 activity underlies many immunoregulatory disorders in PE. Takatsuka S. et al. (2018) showed that IL-9R is selectively expressed on memory B-cells, which can produce IL-9 in a secondary response [12]. IL-9/IL-9R signal transduction initiates development of B-mems and promotes their differentiation into

plasma cells upon repeated exposure to antigen [12]. This agrees well with our data on the increased peripheral blood plasma cells (CD19⁺CD20⁺CD38⁺) in EOPE. Stimulation of B-lymphocytes by IL-5 could also have contributed to this. Experimental studies have proven that IL-5 is a critical cytokine for the homeostatic proliferation of B1-lymphocytes and their immunoglobulin production [7]. We found that both types of PE were characterized by elevated levels of B1-cells (CD20⁺CD5⁺). Most studies link their pathological effects in PE with the production of agonistic angiotensin II type 1 receptor autoantibody (AT1-AA) [6], anti-endothelial cell antibodies [15]. These antibodies induce signaling pathways leading to blood vessel constriction, secretion of anti-angiogenic factors, circulation of endothelial microparticles, etc. [2, 3, 14]. Autoimmunity involving such antibodies affect the vascular endothelium in target organs, including the placenta. According to our data, the level of B1-cells ultimately increased in woman peripheral blood with LOPE. Perhaps the dystrophic and necrotic changes noted in the placenta in LOPE

syncytiotrophoblast of villi with increased deposition of fibrin and fibrinoid, with a reduced volume of intervillous space and the development of placental hypoperfusion reflect these autoimmune reactions. Data from pathomorphological studies suggest that in LOPE, development of fetoplacental insufficiency is defined by chronic disorders of maternal hemocirculatory tract combined with obliteration angiopathy of the stem villous arteries, increased fibrin and fibrinoid deposition in the basal bed, in the peripheral cytotrophoblast and areas of villous epithelial necrosis. The formation of compensatory processes in LOPE ensures the prolongation of pregnancy with the appearance of clinical signs of placental deficiency only at the phase of late fetalization of the placenta.

The study showed that the effect of clinical history factors determines the timing of PE manifestation, disorders in development of various placental structures, and B-lymphocyte immune reactions closely interrelated.

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РОЛЬ КЛЕТОЧНОГО ИММУНИТЕТА В РАННИЙ ПОСЛЕРОДОВЫЙ ПЕРИОД

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Резюме. Функционирование секреторных органов тесно связано с деятельностью иммунной системы. Как хорошо известно, это участие проявляется в том, что на определенных стадиях активности, мигрирующие в орган лимфоидные клетки, могут включаться в процесс регуляции секреторного образования. Кроме того, продукты деятельности иммунной системы и даже ее клеточные элементы могут становиться составляющими ряда секретов. Молозиво и молоко содержит большое количество клеток широкого спектра (до 1/3 объема), из них численность лимфоцитов составляет до 16% от лейкоцитов. Лимфоциты в иммунологической активной форме, попадая с молозивом в организм новорожденного, активизируют систему клеточного иммунитета. Некоторую роль в этом процессе играет транспорт медиаторов лимфокинов. Микрофаги, Т- и В-лимфоциты, проникая по межклеточным пространствам в лимфоидный слой кишечника передают иммунорецепторы пролимфоцитам новорожденного, «вооружая» их активностью к распознаванию генетически чужеродного. Лимфоциты, содержащиеся в молозиве, являются клетками иммунной системы, обеспечивающими клеточный и гуморальный иммунитет. Они представлены в большей степени Т-клетками, В-клетками и клетками киллерами. Т-клетки молока вырабатывают весь спектр иммунорегуляторных белков, таких как интерферон, фактор некроза опухоли-альфа. Эти клетки являются клетками иммунной памяти. Новорожденные, получившие первую порцию молозива не позже, чем через час после рождения, отличаются повышенной численностью лейкоцитов, более выраженными фагоцитозом, что свидетельствует о стимуляции гемо- и лимфоцитоза. При проведении просвечивающей и сканирующей электронной микроскопии в эпителиальном слое кишечника обнаружены клеточные элементы, которые попали туда из просвета кишечника. На микросрезах видно, как клетки лимфоидной природы, раздвигая структуры эпителиального пласта, минуют естественные барьеры и сохраняют при этом свою физиологическую полноценность. Возможность проникновения иммунокомпетентных клеток молозива матери в кровотоки детенышей доказывается при использовании естественной метки клеток самки — полового хроматина. Естественно, что меченные по половому хроматину клетки искали у новорожденных мужского пола. Обнаружение молозивных клеток в кишечной стенке и кровеносном русле детеныша примерно составляет 25% в крови, 1% в лимфе и около 70% в кишечнике. Несомненно, что лейкоциты молозива имеют исключительное значение в создании иммунитета у новорожденных животных.

Ключевые слова: лимфоциты, молоко, молозиво, иммунологическая память, X-хромосома, половой хроматин

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A ROLE FOR CELLULAR IMMUNITY IN EARLY POSTPARTUM PERIOD

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Abstract. The functioning of the secretory organs is closely related to the activity of the immune system. As is well known, this participation is manifested in the fact that at certain stages of activity, the lymphoid cells migrating to the organ can be involved in the regulation of secretion. In addition, the products of the immune system and even its cellular elements can become components of a number of secrets. Colostrum and milk contain a large number of cells of a wide spectrum (up to 1/3 of the volume), of which the number of lymphocytes is up to 16% of leukocytes. Lymphocytes, in an immunologically active form, entering the newborn's body with colostrum, activate the cellular immunity system. The transport of lymphokinin mediators plays a certain role in this process. Macrophages, T- and B-lymphocytes, penetrating through the intercellular spaces into the lymphoid layer of the intestine, transmit immunoreceptors to the prolymphocytes of the newborn, "armed" with their activity to recognize genetically foreign ones. The lymphocytes contained in colostrum are the cells of the immune system that provide cellular and humoral immunity. They are mainly represented by T-cells, B-cells and killer cells. Milk T-cells produce a full spectrum of immune regulatory proteins such as interferon, tumor necrosis factor alpha. These cells are the cells of the immune memory. Newborns who received the first portion of colostrum no later than an hour after birth are characterized by an increased number of leukocytes, more pronounced phagocytosis, which indicates the stimulation of hemo- and lymphocytosis. When carrying out transmission and scanning electron microscopy in the epithelial layer of the intestine, cellular elements were found that got there from the intestinal lumen. Microsections show how cells of a lymphoid nature, pushing apart the structures of the epithelial layer, bypass natural barriers and, at the same time, retain their physiological usefulness. The possibility of penetration of immunocompetent cells of the mother's colostrum into the bloodstream of the young is proved using the natural label of the female's cells – sex chromatin. Naturally, sex chromatin-labeled cells were sought in male newborns. The detection of colostrum cells in the intestinal wall and bloodstream of the young is approximately 25% in the blood, 1% in the lymph, and about 70% in the intestine. There is no doubt that the leukocytes of colostrum are of exceptional importance in creating immunity in newborn animals.

Keywords: lymphocytes, milk, colostrum, immunological memory, X chromosome, sex chromatin

Introduction

Functioning of the body secretory organs is closely related to activity of immune system well recognized to be exerted in involvement of migratory lymphoid cells in controlling secretion. Moreover, immune system cues and even its cellular components may become a part of certain secretions. For instance, colostrum contains substantial amount of immunoglobulins ensuring passive neonatal immunization. Furthermore, breast milk continuously contains leukocytes (neutrophils) and lymphocytes, which amount may markedly rise upon physiological organ-wide reactions. Of note is also to describe a leukocyte infiltration into reproductive organ tissues occurring during certain alterations of hormone status. It is known that in the chain of reactions a final effect against intruding foreign protein (antigen) may not be accomplished by a single cell type, but requires sequential or simultaneous interaction between diverse cells. This phenomenon formed the basis for describing a three-cell paradigm of immunogenesis. Quantitative and qualitative characteristics of immune cells upon a cooperative response are exhibited during

immune reaction. In connection with this, it is worth noting to analyze structural modules underlying lymphoid organ functioning. Stromal components (epithelial cells or fibroblasts) become connected with macrophages bearing certain antigens. Motile immune cells bearing antigen-specific receptors (T- and B-cells) make contacts to stromal components. Hence, both stromal components and motile cells exert strict immunobiological specificity collectively comprising a distinct level of structural organization. It is worth mentioning that functioning of secretory organs becomes involved in activity of immune system. At some stage, structural components of secretory organs may consist of lymphoid cells and create novel structural assemblies [2, 3]. Breast colostrum and milk contain a great amount of broad spectrum cells. The latter consist of epithelial cells, lymphocytes, lactocytes, myoepithelial cells, macrophages, and neutrophils. Major cellular components in colostrum and subsequent breast milk are presented by macrophages, neutrophils and lymphocytes. Neutrophilic leukocytes function in body defense against fungal and bacterial infections. Amount of

such cells is specifically elevated in colostrum period (up to 50% total composition), whereas in mature breast milk it declines (down to 5% total cell count). Lymphocytes in the breast milk and colostrum being the major cell type of immune system execute cellular and humoral immunity. Mainly, they are presented by T-, B- and NK-cells. Breast milk T-cells produce a whole range of immunoregulatory proteins such as interferons, interleukins, tumor necrosis factor- α . Such cells display immunological memory able to account for maternal immunocompetence [1, 11]. While preparing to lactation, lumen of mammary alveoli becomes filled up with leukocytes, which are removed from the mammary gland with the first portions of secreted colostrum entering neonatal offspring digestive tract. Leukocytes comprise up to one the third of the colostrum volume. Upon entrance, active immune colostrum lymphocytes activate neonate cellular immunity. Upon that, some role may be played by lymphokine trafficking [4]. Being in the digestive tract, maternal cells undergo no degradation, as they might be found in the intestinal mucosal smears by electron microimaging. Microphages, T- and B-cells enter the offspring gut lymphoid tissues via intercellular spaces and hand on antigen-specific immune receptors to neonatal prolymphocytes to arm with potential to recognize foreign non-self substances. Neonatal prolymphocytes after acquiring such biochemical cues and undergoing a multi-step life cycle convert into offspring T- and B-cells. However, some time is required for completing their full maturation and accumulation [9]. Not later than an hour after birth, neonates receiving a first portion of colostrum are featured by elevated blood leukocyte count, marked granulocyte phagocytosis against diverse bacteria evidencing about stimulated hemo- and lymphopoiesis.

Such cells are found in the neonatal circulation. In particular, neonatal male calves contained maternal sex chromatin-marked leukocytes. Sex chromatin (Barr body) is bound to X-chromosome being normally observed in somatic cells solely in female subjects [8, 10]. It raised a question about presence of durable maternally-transferred cellular immunity in neonates. Hence, it posed a need to study maternal immune cells particularly leukocytes exerting resistance to degradation in the neonate digestive tract and able to penetrate across intestinal walls and enter circulation to provide long-term protection as well as contribute to establishing host own immunity.

Objectives and Tasks

Our study was aimed at revealing immune cell types in breast colostrum and neonatal gut lumen as well as their penetration across intestinal mucosa and detection in peripheral blood and bone marrow after colostrum feeding. The task of the study was to examine cell composition in immune organs of mouse pups after colostrum feeding. For this, there were investigated mouse organs playing a crucial role in whole body immune reactions: red bone marrow

and Peyer's patches. In addition, peripheral blood was also collected for analysis.

Materials and methods

The study was conducted with two-day-old inbred mouse male pups after colostrum feeding. Mice were reared at standard conditions in animal facility. All procedures were performed in accordance with requirements for humane use of laboratory animals. Mouse Peyer's patches are found in the ileum as clustered elongated spots of lymphoid tissue. Tissue smears were obtained by attaching dissected Peyer's patches to water-washed and ethanol-ether-defatted glass slide. Red bone marrow was collected as follows: femoral bone was isolated from adjacent muscles and cracked in the middle to collect small portion of bone marrow by tapping onto a glass slide. Peripheral blood was sampled by cutting off tail end. Bone marrow, Peyer's patch and peripheral blood smears were stained according to the Pappenheim method using ready-to-use May-Grunwald solution and liquid Romanowsky–Giemsa dye. Next, tissue smears were air dried and put into sample cell containing May-Grunwald solution for 3 min. After that, smears were washed out with distilled water and put into sample cells containing dissolved Romanowsky–Giemsa dye for 20 min. After staining, smears were washed out with diluted water and air-dried. Sample imaging was performed by using immersion optical microscopy (objective $\times 100$). Mouse pup peripheral blood samples were air dried and stained with Romanowsky–Giemsa dye: work solution was prepared *ex tempore* based on taking 1 dye drop per 1 ml distilled water. Staining of samples was performed for 20 min which were subsequently washed out with distilled water and air dried. Sample imaging was performed by using immersion optical microscopy (objective $\times 100$).

Sample ultrastructure was analyzed by using fragments of certain regions of the digestive tract straightened on template and fixed with 1.5% glutaraldehyde in 0.1M cacodylate buffer. Transmission electron microscopy was performed after samples were postfixed in 1.5% osmium tetroxide and contrasted by Reynolds method followed by imaging on Hitachi-H-300 microscope. Cells located about at the middle of gastric and intestinal folds were analyzed. However, it is worth noting that no prominent morphofunctional changes were observed while comparing cells at various levels in tissue folds.

Scanning electron microscopy was performed by using samples fixed in glutaraldehyde, dehydrated and underwent CO₂ critical point drying in HCP-2 Hitachi device. Samples underwent gold sputtering followed by imaging in Hitachi-H-300 scanning microscope.

Results and discussion

While conducting transmission electron microscopy of the intestinal epithelial layer, it was found that it contained cellular defense components,

which penetrated from the gut lumen. In particular, lymphoid lineage cells by pushing apart structures in the epithelial layer moved across its apical and basolateral membranes thereby bypassing natural physiological and anatomical barriers while preserving own morphological integrity (Figure 1).

Ultrathin sections of mouse pup intestine revealed structurally diverse immune cells. In particular, it was shown that lymphocytes exerted heterogeneous size and ultrastructure. It may be accounted for by varying maturation degree, life span as well as diverse functions and antigen-specificity. Most of circulating lymphocytes are featured with a large nucleus, high nucleoplasmic ratio, and underrepresented cytosolic organelles. Cells structurally resembling small lymphocytes were most common in the blood stream.

While examining small mouse pup intestinal microsections by using electron microscopy it was found that they contained cells corresponding to small dark lymphocytes. Such cells detected by us had round, sometimes notched, nucleus with heterochromatin presented as large clumps. Sometimes, heterochromatin was distributed homogeneously across entire nucleic region. The cell nucleus is located at the center being surrounded by a thin rim of fine-grained light cytosol as well as a nucleolus or its fragments (Figure 2). Nuclear membrane is clearly observed, with a dense chromatin located nearby. The cells contain small amount of cytosol and organelles, with few large spherical mitochondria and pinocytotic vesicles were found. Endoplasmic network is observed as small isolated sheets. In addition, cytosol also contained large amount of ribosomes.

In addition, intestinal ultrathin sections were shown to contain cells ultrastructurally resembling plasma cells, which are clearly differed from other immune cells. Mature plasma cells possess eccentric

round nucleus, with small ring-shaped nucleolus. Chromatin is located peripherally close to the nuclear membrane. Ribosomes are assembled into polysomes found as clustered rosettes. Mitochondria were of spheric shape. Golgi apparatus was large that surrounded centrosome and formed extended perinuclear structure. Extensively developed granular endoplasmic network was prominently observed inside cells (Figure 3).

In addition, we also found macrophages in the intestinal mucosa and submucosa. Macrophage beanlike or irregular nuclei were of average size and contained chromatin clumps, whereas cytosol contained large amount of lysosomes, phagosomes and pinocytotic vesicles. In contrast, amount of mitochondria, endoplasmic network and Golgi apparatus was decreased. While analyzing intestinal smears from newborn mouse pups by light microscopy, we found on the surface of intestinal mucosa and intestinal sections a great number of leukocyte lineage cells (lymphocytes and neutrophils) with preserved functional morphological structure.

Further investigation of intestinal microsections with electron microscopy allowed us also to visualize maternal immune cells, which attach as if pushing apart epithelial layer and penetrate into mouse pup intestinal wall while preserving own morphological integrity (Figure 4).

Ultrathin intestinal sections contain cells ultrastructurally resembling plasma cells bearing small eccentric nucleus and large Golgi apparatus. Clustered ribosomal rosettes are found in the cytosol almost entirely covered with well developed enlarged granular endoplasmic network. While examining mouse pup blood samples, it was found that leukocytes contained Barr bodies. Intensity of X-chromatin-specific chromatin staining was higher compared to



Figure 1. Transmission electron microscopy of the intestinal epithelial layer in newborn mouse pup after colostrum feeding. A maternal leukocyte penetrating across intestinal epithelial layer is visualized

Note. Magnification $\times 3,000$.

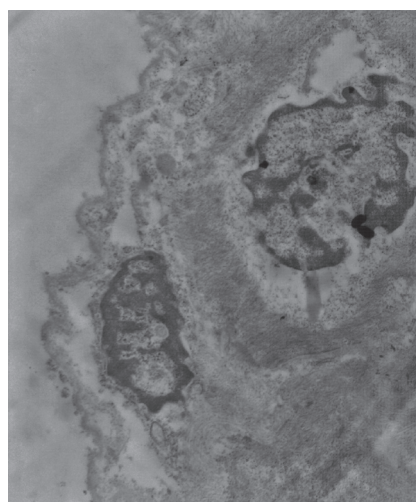


Figure 2. Transmission electron microscopy of the intestinal epithelial layer in newborn mouse pup. Maternal leukocytes penetrating at the site of intestinal serous membrane are depicted

Note. Magnification $\times 3,000$.

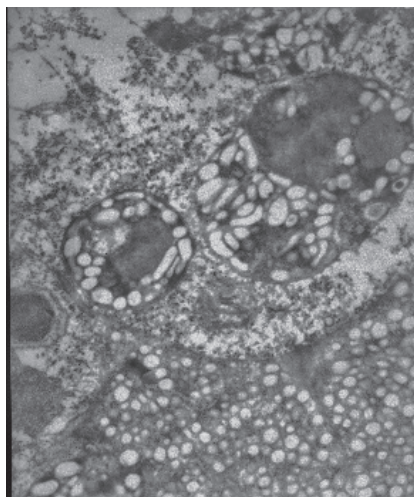


Figure 3. Transmission electron microscopy of plasma cells inside intestinal epithelial layer in newborn mouse pup
Note. Magnification $\times 3,000$.

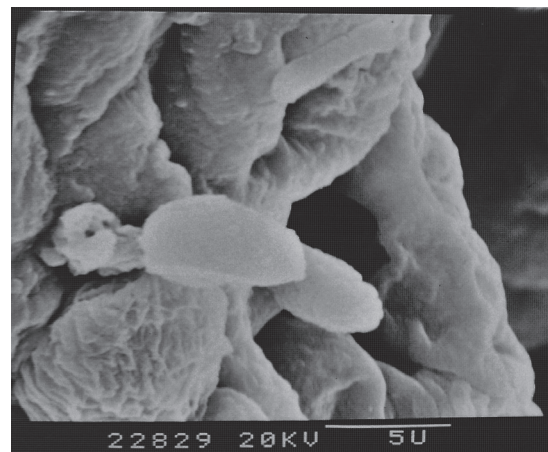


Figure 4. Scanning electron microscopy of intestinal surface. Maternal leukocytes penetrating into the intestinal epithelium are depicted
Note. Magnification $\times 3,000$. Scale – 5 μm .

other chromatin areas, whereas any form of clear-cut sex chromatin-containing bodies was identified.

While conducting morphology analysis of bone marrow leukocyte lineage cells and gut lymphoid assemblies, we found sex X-chromatin-bearing immune cells (Figure 5).

Thus, our study allowed to unravel that maternal leukocytes with colostrum intake entered newborn mouse pup intestine and were able not only to cross the gut epithelium and enter the circulation, but also traffick towards the central and peripheral lymphoid tissues and subsequently take part in formation of antigen-specific T and B-cells, interact with antigen-presenting cells by contributing to establishment of neonatal immune system in mouse pups and durable long-lasting immunity.

A potential for colostrum immune cells to enter newborn subject circulation is verified by detecting natural maternally-derived specific marker called sex chromatin. In particular, this technique is based on examining structural inclusion located inside cell nuclei – sex X-chromatin, which is lacked in male-derived cells. Hence, such approach was applied to identify sex chromatin-tagged leukocytes in male newborn subjects. Detecting maternal colostrum-derived cells in neonatal intestinal wall and circulation revealed that they comprised around 25, 1 and 70% total cell composition in the peripheral blood, lymph and gut, respectively. Undoubtedly, colostrum-derived leukocytes play a pivotal role in establishing local and systemic immunity in newborn animals. Previous evidence demonstrated that after colostrum feeding blood leukocyte count in animal offsprings becomes elevated primarily due to thymus-derived lymphocytes. Newborn lymphatic system undergoes certain reorganization to gain immune-related education. At least, it may be illustrated by a long established fact when granular leukocytes

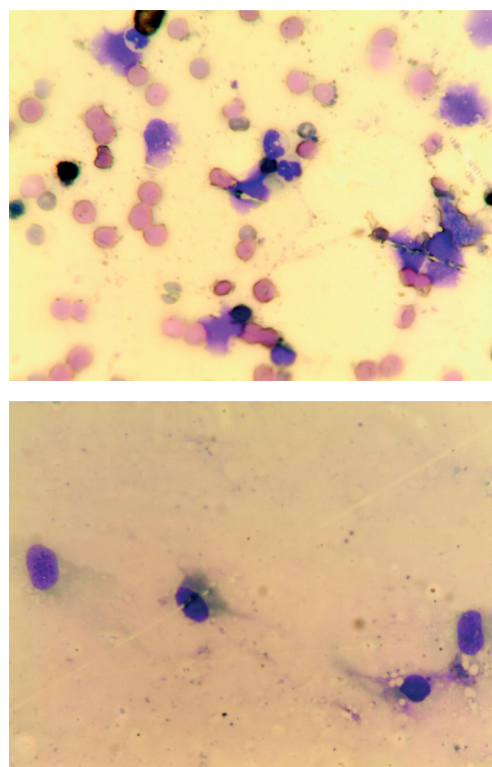


Figure 5. Barr bodies in the cells of red bone marrow smears from newborn pups

such as eosinophils and neutrophils emerged in digestive tract mucosa in late-gestation fetuses. Such cell types appeared earlier and became enriched in high numbers in the small intestine, especially in the ileum. Moreover, this anatomical region in late-gestation sheep and swine fetuses was found to contain unique clumpy leukocytes. Their generation begins with migration of leukocytes from mucosal connective tissue into the space between epithelial

cells at the villus base and crypts. Such lymphocytes lose basophilic properties in the protoplasm and start producing specific eosinophilic granules, which is paralleled with increased cell size. Moreover, it was shown that such granules lie free between epithelial cells. In addition, it also resulted in describing lymphocyte efflux from inter-epithelial space into mucosal connective tissue. However, plasma cells emerge in sheep and swine intestinal mucosa before birth, whereas in cattle they were detected postnatally, perhaps due to colostrum intake containing a marked immunoglobulin amount [12].

During lactation, maternal immune system is extremely oriented at defending and maintaining neonatal immune status within the first days of life. It is accomplished via immediate control over

neuroendocrine regulation. All nutrients and immunoglobulins necessary for body growth and development are provided to neonates via colostrum and breast milk. However, it should be taken into consideration that immunoglobulins as defense molecules enter colostrum only at the onset of lactation period. Later, their level and potential to cross gut epithelial barrier profoundly decline. However, efflux of maternal cells into colostrum and mature breast milk is sustained over entire lactation period, which we showed to be stimulated via oxytocin. Hence, the data of our studies allow to assume that maternal cellular defense components serve as constituents both in colostrum and breast milk immunity. As a result, a newborn subject becomes armed with durable and long-lasting immunity upon natural breastfeeding [5, 6, 7].

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РОЛЬ ЦИТОТОКСИЧЕСКИХ Т-ЛИМФОЦИТОВ В ПАТОГЕНЕЗЕ ПРЕЖДЕВРЕМЕННЫХ РОДОВ

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Резюме. В настоящее время выявлено существование широкого спектра субпопуляций CD8⁺Т-лимфоцитов, среди которых выделяют субпопуляции наивных и эффекторных клеток, а также клеток памяти. Считается, что CD8⁺Т-лимфоциты являются популяцией лимфоцитов, обладающей высокой цитотоксической активностью, что имеет чрезвычайное значение во время беременности. Учитывая, что каждая субпопуляция характеризуется совокупностью продуцируемых медиаторов, поверхностных и внутриклеточных маркеров, можно предположить их роль в патогенезе преждевременных родов. Это определило необходимость исследования роли наивных клеток, эффекторных клеток и клеток памяти в развитии спонтанных преждевременных родов. Данные о содержании наивных CD8⁺-лимфоцитов в периферической крови женщин с угрожающими преждевременными родами практически отсутствуют. Установлено, что инфильтрация CD8⁺-лимфоцитов в зоне маточно-плацентарного контакта была связана с развитием своевременных родов. Хронический хориоамнионит является наиболее распространенным состоянием при идиопатических преждевременных родах и характеризуется инфильтрацией материнских CD8⁺Т-клеток в хориоамниотические мембраны. В настоящее время считается, что хронические воспалительные поражения плаценты представляют собой материнское антифетальное отторжение. Это послужило поводом для исследования роли этих клеток в патогенезе преждевременных родов.

Цель — установить возможный патогенетический механизм преждевременных родов у женщин с угрожающими преждевременными родами на основании выявленных особенностей дифференцировки и функциональной активности CD8⁺-лимфоцитов на системном уровне.

Обследование женщин производилось на базе Федерального государственного бюджетного учреждения «Ивановский научно-исследовательский институт материнства и детства имени В.Н. Городкова» Министерства здравоохранения России. Всего было обследовано 126 женщин, которые ретроспективно были подразделены на 2 основные группы — женщины с угрожающими преждевременными родами (n = 68), которые была подразделена на 2 подгруппы — с исходом беременности преждевременные роды (n = 30) и своевременные роды (n = 38). В группу контроля вошли 58 женщин с неосложненным течением беременности и родившие своевременно. В популяции CD8⁺-лимфоцитов определяли содержание центральных — Tcm (CD45RA⁻CD62L⁺), претерминально-дифференциро-

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ванных — Tem (CD45RA⁻CD62L⁻) и терминально-дифференцированных — Temra (CD45RA⁺CD62L⁻) клеток памяти. Во всех популяциях клеток памяти определяли содержание клеток, внутриклеточно продуцирующих Granzyme B. Исследования проводили с помощью моноклональных антител (мАТ) методом проточной цитофлюориметрии на цитометре FACSCanto II с использованием программного обеспечения FACSDiva (Becton Dickinson, США).

Проведен анализ особенностей относительного содержания CD8⁺-лимфоцитов в основной группе женщин в зависимости от исхода беременности. При сравнении пациенток, имеющих клинику угрожающих преждевременных родов, беременность которых завершилась преждевременно, выявлено более высокое содержание CD8⁺-лимфоцитов, чем в группе женщин, родивших своевременно, что свидетельствует о высокой стимуляции цитотоксических Т-лимфоцитов у этой группы женщин. При угрожающих преждевременных родах происходит повышение содержания в периферической крови наивных CD8⁺-лимфоцитов. Данные о содержании наивных CD8⁺-лимфоцитов в периферической крови женщин с угрожающими преждевременными родами практически отсутствуют. Повышение уровня CD8⁺Tn более выражено в подгруппе женщин с благоприятным исходом беременности. Учитывая этот факт, можно предположить, что у женщин с развившимися преждевременными родами более низкий CD8⁺ Tn связан с их усиленной дифференцировкой в эффекторные Т-лимфоциты, с последующей их миграцией в зону плацентации. Этот процесс мог определять отмеченное нами снижение уровня терминально-дифференцированных гранзим-продуцирующих CD8⁺-лимфоцитов в подгруппе женщин с исходом беременности преждевременные роды, что совпало с литературными данными.

Ключевые слова: беременность, преждевременные роды, Т-лимфоциты

ROLE OF CYTOTOXIC T-LYMPHOCYTES IN THE PATHOGENESIS OF PRETERM BIRTH

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Abstract. Currently, the existence of a wide range of subpopulations of CD8⁺T-lymphocytes has been revealed, among which there are subpopulations of naive and effector cells, as well as memory cells. CD8⁺T-lymphocytes are thought to be a population of lymphocytes with high cytotoxic activity, which is of extreme importance during pregnancy. Given that each subpopulation is characterized by a set of produced mediators, surface and intracellular markers, we can assume their role in the pathogenesis of preterm birth. This determined the need to investigate the role of naive cells, effector cells, and memory cells in the development of spontaneous preterm birth. Data on the content of naive CD8⁺-lymphocytes in the peripheral blood of women with threatened preterm birth are practically absent. It was found that the infiltration of CD8⁺-lymphocytes in the area of utero-placental contact was associated with the development of timely delivery. Chronic chorioamnionitis is the most common condition in idiopathic preterm birth and is characterized by the infiltration of maternal CD8⁺T-cells into the chorioamniotic membranes. Currently, it is believed that chronic inflammatory lesions of the placenta represent maternal antifetal rejection. This led to the study of the role of these cells in the pathogenesis of preterm birth. Purpose. To establish a possible pathogenetic mechanism of preterm birth in women with threatened preterm birth on the basis of the revealed features of differentiation and functional activity of CD8⁺-lymphocytes at the systemic level

Materials and methods. The survey of women was carried out on the basis of the Federal State Budgetary Institution “V. Gorodkov Ivanovo Research Institute of Maternity and Childhood” of the Ministry of Health of the Russian Federation. A total of 126 women were examined, which were retrospectively divided into 2 main groups — women with threatened preterm birth (n = 68), which was divided into 2 subgroups — with the outcome of pregnancy preterm birth (n = 30) and timely delivery (n = 38). The control group included 58 women with uncomplicated pregnancy and who gave birth on time. In the CD8⁺-lymphocyte population, the content of central — Tcm (CD45RA⁻CD62L⁺), preterminally differentiated-Tem (CD45RA⁻CD62L⁻) and terminally differentiated-Temra (CD45RA⁺CD62L⁻) memory cells was determined. In all memory cell

populations, the content of cells producing Granzyme B intracellularly was determined. The studies were performed using monoclonal antibodies (mAT) by flow cytometry on a FACSCanto II cytometer using the FACSDiva software (Becton Dickinson, USA).

The analysis of the features of the relative content of CD8⁺-lymphocytes in the main group of women, depending on the outcome of pregnancy, was carried out. When comparing patients with a clinic of threatened preterm birth, whose pregnancy ended prematurely, a higher content of CD8⁺-lymphocytes was revealed than in group c of women who gave birth in a timely manner, which indicates a high stimulation of cytotoxic T-lymphocytes in this group of women. With threatening preterm birth, there is an increase in the content of naive CD8⁺-lymphocytes in the peripheral blood. Data on the content of naive CD8⁺-lymphocytes in the peripheral blood of women with threatened preterm birth are practically absent. The increase in CD8⁺Tn levels is more pronounced in the subgroup of women with a favorable pregnancy outcome. Given this fact, it can be assumed that in women with preterm birth, a lower CD8⁺Tn is associated with their increased differentiation into effector T-lymphocytes with their subsequent migration to the placental zone. This process could determine the observed decrease in the level of terminally differentiated granzyme-producing CD8⁺-lymphocytes in a subgroup of women with a pregnancy outcome of preterm birth, which coincided with the literature data.

Keywords: pregnancy, preterm birth, T-lymphocytes

Introduction

Preterm birth is of great interest to researchers around the world, as its pathogenesis remains unexplored. Since pregnancy is a process covering long-lasting development of semi-allogeneic fetus in the uterus, a unique immunological situation is established in the “mother-fetus” interface. It is well known that the placenta is not an absolute barrier to the body cells, and the results of numerous studies show that during pregnancy, fragments of chorionic villi and fetal antigens enter maternal peripheral bloodstream. This is not accompanied by a pathological response from maternal immune system, since its emerging immune adaptation at the “mother-fetus” system allows semi-allogeneic fetus avoid rejection. Of all the suspected causes associated with spontaneous preterm birth, one of the most fundamental is pathological inflammation. Most immunological studies have focused on the role of innate immunity in the mechanisms leading to preterm birth. Indeed, the stimulation of neutrophils and macrophages by administered endotoxin or the activation of invariant natural killer T-cells by alpha-galactosylceramide causes premature birth [1, 6]. However, pathological inflammation can also be mediated by T-cells, a cellular component of the adaptive immune system. In some studies, it was found that T-cell infiltration in the area of utero-placental contact (i.e., the decidual membrane) was associated with the development of timely delivery [10]. Chronic chorioamnionitis most commonly results in idiopathic preterm birth being characterized by the infiltration of maternal CD8⁺T-cells into chorioamniotic membranes. Emerging lesion is often accompanied by aseptic inflammation associated with the infiltration of the placental basal layer by lymphocytes or plasma cells [8]. It is currently believed that chronic inflammatory lesions in the placenta represent maternal anti-fetal rejection reaction. Since the adaptation of maternal immune system is involved in the processes that ensure suc-

cessful course of pregnancy, the cause of premature birth can be coupled to impaired immunological tolerance from the very beginning of pregnancy, starting with disturbed embryo implantation. Early immune disorders can lead to impaired maternal immunological tolerance and, as a result, to excessive inflammation [4]. This led to the study a role of such cells in the pathogenesis of preterm birth, and **the aim was** to uncover potential pathogenetic mechanism of preterm birth in women with threatened preterm birth on the basis of the identified features of CD8⁺-lymphocyte differentiation and functional activity at the systemic level.

Materials and methods

The females enrolled to the study were examined at the Federal State Budgetary Institution “V. Gorodkov Ivanovo Research Institute of Maternity and Childhood” of the Ministry of Health of the Russian Federation. A total of 126 women were examined, which were retrospectively divided into 2 main groups – women with threatened preterm birth (n = 68), which was further divided into 2 subgroups – females with differed outcome of pregnancy such as preterm birth (n = 30) and timely delivery (n = 38). The control group included 58 women with uncomplicated pregnancy, whose anamnesis was not burdened by the presence of premature birth, habitual miscarriage, late spontaneous miscarriages, finished with timely delivery. The exclusion criteria were: multiple pregnancy, severe extragenital pathology, acute/exacerbation of chronic infection and acute allergic reactions at the time of examination, pre-eclampsia/eclampsia, prenatal amniotic fluid leakage, placental abruption. The study material was presented by peripheral venous blood samples. In the CD8⁺-lymphocyte population, percentage of central – Tcm (CD45RA⁻CD62L⁺), preterminally differentiated-Tem (CD45RA⁺CD62L⁻) and terminally differentiated-Temra (CD45RA⁺CD62L⁻) me-

memory cells were determined. In all memory cell populations, percentage of perforin- and granzyme B-producing cells was determined. The studies were performed using monoclonal antibodies (mAT) for flow cytometry on a "FACSCanto II" cytometer using the "FACSDiva" software ("Becton Dickinson", USA). Monoclonal antibodies anti-CD8, anti-CD45RA, anti-CD62L, anti-Perforine, anti-GranzymeB (eBioscience) were used. Cell staining and fixation were carried out by routine protocol in accordance with the manufacturer's instructions. Percentage of cells producing intracellular Granzyme B in the CD8⁺-lymphocyte population was evaluated by using the intracellular cell staining procedure with the commercial kit "FIX & PERM cell permeabilization reagents" (Invitrogen, USA) for permeabilization of the cell membrane. Statistical and mathematical data processing was carried out by using the package of licensed programs "Microsoft Office 2013", "Statistica for Windows 12.0, StatSoftInc.", "MedCalc v7.4.4.1". The normality test of the sample data was determined using the Shapiro-Wilk and Kolmogorov-Smirnov criteria. Here, data distribution in the studied groups differed from the normal distribution (Gauss). To assess significance of differences in the compared values between the study groups, the Mann-Whitney U-test and the Fisher exact two-tailed two-point test were used (at $p < 0.05$, the differences were considered significant). The data are presented as Me – median, indicating 25% ($Q_{0.25}$) and 75% ($Q_{0.75}$) quartile, indicating the number of studies performed in the group (n).

Results and discussion

The analysis of the obtained data showed that percentage of CD8⁺-lymphocytes in patients with threatened preterm birth was higher than in the control group ($p = 0.000$). We also assessed percentage of CD8⁺-lymphocytes in the main group depending on the outcome of pregnancy. While comparing patients with a clinical picture of threatened preterm birth resulting in preterm vs. term delivery, a higher level of CD8⁺-lymphocytes was revealed (33.5 (28.5–37.7) %;

26.8 (23.1–29.7) %, $p = 0.033$). Distribution of naive CD45RA⁺CD62L⁺ (TN)-cells, central (TCM) CD45RA⁺CD62L⁺-cells, preterminally differentiated (TEM) CD45RA⁺CD62L⁺-cells, and terminally differentiated (TEMRA) CD45RA⁺CD62L⁺-cells among peripheral blood cytotoxic T-lymphocytes are shown in Table 1.

In the group of women with threatened preterm birth, a high level of naive cells in the CD8⁺-lymphocytes was revealed, compared with pregnant women in the control group ($p = 0.000$). However, no differences between these groups in percentage of central, preterminal and terminal differentiated cells were found ($p > 0.05$). In the subgroup of women with threatened preterm birth and a favorable pregnancy outcome, an increased percentage of Tn CD8⁺-lymphocytes was observed compared to control group ($p = 0.022$). A comparison of TCM, TEM, and TEMRA levels in the groups of women with threatened preterm birth revealed no significant differences depending on the outcome of pregnancy ($p > 0.05$). Significant differences in percentage of central, preterminal and terminal differentiated cells in the subgroups of women with the outcome of pregnancy-premature and timely delivery were not revealed. ($p > 0.05$). We also analyzed the features of intracellular Granzyme B production in CD8⁺-lymphocytes in the studied groups. There were found no significant differences in percentage of Granzyme B-producing cells in all CD8⁺ memory cell subsets in control and main group of women with threatened preterm birth ($p > 0.05$). A comparatively analyzed percentage of Granzyme B-producing CD8⁺TCM, CD8⁺TEM, and CD8⁺TEMRA-cells in a subgroup of women with pregnancy resulting in term delivery and control revealed no significant differences ($p > 0.05$ in all cases). Women with a preterm pregnancy had decreased percentage of GranzymeB-producing terminal differentiated cells, compared with women having term delivery, as well as compared with the control group ($p = 0.01$ and $p = 0.001$, respectively). No significant differences in percentage of Granzyme B-producing CD8⁺TCM and CD8⁺TEM between subgroups with the outcome of preterm pregnancy

TABLE 1. CONTENT OF NAIVE CELLS AND CELLS IN THE POPULATION OF CD8⁺-LYMPHOCYTES IN THE PERIPHERAL BLOOD IN WOMEN WITH UNCOMPLICATED PREGNANCY AND WITH THREATENED PRETERM BIRTH

Indicator, %	Threatened preterm birth n = 68	Threatened preterm birth		Control group n = 58
		Preterm birth n = 30	Timely delivery n = 38	
T _N	41.35 (32.7–51.00) $p = 0.001$	37 (29.3–48.4)	40.7 (33.3–46.4) $p = 0.022$	32.9 (28.5–41.1)
T _{CM}	11.1 (8.14–15.50)	11.1 (9.0–13.5)	10.1 (7.9–14.2)	11.9 (8.7–15.3)
T _{EM}	17.4 (30.0–30.8)	22.8 (16.9–32.1)	25.9 (19.15–30.30)	25.6 (19.9–34.2)
T _{EMRA}	19 (13.4–29.6)	18.5 (14.3–30.1)	24 (16.6–30.6)	22.4 (14.6–29.6)

Note. P, the statistical significance of the differences is indicated in comparison with the indicators of the control group.

and term delivery ($p > 0.05$ in all cases). An important mechanism of immunoregulation during pregnancy is the activation of T-lymphocytes altering modality of their differentiation and functional activity [13, 14]. In most studies, increased functional activity highlighted by excessive production of granzyme B molecules in the population of cytotoxic T-lymphocytes is associated with early spontaneous miscarriage, preeclampsia, and fetal growth retardation [4]. It is known that contact with an antigen triggers a cascade of reactions promoting the differentiation of naive lymphocytes into effector cells and memory cells. Naive T-cells (TN) exert low cytotoxic activity and circulate in the maternal body seeking out for antigens, detection of which elicits T-cell differentiation into effector cells migrating to inflammatory or alloimmune tissues [3]. A number of studies revealed that effector T-cells are present in the area of utero-placental contact (decidual membrane). It is proved that they are involved in the development of term delivery in physiologically occurring pregnancy [10, 13]. Virtually no data on percentage of naive $CD8^+$ -lymphocytes in the peripheral blood of women with threatened preterm birth are available. This accounted for the need to investigate a role of naive cells, effector cells, and memory cells in the development of spontaneous preterm birth. According to our study, threatened preterm birth is coupled to increased percentage of naive $CD8^+$ -lymphocytes in the peripheral blood. Due to the fact that partial thymus involution as a natural process occurs during pregnancy, we are unable to unambiguously conclude about the nature of differentiation of naive $CD8^+$ -lymphocytes in threatened preterm labor (thymic/extrathymic). The most likely factors stimulating the differentiation of naive cytotoxic T-lymphocytes are the expression of specific HLA antigens, as well as the particles of cytotrophoblast cells entering the maternal bloodstream in early pregnancy [4]. Thus, recent studies have shown that fetal T-cells are activated in different subgroups of preterm labor and can cause various inflammatory reactions in the amniotic cavity, leading to preterm birth [5]. The

level of such particles increases with all complications of the gestational process. The increase in $CD8^+Tn$ levels is more pronounced in the subgroup of women with a favorable pregnancy outcome. Given that, it can be assumed that in women with preterm birth, a lower $CD8^+TN$ is associated with their increased differentiation into effector T-lymphocytes and their subsequent migration to the placental zone. This process could determine the observed decrease in the level of terminally differentiated granzyme-producing $CD8^+$ -lymphocytes in a subgroup of women with a preterm birth. In some studies, it has been shown that percentage of effector $CD8^+$ memory cells increase in the decidual membrane during spontaneous preterm birth [7]. Few data on the mechanisms of migration of $CD8^+T$ -lymphocytes to the placenta are available. However, several specific molecules can be identified, the expression of which can contribute to the selective migration of cytotoxic lymphocytes into the placenta and amniotic fluid. According to the publications, decidual stromal cells express CXCR3 ligands, produce CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC), and $CD8^+$ -cells are characterized by increased expression of CXCR3 and CCR5 in threatened preterm labor [11]. $CD8^+T$ -cell migration may be facilitated by the expression of CXCL9 in the placenta and in the chorioamniotic membrane [9]. Increased production of this chemokine in the placenta and fetal membranes was observed in preterm labor. In addition, preterm birth was associated with an increased content of CXCL11 in the amniotic fluid during the second trimester of pregnancy [2, 12].

Thus, in the peripheral blood of women with threatened preterm birth, there is an increased differentiation of naive $CD8^+$ -lymphocytes, which in the group of women with an unfavorable outcome of pregnancy such as preterm birth, is accompanied with decreased level of terminally differentiated cells with high cytotoxic potential. Perhaps, such changes result from excessive fetal antigen-cell stimulation and subsequent migration to the placenta and amniotic fluid, inducing premature birth.

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ОСОБЕННОСТИ КОЛИЧЕСТВЕННОЙ ЭКСПРЕССИИ РЕЦЕПТОРОВ CHECKPOINT-МОЛЕКУЛ PD-1 И TIM-3 НА CD4⁺ И CD8⁺ Т-КЛЕТКАХ ПРИ РАКЕ МОЛОЧНОЙ ЖЕЛЕЗЫ РАЗНОЙ СТЕПЕНИ ПРОГРЕССИИ

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Резюме. Во время течения хронических вирусных инфекций или роста опухоли из-за постоянного присутствия антигена и воспаления наступает дисфункциональное состояние Т-клеток, называемое истощением (exhaustion). К факторам, связанным с истощением Т-клеток, относят повышение экспрессии различных ингибиторных рецепторов, известных также как checkpoint-молекулы, которое приводит к подавлению пролиферации и продукции таких медиаторов, как IL-2, IFN γ и TNF α .

Рецептор TIM-3 представляет собой иммунорегуляторный рецептор, открытый в 2002 г., который экспрессируется на различных иммунных клетках, включая дендритные клетки, макрофаги и Т-клетки, и опосредует супрессивную активность на иммунных клетках. Устойчивая экспрессия рецептора PD-1 на Т-лимфоцитах также ассоциирована с фенотипом истощения, при этом остается неясным, как экспрессия данных ингибиторных рецепторов в норме отличается от таковой в патологических состояниях организма, для которых характерно увеличения числа истощенных Т-клеток.

Целью настоящего исследования стало определение относительного и абсолютного количества Т-клеток, экспрессирующих PD-1 и TIM-3, а также количества молекул PD-1 и TIM-3 на поверхности CD4⁺ и CD8⁺ Т-клеток у здоровых людей и пациентов с раком молочной железы (РМЖ). Группа пациентов с РМЖ была условно разделена на две группы в зависимости от степени прогрессии заболевания на пациентов с первичным (отсутствие метастаз) и прогрессирующим РМЖ (наличие метастаз).

В результате исследования показано, что у больных раком молочной железы наблюдается увеличение абсолютного количества PD-1-позитивных CD4⁺ Т-клеток. Абсолютное количество молекул на клетку также выше у пациентов с РМЖ по сравнению со здоровыми донорами. Для больных показана тенденция к увеличению абсолютного количества TIM-3-позитивных CD4⁺ Т-клеток по сравнению со здоровыми донорами и в ряду от первичного заболевания к прогрессирующему РМЖ.

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Таким образом, различия в характере экспрессии checkpoint-молекул TIM-3 и PD-1 наблюдаются при сравнении нормы и злокачественной патологии молочной железы, и могут стать важным маркером функционального состояния Т-лимфоцитов у больных РМЖ.

Ключевые слова: PD-1, TIM-3, рак молочной железы, рецепторы checkpoint-молекул, истощенные Т-лимфоциты, количественная экспрессия рецепторов

QUANTITATIVE EXPRESSION FEATURES OF PD-1 AND TIM-3 CHECKPOINT MOLECULE RECEPTORS ON CD4⁺ AND CD8⁺ T-CELLS IN BREAST CANCER OF VARYING PROGRESSION DEGREES

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Abstract. During the course of chronic viral infections or tumor growth, due to the constant presence of antigen and inflammation, a dysfunctional state of T-cells called exhaustion occurs. Factors associated with T-cell exhaustion include an increase in the expression of various inhibitory receptors, also known as checkpoint molecules, which leads to inhibition of the proliferation and production of mediators such as IL-2, IFN γ , and TNF α .

The TIM-3 molecule is expressed on a variety of immune cells, including dendritic cells, macrophages, and T-cells, and mediates suppressive activity on immune cells. Sustained expression of the PD-1 receptor on T-lymphocytes is also associated with the exhaustion phenotype, while it remains unclear how the expression of these inhibitory receptors normally differs from that in pathological conditions of the body, which are characterized by an increase in the number of exhausted T-cells.

The aim of the study was to determine the relative and absolute number of T-cells expressing PD-1 and TIM-3, as well as the number of PD-1 and TIM-3 molecules on the surface of CD4⁺ and CD8⁺ T-cells in healthy donors and breast cancer (BC) patients. Group of BC patients were conditionally divided into two groups depending on the degree of disease progression into patients with primary (without metastases) and metastatic BC.

As a result of the study, it was shown that an increase in the absolute number of PD-1⁺CD4⁺ T-cells is observed in breast cancer patients. The absolute number of molecules per cell is also higher in BC patients compared to healthy donors. For patients, a tendency towards an increase in the absolute number of TIM-3⁺CD4⁺ T-cells was shown in comparison with healthy donors and in a row from primary disease to metastatic BC.

Thus, differences in the expression pattern of TIM-3 and PD-1 checkpoint molecules are observed when comparing the norm and malignant pathology of the breast, and can become an important marker of the functional state of T-lymphocytes in BC patients.

Keywords: PD-1, TIM-3, breast cancer, checkpoint molecule receptors, exhausted T-lymphocytes, quantitative expression of receptors

Introduction

Currently, an important role in the development of immune-mediated diseases, including malignant neoplasms, is assigned to suppressor populations of immunocompetent cells (Shou et al., 2016). There are many mechanisms for malignant tumor genera-

ted and maintained immunosuppressive state. Thus, a malignant neoplasm attracts as well as promotes activation and differentiation of immunocompetent cells with a regulatory – immunosuppressive phenotype in its microenvironment. In addition, tumor cells express a variety of membrane-bound and soluble molecules that alter the functional state of infiltrating

immune cells to act as tumor-supporting cells (Ager et al., 2021).

The TIM-3 receptor is an immunoregulatory receptor, discovered in 2002, that is expressed on a variety of immune cells, including dendritic cells, macrophages, and T-cells. TIM-3 mediates suppressive activity on immune cells through its ligands, including phosphatidylserine, CEACAM-1, and galectin-9 ligand (Li et al., 2021). The signaling triggered by it on cytotoxic T-cells leads to the development of an exhaustion phenotype. The programmed cell death protein 1 (PD-1) is also expressed on the surface of activated T-cells and regulates the immune response at the effector stage. PD-1 plays an important role in the balance of protective immunity and immunopathology, homeostasis and tolerance; however, in case of tumor growth, immunosuppression becomes the main PD-1 activity. The effect that PD-1 and TIM-3 molecules have on cells depend on many parameters, such as the level of soluble mediators, the percentage of cells carrying receptors, the ratio between the percentage and density subpopulations, and intracellular proteins of the target cell. Currently, close attention of both fundamental science and clinical immunology have been paid to such an important factor for the regulation of the biological properties of immunoregulatory proteins such as the expression density of surface receptors (Sennikov et al., 2019). For an informative assessment of the expression level, it is necessary to accurately count the number of binding sites on the cell surface.

Thus, it can be assumed that impaired cell functional activity resulting from increased expression of PD-1 and TIM-3 may be associated not only with increased number of positive cells, but also with the number of cognate receptors on the cell surface, so that if a certain level of receptors is exceeded, then cell loses the ability to execute effector functions.

The aim of the study was to determine the relative and absolute number of T-cells expressing PD-1 and TIM-3, as well as the number of PD-1 and TIM-3 molecules on the surface of normal and breast cancer CD4⁺ and CD8⁺ T-cells. Assessing the number of PD-1 and TIM-3 molecules will help to determine the threshold level of exhaustion for cell functional properties and impairment of cytotoxic functions in patients with breast cancer (BC).

Materials and methods

Donors and patients

Peripheral venous blood cells were collected from 25 patients with primary breast cancer, 15 patients with metastatic breast cancer (presence of metastases), as well as healthy women without clinically and instrumentally diagnosed breast pathology (25 people). The study was conducted with the informed con-

sent of all healthy donors and patients. The study was approved by the local ethics committee of RIFCI.

Venous blood from patients with primary breast cancer (T 1-3 N 0-2 M 0) was collected before surgery (radical mastectomy or radical breast resection) or from patients with metastases before the course of chemotherapy. Blood sampling from donors and patients was carried out in vacuum tubes added with anticoagulant EDTA (Improvacuter, China). Peripheral blood mononuclear cells (PBMCs) were isolated under sterile conditions using a Ficoll-Urographin gradient ($\rho = 1.077$ g/l) using a standard method.

Cell phenotyping and counting TIM-3 and PD-1 molecules

The phenotypic characteristics of T-lymphocytes were assessed by flow cytometry on a FACSVerse cytometer (BD, USA) using monoclonal antibodies (Biolegend, USA): CD3-PerCP-Cy5. (clone OKT3, Cat # 317336), CD4-PE-Cy7 (clone RPA-T4, Cat # 300512), CD8-FITC (clone RPA-T8, Cat # 301006), TIM-3-PE (clone F38-2E2, Cat # 345006), PD-1-PE (clone NAT105, Cat # 367404). A BD QuantiBRITE PE kit (BD Biosciences, USA) was used to create a calibration curve and convert the fluorescence intensity values of cells expressing the corresponding marker into absolute values of the receptor number, containing 4 fractions of lyophilized beads, each of which carries a different level of phycoerythrin.

The study of the expressed TIM-3 and PD-1 was carried out with the same parameters of the voltage of the photomultiplier tube according to the PE detector as in the analysis of the calibration beads, which allowed to convert the values of the fluorescence intensity into the number of PE molecules per cell. Further, the number of PE molecules per cell was converted to the number of antibody molecules per cell using the known ratio of PE molecules per antibody, equal to 1:1. Cytometer settings were checked weekly using Cytometer Setup and Tracking (CS&T) beads (BD Biosciences, USA).

Statistical methods

Results are presented as median and interquartile range. For statistical data processing, ANOVA and Tukey's test were used in GraphPadPrism 6 software. Differences were considered significant at $p < 0.05$. Correlations between the studied parameters were established using the Spearman correlation coefficient (at $p \leq 0.05$).

Results and discussion

Determining amount of PD-1- and TIM-3- positive cells in peripheral blood

To determine the number of PD-1- and TIM-3⁺ cells, populations of CD4⁺ and CD8⁺ T-lymphocytes were isolated from the CD3⁺ T-lymphocyte popu-

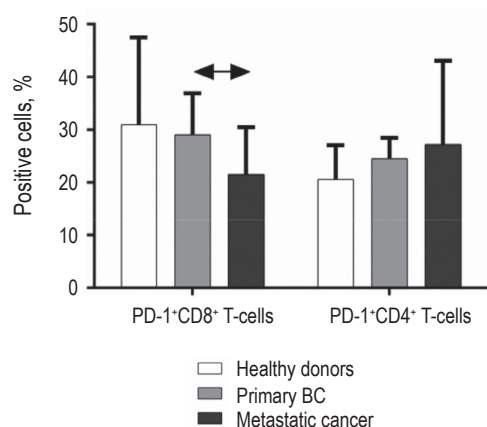


Figure 1. Relative number of PD-1⁺-cells in healthy donors (n = 25), patients with primary breast cancer (n = 25), and patients with metastatic breast cancer (n = 15)

Note. Data are presented as median and range of quartiles. Arrows indicate statistically significant differences, $p < 0.05$.

lation, in which the relative and absolute number of target cells were determined. For the index of PD-1⁺-cells, significant differences were obtained between the groups of CD8⁺ T-lymphocytes from patients with primary and metastatic breast cancer (Figure 1). For TIM-3, no significant differences were found.

Assessing absolute number of cells expressing the markers examined showed significant differences between the groups of patients with primary and metastatic breast cancer, respectively, in the number of PD-1⁺CD8⁺ T-cells, as well as differences between the cells of healthy donors and patients in the number of PD-1⁺CD4⁺ T-cells (Figure 2A). For TIM-3⁺-cells, significant differences in the absolute number of cells were found only for CD4⁺-cells: between the groups of apparently healthy donors and patients with metastatic breast cancer, as well as between groups of patients with primary and metastatic breast cancer (Figure 2B).

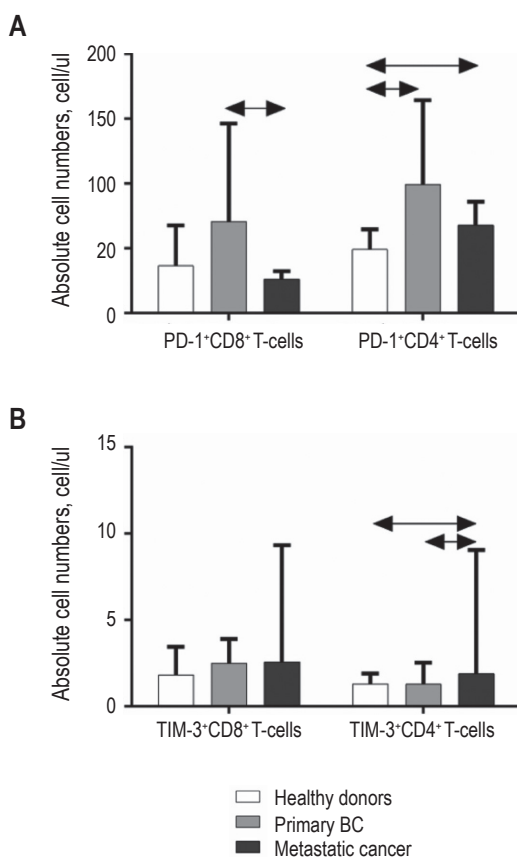


Figure 2. Absolute number of (A) PD-1- and (B) TIM-3⁺-cells in healthy donors (n = 25) and patients with primary (n = 25) and metastatic breast cancer (n = 15)

Note. As for Figure 1.

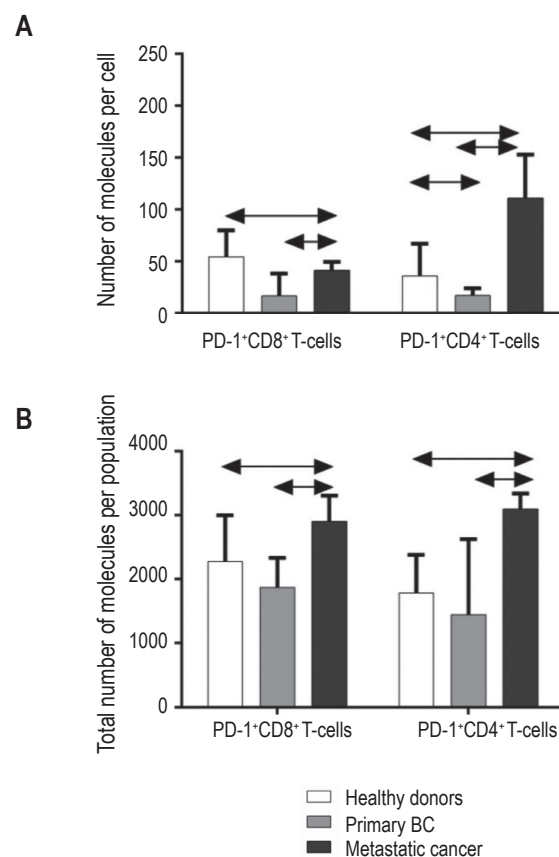


Figure 3. (A) Total number of PD-1 molecules on the surface of CD4⁺ and CD8⁺ T-cells and (B) the number of PD-1 molecules per 1 cell on the surface of CD4⁺ and CD8⁺ T-cells in healthy donors (n = 25), patients with primary breast cancer (n = 25) and patients with metastatic breast cancer (n = 15)

Note. As for Figure 1.

Determining the number of PD-1 and TIM-3 molecules in peripheral blood T-cells

While studying the total number of molecules on T-lymphocytes, it was shown that T-lymphocytes from both healthy donors and breast cancer patients carry approximately the same number of TIM-3 molecules, i.e. showing no significant differences. The total number of PD-1 molecules on CD4⁺ and CD8⁺ T-cells was increased in patients with metastatic breast cancer compared with the other two groups (Figure 3A).

In addition to the total number of molecules expressed on the surface of total CD4⁺ and CD8⁺ T-cells, we also calculated the parameter corresponding to the number of PD-1- and TIM-3 molecules per one cell for each population. With regard to the PD-1 molecule, it was found that the number of molecules in terms of the number of CD8⁺ T-cells in patients with metastatic breast cancer is significantly lower than in healthy donors. At the same time, the opposite trend is observed for CD4⁺-cells from these two groups – the number of PD-1 per cell for CD4⁺ T-cells in patients with advanced cancer was significantly higher than that for CD4⁺-cells from healthy donors (Figure 3B). The number of PD-1 molecules per cell in the group of patients with primary breast cancer was significantly lower both in CD4⁺ and CD8⁺ T-cells than that in the group of patients with metastases.

To clarify the mutual influence of the studied subpopulations, we carried out a correlation analysis for each of the parameters. To calculate the correlations, the Spearman coefficient and the p-level of significance were used. The presence of a relationship between the values was considered significant at $p < 0.05$, which corresponded to Spearman's coefficient from 0.4 to 1 (moderate and high tightness of the relationship).

In healthy donors, the maximum number of correlations is determined between PD-1- and TIM-3-positive CD4⁺ T-cells (for parameters, the absolute and relative number of cells, total number of molecules on the cells) and PD-1- and TIM-3-positive CD8⁺ T-cells (for the parameters, the absolute number of cells, the total number of molecules on the cells, the number of molecules per cell), there are also correlations between PD-1- and TIM-3 in

different subpopulations (correlations between the relative numbers of PD-1⁺CD8⁺ and TIM-3⁺CD4⁺, absolute number of PD-1⁺CD4⁺ and TIM-3⁺CD8⁺-cells). In breast cancer patients, fewer correlations were observed particularly being found between the absolute number of PD-1⁺CD8⁺ and TIM-3⁺CD8⁺-cells, PD-1⁺CD4⁺ and TIM-3⁺CD8⁺-cells, between the total number of molecules per cell for PD-1⁺CD8⁺ and TIM-3⁺CD8⁺-cells.

Thus, changes in the absolute number of PD-1 and TIM-3-positive cells and the number of molecules are shown in breast cancer patients. In particular, in breast cancer patients compared with healthy people, the absolute number of CD4⁺-cells expressing PD-1 is higher, and the absolute number of PD-1 molecules per cell also increases, while the most pronounced differences between health and pathology are found while comparing healthy donors with a group of patients with metastatic breast cancer. Also, patients tended to increase absolute number of TIM-3⁺CD4⁺ T-cells compared to healthy donors. At the same time, a correlation was shown in the expression pattern of the studied markers between PD-1- and TIM-3-positive CD4⁺ T-cells, as well as between PD-1- and TIM-3-positive CD8⁺ T-cells, which also indicates an important prognostic role for evaluating the expression and co-expression of such checkpoint molecules and the need for further studies regarding an influence of T-cell phenotype on related function in the course of breast cancer.

The development of methods for analyzing the features of the expressed checkpoint molecules on peripheral blood T-cells can serve as the basis for the development of minimally invasive methods that might allow with a high probability to assess the prognosis of oncological disease without using histological archival material. It is assumed that it is the expression of suppressor co-stimulating molecules on antigen-presenting cells that fundamentally differs in healthy donors and patients with breast cancer and could account for isolating factors to maximize the overexpression of suppressor molecules: soluble mediators, tumor-associated antigens, or contact interaction of immunocompetent and tumor cells.

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ЭМБРИОГЕНЕЗ НЕЙРОНАЛЬНЫХ ЭЛЕМЕНТОВ (ГЛИОБЛАСТОВ И ГАМКА-РЕЦЕПТОРОВ) НЕЙРОИММУННОЙ СИСТЕМЫ МОЗГА ЧЕЛОВЕКА ПРИ ПРЕНАТАЛЬНОМ ВЛИЯНИИ АЛКОГОЛЯ

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Резюме. Воздействие алкоголя вызывает дисбаланс нейроиммунной функции и приводит к нарушению развития мозга. Алкоголь активирует сигнальные пути врожденного иммунитета в мозге. Нейроиммунные молекулы, экспрессируемые и секретируемые глиальными клетками головного мозга (микроглия, олигодендроглия), изменяют функцию нейронов и стимулируют в дальнейшем развитие алкогольного поведения. В передаче нейроиммунных сигналов участвуют различные сигнальные пути и клетки мозга. Глиальные клетки являются основными источниками иммунных медиаторов в головном мозге, которые отвечают на иммунные сигналы в центральной нервной системе и выделяют их. Целью настоящего исследования было изучение нейрональных элементов: морфометрических параметров глиобластов, синаптических структур и свойств синаптосомальных ГАМКА-бензодиазепиновых рецепторов нейроиммунной системы в эмбриогенезе мозга человека при перинатальном воздействии алкоголя. Выявлены изменения глиобластов в ткани мозга эмбрионов и плодов человека в условиях хронической пренатальной алкоголизации с увеличением срока беременности по сравнению с контрольными подгруппами: достоверное увеличение среднего количества глиобластов, длины периметров пресинаптических терминальных структур, постсинаптической плотности, пресинаптические терминальные области были значительно меньше ($p < 0,01$) в исследуемой группе, чем в контрольной группе сравнения. Воздействие этанола приводит к снижению аффинности ГАМКА-бензодиазепиновых рецепторов, что влияет на нейрональную пластичность, связанную с развитием и дифференцировкой клеток-предшественников (глиобластов и нейробластов) в период эмбриогенеза головного мозга человека и приводит к подавлению ГАМКергической функции в головном мозге. Это вызывает нарушение взаимосвязей эмбриональных клеток в головном мозге, приводит к чрезмерным явлениям апоптоза из-за активации глиальных клеток нервной ткани, нарушению нейроиммунной

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функции в развивающемся мозге, изменениям нейрональных цепей, а также к изменению баланса возбуждающих и тормозных эффектов, что оказывает влияние на функциональную активность в центральной нервной системе. Активация глии — это компенсаторная реакция, вызванная нейропластическими изменениями, направленная на адаптацию развивающегося мозга эмбриона и плода в условиях нейротоксичности и гипоксии под воздействием пренатальной алкоголизации материнского организма и влияния этанола на плод. Динамика изменений глиальных элементов и рецепторной активности в нервной ткани эмбрионов и плодов человека в условиях пренатального воздействия алкоголя свидетельствует о более выраженном влиянии алкоголя на самые ранние стадии развития эмбриона человека, что имеет большое практическое значение при планировании беременности и недопустимость алкоголизации матери во избежание негативных последствий у потомства.

Ключевые слова: эмбриогенез, нейроиммунная система, головной мозг, алкоголь, глиобласт, ГАМКА-рецептор, синапс

EMBRIOGENESIS OF NEURONAL ELEMENTS (GLIOBLASTS AND GABAA RECEPTORS) IN THE HUMAN BRAIN NEUROIMMUNE SYSTEM UNDER PRENATAL ALCOHOL EXPOSURE

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Abstract. Exposure to alcohol causes imbalances in neuroimmune function and impaired brain development. Alcohol activates the innate immune signaling pathways in the brain. Neuroimmune molecules expressed and secreted by glial cells of the brain (microglia, oligodendroglia) alter the function of neurons and further stimulate the development of alcoholic behavior. Various signaling pathways and brain cells are involved in the transmission of neuroimmune signals. Glial cells are the main sources of immune mediators in the brain, which respond to and release immune signals in the central nervous system. The aim of this study was to study neuronal elements: morphometric parameters of glioblasts, synaptic structures and properties of synaptosomal GABAA-benzodiazepine receptors of the neuroimmune system in the embryogenesis of the human brain under perinatal exposure to alcohol. Changes in glioblasts in the brain tissue of human embryos and fetuses were revealed under conditions of chronic prenatal alcoholization with an increase in gestational age compared with control subgroups: a significant increase in the average number of glioblasts, the length of the perimeters of presynaptic terminal structures, postsynaptic density, presynaptic terminal regions were significantly less ($p < 0.01$) in the study group than in the control comparison group. Exposure to ethanol leads to a decrease in the affinity of GABAA-benzodiazepine receptors, which affects neuronal plasticity associated with the development and differentiation of progenitor cells (glioblasts and neuroblasts) during embryogenesis of the human brain and leads to suppression of GABAergic function in the brain. This causes a disruption in the interconnection of embryonic cells in the brain, leads to excessive apoptosis due to the activation of glial cells of the nervous tissue, disruption of neuroimmune function in the developing brain, changes in neuronal circuits, as well as a change in the balance of excitatory and inhibitory effects, which affects the functional activity in the central nervous system. Glial activation is a compensatory reaction caused by neuroplastic changes aimed at adapting the developing brain of the embryo and fetus under conditions of neurotoxicity and hypoxia under the influence of prenatal alcoholization of the maternal organism and the effect of ethanol on the fetus. The dynamics of changes in glial elements and receptor activity in the nervous tissue of human embryos and fetuses

under conditions of prenatal exposure to alcohol indicates a more pronounced effect of alcohol on the earliest stages of human embryo development, which is of great practical importance in planning pregnancy and the inadmissibility of alcoholization of the mother in order to avoid negative consequences in offspring.

Keywords: embryogenesis, neuroimmune system, brain, alcohol, glioblast, GABAA receptor, synapse

Introduction

Exposure to ethanol during pregnancy causes fetal alcohol spectrum disorders (FASD). The most severe form of FASD is fetal alcohol syndrome (FAS), characterized by growth retardation, facial abnormalities, and neurobehavioral changes. In some patients with FASD, only a fraction of the FAS characteristics, such as cognitive and behavioral disorders not associated with facial dysmorphias, are detected. High and moderate prenatal alcohol exposure is associated with behavioral and cognitive problems in childhood and adolescence associated with mood disorders, working memory deficits, attention deficits, increased aggression, and behavior changes [6, 16, 17]. Important brain regions and neuronal circuits involved in the development of alcohol use disorders (AUD) have been identified [10]. Exposure to alcohol causes imbalances in neuroimmune function and leads to impaired brain function. Alcohol activates innate immune signaling pathways in the brain and stimulates alcohol consumption [4, 11]. Neuroimmune molecules expressed and secreted by cerebral glial cells alter neuronal function and stimulate alcoholic behavior [11, 16]. New insights into molecular mechanisms underlying AUD have led to the identification of new therapeutic targets associated with immunity. Various indigenous pathways and brain cells are involved in the transmission of neuroimmune signals [5, 13, 18]. The innate immune system in the central nervous system (CNS) is concentrated in brain cells that are capable of recognizing and responding to changes in the neuronal microenvironment. Microglia and astrocytes are the main immune mediators in the brain that respond to and release immune signals in the central nervous system [11, 12, 13, 14, 15]. Chemokines, cytokines, and pathogen-associated molecular patterns activate various families of cerebral immune receptors. Microglia activation includes morphological transformation into a phagocytic, macrophage-like cell being visualized by increased size of cell bodies, decreased duration of processes and increased immunoreactivity. The investigation of developing disorders of the neuroimmune system shape the basis for designing effective pharmacotherapeutic agents. Alcohol affects the neuronal regulation of innate immunity in brain cells by altering gene expression and molecular pathways regulating neuroinflammation.

The aim of the study consists in examining neuronal elements: glioblast morphometric parameters, synaptic structures and properties of synaptosomal GABAA/benzodiazepine receptors of the neuroimmune system in embryogenesis of the human brain under perinatal alcohol exposure.

Materials and methods

The study involved women aged 25 to 41 years (mean – 37 years), divided into two groups. The first group included participants, without somatic or mental pathology, who did not drink alcohol before (within the 1 month before conception) and during pregnancy. The second group consisted of women who suffered from I-II degree alcoholism for 3 to 13 years. All participants in the study had previously undergone treatment for alcoholism, but in the period preceding collection of biomaterial for the study, none of them received disulfiram. The material was obtained during operations for the artificial termination of pregnancy in maternity hospitals and gynecological departments of hospitals in the city of Tomsk. All procedures were carried out in accordance with the conditions and instructions of the ethics committee and do not contradict to the Declaration of Helsinki 1975 or the provisions of the 2000 revision. During the operations, samples of embryonic material were obtained ranging from 8 to 15 weeks of intrauterine development. Based on the gestational age and confirmed alcohol consumption by mothers, 2 groups were formed, divided into subgroups. Group Alcohol (A) was formed by collecting biomaterial obtained from women suffering from alcohol dependence and included 2 subgroups: A1 – embryos at the age of 8-9 weeks of gestation and A2 – at the age of 10-11 weeks of gestation. Both subgroups included 6 samples. The Control group (K) was formed in a similar way from apparently healthy women: K1 – 9 weeks and K2 – 10-11 weeks, 7 samples per each group.

Microscopic and morphometric assay

The study of the samples was carried out by using light microscope Axio Scope A1 (Carl Zeiss, Germany) with sample preparation: preliminary fixation in 0.5%- glutaraldehyde solution in 0.1 M sodium phosphate buffer (pH 7.3-7.4) and additional fixation in 1%-osmium oxide solution. Subsequent processing included dehydration in alcohols of ascending concentrations and embedding in epoxy

resins (Araldite). Next, the obtained samples were cut by using an Ultracut-E ultratome (Reichert, Austria) into semi-thin sections (0.5-1 μm) and stained with toluidine blue (Nissl's dye) according to the standard technique. In this study, we used slice cut at the level of the intermediate layer. During the subsequent photography, a Canon G10 digital camera was used. Sections were also stained by hand using saturated aqueous solution of uranyl acetate (1.5 h at 56 °C) and a 2% solution of lead citrate (30 min at room temperature) and viewed under a JEM 100CX electron microscope (Jeol). The cells of the intermediate layer of the GM cortex, which were isolated by focusing on the ventricular layer, were examined electronically-microscopically.

The analysis of changes in morphometric parameters was used to identify quantitative changes among glioblasts of the human cerebral cortex at different stages of intrauterine development, as well as to reveal a correlation between the degree of differentiation of the listed tissue components and degree of the pathological factor (alcohol) impact on the process of tissue development. For this, the AxioVision 4.8 software was used, in which the diameter and area of each individual element, as well as the average number of units of cellular or vascular structures per unit of cut area, were determined.

Electron microscopy examined in the intermediate layer of the forebrain wall, neuro- and glioblasts (including microglia cells). Morphometric analysis was carried out by using photographs of negatives obtained under electron microscope. Images were scanned at 300 dpi and saved in TIFF format, processed with ScionImage for Windows developed at the National Institutes of Health (Scion Corporation), used to assess the regions of presynaptic terminals, their perimeters and the length of postsynaptic densities. In each case, 15-20 photomicrographs of the intermediate layer were analyzed. The measurement results are presented in relative units: the number of pixels to estimate the length and the number of image pixels squared to estimate the areas of synaptic structures.

Radioreceptor assay

Parameters of GABAA / benzodiazepine receptors were studied by the radio-receptor assay of [^3H]-flunitrazepam (Amersham) to the synaptosomal membranes of the brain samples tested at concentration range of 0.2-10 nM. The protein concentration in the synaptosomal membranes was 0.3 mg/ml. Non-specific binding was determined in the presence of a non-radioactive ligand at a concentration of 10 μM . Radioactive assay of the amount of bound ligand was performed in a scintillation beta-counter "Rack-beta" ("LKB"). The constant of dissociation of the radio-ligand complex (K_d) and the maximum number of specific binding sites (B_{max}) were determined by studying kinetic binding in Scatchard coordinates. To determine the statistical significance, the Statistica 10 program was used with the analysis of the Mann-Whitney test (significant differences at ($p < 0.05$), Student's t-test ($p < 0.01$), correlational relationships were assessed by Spearman analysis.

Results and discussion

The study of the patterns of developing glial cells in the embryonic brain revealed that the numerical indicator of the average area of glioblasts after prenatal alcohol exposure (in the main studied subgroup A1) was significantly lowered compared to control study group (in group K1) (table). The average perimeter of cells significantly differed between groups A1 and K1, where the perimeter of glioblasts in the control group at this time of development (K1) also exceeded that in the main group (A1). The number of cells in the studied samples of the embryonic brain tissue in the main group (A1) significantly exceeded the number of cells in the samples of the control group (K1) (table). Thus, we found that under prenatal alcoholization, the growth of glioblasts in the brain tissue of human embryos at the gestational age of 8-9 weeks was retarded, whereas the number of cells increases, which we consider as a compensatory reaction due to decreased size of glioblasts in the brain tissue of embryos after prenatal alcohol exposure.

TABLE 1. DYNAMICS OF THE NUMERICAL INDICATORS OF GLIOBLASTS IN THE STUDIED GROUPS

Analyzed parameter	Average area, μm^2	Average perimeter, μm	Number of cells per 1 mm^2 , pcs
Control 1 (C1)	32.1	23.9	75
Control 2 (C2)	13.3*	15.0*	160
Alcohol 1 (A1)	21.3*	19.1*	121
Alcohol 2 (A2)	12.9**	14.8**	263

Note. *, significant differences compared to Control 1 ($p < 0.05$). **, significant differences compared to Alcohol 1 ($p < 0.05$).

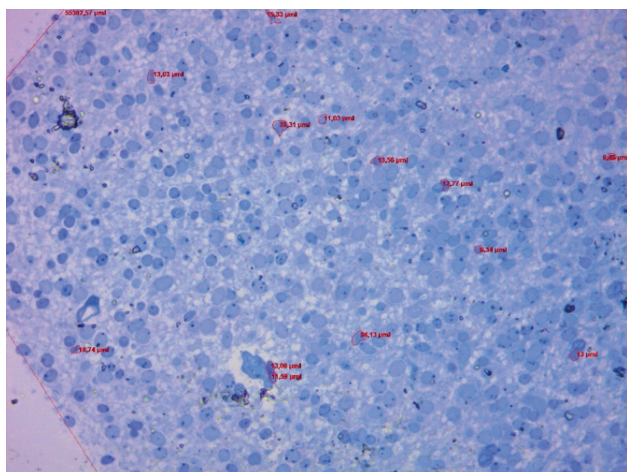


Figure 1. Photo shows a section of the brain tissue of a human embryo at 8-9 weeks of intrauterine development in the process of visualization and obtaining digital indicators of the perimeter of glioblasts and the total perimeter of the section in the AxioVision 4.8 program

Note. Glioblasts, which are asymmetric hyperchromic cells, are highlighted in red. The photo also shows rounded normochromic neuroblasts. Staining with toluidine blue, magnification $\times 400$.

Regarding human fetal development at 10-11 weeks of gestation, we revealed no significant differences in the size of glioblasts (average area and perimeter of cells) in the control (K2) and main (A2) study groups; however, the tendency to an increase in the average number of glioblasts per unit area in tissue the embryonic and fetal brain exposed to the prenatal alcohol (in the main subgroups (A1 and A2)) was preserved at all examined stages of development (Table 1, Figure 1). These changes may indicate at retarded development of cellular components with a compensatory increase in their number that might be regarded as a protective, compensatory reaction of glia to prenatal ethanol exposure.

The results obtained indicate at morphometric changes in the human embryonic and fetal brain tissue under conditions of chronic prenatal alcoholization: along with increasing gestational age, a significant increase in the average number of the studied structures vs. control subgroups was observed. The dynamics of changes in glial elements in the nervous tissue of human embryos and fetuses exposed to prenatal alcohol indicates a more pronounced effect of alcohol at the earliest stages of human embryonic development, which is of great practical importance in planning pregnancy and the inadmissibility of maternal alcoholization in order to avoid negative consequences in offspring.

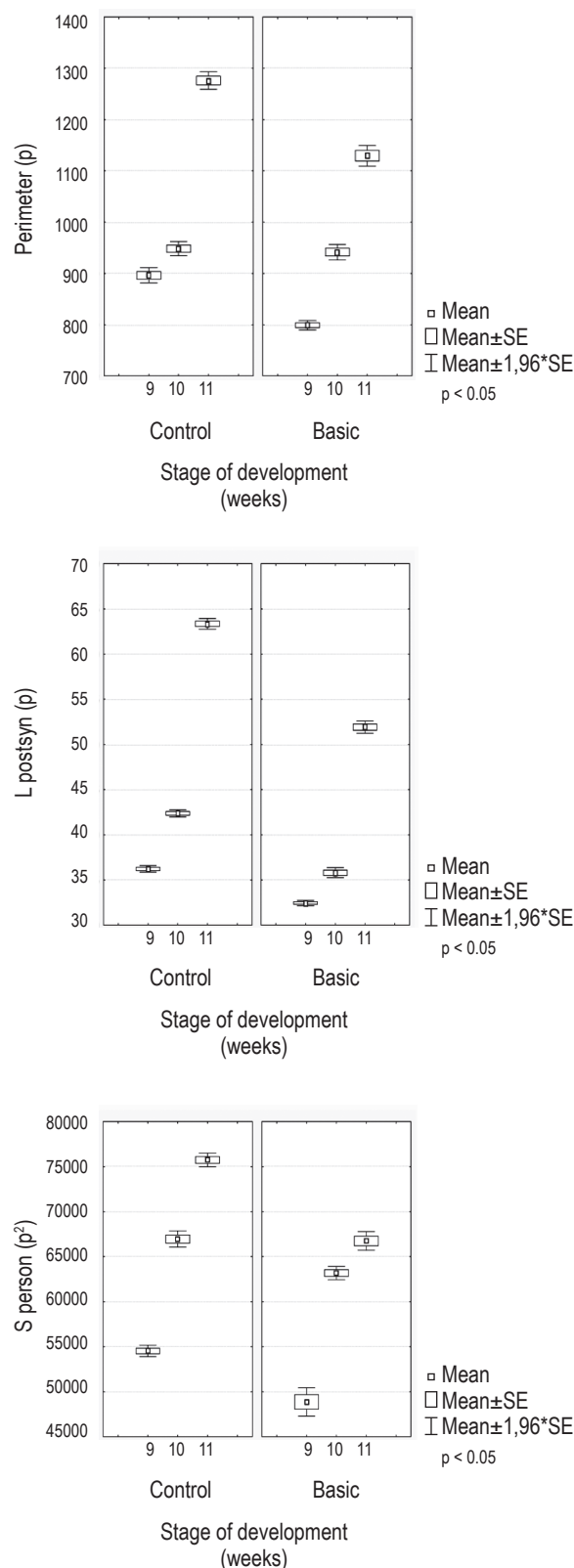


Figure 2. Statistical analysis of morphometric indices of presynaptic terminals (S, presynaptic terminal area; L, postsynaptic density length; P, presynaptic terminal perimeter) in the human embryonic brain at various stages of development in the control and main (prenatal alcoholization) groups

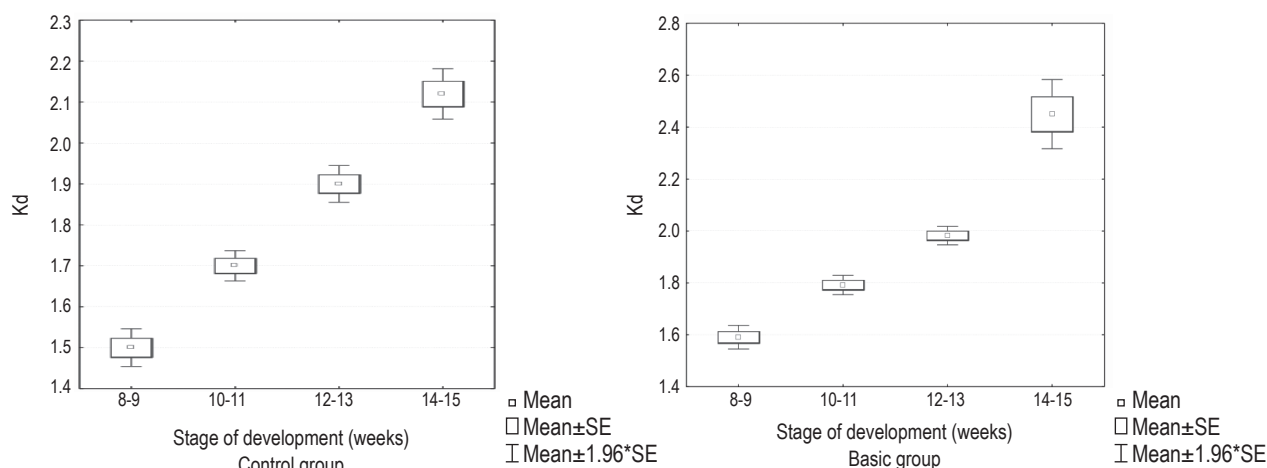


Figure 3. Statistical analysis of the binding parameter (Kd) of $[^3\text{H}]$ flunitrazepam with membranes of synaptosomes of the human embryonic brain in the control and experimental groups

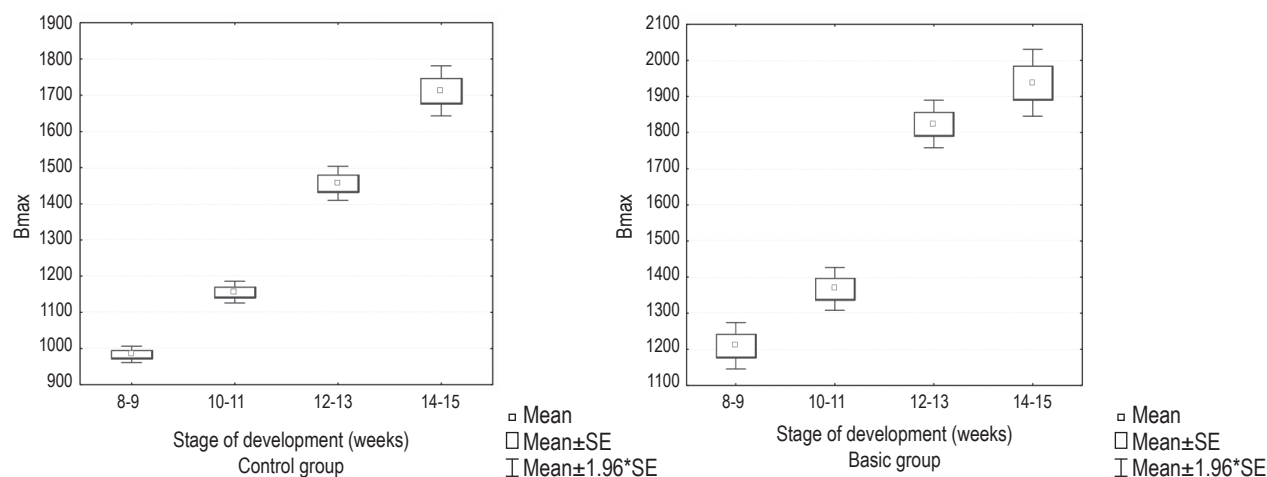


Figure 4. Statistical analysis of the binding parameter (Bmax) of $[^3\text{H}]$ flunitrazepam with membranes of synaptosomes of the human embryonic brain in the control and experimental groups

Electron microscopic examination with the assessment of morphometric parameters of cerebral tissue synaptic structures

The quantitative morphometric parameters of synaptic contacts of the developing human brain in the main and control groups were significantly differed. Alcohol has a modulating effect on the formation of intercellular contacts.

We observed that the length of presynaptic terminal perimeters, postsynaptic densities, presynaptic terminal areas were significantly shortened ($p < 0.01$) in the study group vs. controls group (Figure 2).

Radioreceptor assay of GABAA / benzodiazepine receptors of synaptic structures of the brain tissue

Radioreceptor analysis of binding the selective ligand 3H flunitrashpam with the fraction of the brain tissue sample membranes from human embryos and

fetuses in normal conditions and after prenatal alcohol exposure showed that the properties of benzodiazepine receptors (BDR) in synaptosomal membranes of GM embryos and human fetuses were distinct at different developmental periods (Figure 3, 4).

In the control group, the affinity of receptors in the brain of human embryos and fetuses decreased during the development period of 8-9-12-13 weeks: dissociation constant (Kd) increased from 1.5 to 2.12 nM was noted. In the experimental group, the affinity of benzodiazepine receptors in the brain of embryos and fetuses was lower at all studied developmental stages compared to the control group, which was expressed as increased absolute values of Kd from 1.59 to 2.45 nM. The dynamics of changes was not linear in the experimental group. The number of receptors in the human embryonic and fetal brain

from the control group during developmental period of 8-9-12-13 weeks increased with some deceleration at the age of 10 weeks of gestation. The number of receptors in the experimental group was significantly higher than in the control at all studied stages of development of embryos and fetuses, which can be compensatory in nature with decreased receptor affinity. The dynamics of changes in the number of receptors was not linear, which was more prominent in the experimental group.

The data obtained indicate about a structural and functional relationship between the development of receptors and the synaptogenesis of young cellular elements of the human cerebral cortex upon maternal pregnancy-related alcoholism, which is expressed in the simultaneous appearance of structural synaptic and benzodiazepine receptor components overlapped with maturation delay at the week 10 of development.

Consequently, the ability of normal functioning and allosteric modulation of the GABAA/benzodiazepine receptor complex in synaptic membranes, regulating the general processes of inhibition in the central nervous system *вусдштуы*. We have found that alcohol consumption by mothers during pregnancy affects the properties of GABAA/benzodiazepine receptors and may subsequently affect the development of the embryonic and fetal central nervous system.

The observed decrease in the formation of synaptic structures in the fetal and embryonic brain at different stages of development influenced by maternal alcoholism vs. normal and decreased affinity of GABAA/benzodiazepine receptors with increased receptor density can be considered as a compensatory reaction aimed at adaptation of the embryonic and fetal nervous system to conditions of functional insufficiency in GABAergic neurotransmission (Table 2).

TABLE 2. CORRELATION ANALYSIS OF MORPHOMETRIC PARAMETERS OF SYNAPTIC STRUCTURES AND THE NUMBER OF BENZODIAZEPINE RECEPTORS IN THE BRAIN TISSUE OF EMBRYOS (BDR) AT DIFFERENT DEVELOPMENTAL STAGES, $M \pm m$

Development period (weeks)	Control group				
	The number of sites of specific binding of [^3H]-flunitrazepam with BDR (Bmax), fmol/mg protein	Perimeter of the presynaptic terminal region (P), $M \pm m$ Pixel	Area of the presynaptic terminal region (S), $M \pm m$ Pixel ²	Presynaptic length terminal area (L), $M \pm m$ Pixel	Number of samples (n), pcs Pixel
8-9	984.22±34.92	896.28±63.7 r = 0.80 p = 0.0006	54521±2673 r = 0.79 p = 0.0003	36.21±1.56 r = 0.89 p = 0.0004	9
10-11	1156.00±43.06	948.19±58.2 r = 0.77 p = 0.0004	66964±3833 r = 0.62 p = 0.0002	42.37±1.70 r = 0.87 p = 0.0008	8
12-13	1456.29±63.93	1276.02±73.08 r = 0.83 p = 0.0008	75742±3207 r = 0.76 p = 0.0001	63.33±2.51 r = 0.91 p = 0.0003	7
	Experimental group				
8-9	1210.00±98.36	798.90±40.09 r = 0.78 p = 0.0004	48861±6773 r = 0.64 p = 0.0002	32.45±1.23 r = 0.85 p = 0.0007	9
10-11	1367.40±96.08	941.56±64.44 r = 0.82 p = 0.0006	63178±3168 r = 0.71 p = 0.0001	35.80±2.37 r = 0.88 p = 0.0005	10
12-13	1824.13±94.79	1129.00±86.87 r = 0.79 p = 0.0004	66750±4436 r = 0.70 p = 0.0003	51.90±2.88 r = 0.83 p = 0.0008	8

Our data show that exposure to ethanol reduces the GABAA / benzodiazepine receptor affinity, which affects neuroplasticity associated with the group (не понятно), differentiation and development of glioblasts and neuroblasts (progenitor cells) during embryogenesis, which causes disruption of the brain embryonic cell interconnections leading to excessive apoptosis phenomena due to activated glial cells, impaired neuroimmune function, changes in neural circuits, altered balance of excitatory / inhibitory effects. Glial activation is a compensatory response caused by neuroplastic changes aimed at adapting the developing embryonic and fetal brain under conditions of neurotoxicity and hypoxia due to ethanol exposure. Microglia provide a fundamental basis for development of normal processes in the nervous system, including neurogenesis [1, 2, 9, 19], as well as the formation, growth and functioning of neuronal elements, such as synapses and receptors [12, 20]. Brain functions are coordinated by the interaction of the main elements in neural circuits: glial elements, receptors and synapses. Cerebral glial cells execute an

immune function, contact with synaptic neuronal cell elements, expanding and contracting their processes.

Conclusion

Glia plays a significant role in the developing brain, synapse pruning and regulating the function of nerve circuits. Disturbing glial homeostatic role can lead to neuronal dysfunction and developing pathology. The activation of glial cells during the period of toxic effects or other pathology is a necessary cerebral protective function. Glial cells control neuronal excitation, regulation of synaptic plasticity and release of neurotrophic factors [2, 3, 7]. They surround the somas of highly active neurons in order to reduce excitation, as well as migrate and displace synapses with inhibitory function in neurons of the cerebral cortex to increase the expression of neuroprotective molecules. Thus, changes in microglial activity cause disruption of the normal neuronal activity and increased neurotoxicity caused by ethanol acting on various neuroimmune molecules.

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ХИМЕРИН УЧАСТВУЕТ В РЕГУЛЯЦИИ КОНТРОЛЯ КАЧЕСТВА МИТОХОНДРИЙ У БОЛЬНЫХ ОЖИРЕНИЕМ

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Резюме. У больных ожирением выявлены взаимосвязи содержания химерина в плазме крови с экспрессией генов *TFAM*, *Drp1*, *MFN2*, *SOD*, *BAX*, ответственных за контроль качества митохондрий, в инсулинзависимых тканях (жировая ткань, печень). Установлены тканеспецифические особенности экспрессии генов (*TFAM*, *Drp1*, *MFN2*, *SOD*, *BAX*), числа копий мтДНК в исследуемых депо у больных ожирением. Доказано, что изменение (снижение) числа копий мтДНК в инсулинзависимых тканях может оказывать протекторное действие на митохондрии, в условиях повышенного окислительного стресса.

Выявлено, что у больных без СД 2-го типа повышение продукции химерина способствует активации антиоксидантной системы в висцеральной жировой ткани, но не в печени. Напротив, у всех больных ожирением с СД 2 типа регистрировалось снижение (в сравнении с больными без СД 2 типа) плазменного уровня химерина.

Таким образом, низкое содержание химерина в плазме крови у больных с СД 2-го типа опосредует формирование дисфункции митохондрий в инсулинзависимых тканях (жировая ткань, печень).

Ключевые слова: ожирение, СД 2 типа, митохондрии, химерин, печень, жировая ткань, мтДНК

CHEMERIN AS A POTENTIAL REGULATOR OF MITOCHONDRIAL QUALITY CONTROL IN OBESE PATIENTS

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Abstract. In obese patients, the relationship between the content of chemerin in blood plasma and the expression of genes *TFAM*, *Drp1*, *MFN2*, *SOD*, *BAX*, responsible for quality control of mitochondria, in insulin-dependent tissues (adipose tissue, liver) was revealed. The tissue-specific features of gene expression (*TFAM*, *Drp1*, *MFN2*, *SOD*, *BAX*), the number of mtDNA copies in the studied depots in obese patients were established. It has been proven that a change (decrease) in the number of mtDNA copies in insulin-dependent

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tissues can have a protective effect on mitochondria under conditions of increased oxidative stress. It was found that in patients without type 2 diabetes, an increase in chemerin production promotes the activation of the antioxidant system in the visceral adipose tissue but not in the liver. On the contrary, all obese patients with type 2 diabetes showed a decrease (compared with patients without type 2 diabetes) in the plasma level of chemerin. Thus, the low content of chemerin in the blood plasma in patients with type 2 diabetes mediates the formation of mitochondrial dysfunction in insulin-dependent tissues (adipose tissue, liver).

Keywords: obesity, T2DM, mitochondria, chemerin, liver, adipose tissue, mtDNA

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Introduction

Obesity has been shown to directly or indirectly cause type 2 diabetes mellitus (T2DM), cardiovascular and oncological diseases. Russia poses the fourth and fifth place worldwide in the number of overweight people and patients with type 2 diabetes, respectively. Epidemiological forecasting shows that by 2025, 40% of males and 50% females will be overweight and obese. Abdominal obesity is associated with dysfunction of adipose tissue (AT) and accumulation of free fatty acids (FFA) outside of it; it all leads to development of nonalcoholic fatty liver disease (NAFLD), atherosclerosis, and dysfunction of pancreatic cells.

A high FFA content activates cell oxidative stress and increases reactive oxygen species (ROS). ROS damage mitochondrial DNA (mtDNA), disrupt mitochondrial quality control (MQC) processes (dynamics, biogenesis, mitophagy). Mitochondrial dysfunction in insulin-dependent tissues contributes to insulin resistance (IR).

Chemerin – a cytokine known as tazarotene-induced gene 2 (*TIG2*) or retinoic acid responder receptor 2 (*RARRES2*), is secreted by preadipocytes, adipocytes, hepatocytes and leukocytes [5] and involved in the regulation of angiogenesis, adipogenesis, inflammation, and energy metabolism.

Mitochondria are essential regulators of cellular energy homeostasis. The number of mitochondria in cells depends on the level tissue metabolic activity. Tissue-specific features of mitochondrial quality control in insulin-dependent tissues (AT, liver) are of particular interest. Thus, the study aimed to identify the role of chemerin in regulating mitochondrial quality control processes in insulin-dependent tissues of obese patients.

Materials and methods

There were enrolled 264 obese patients, among which 154 had T2DM (42.90 ± 10.10 years; 42 males

and 112 females), and 110 obese patients had no carbohydrate metabolism disorders (41.80 ± 7.00 years; 50 males and 60 females) (Table 1). The control group consisted of 38 apparently healthy donors with normal anthropometric and biochemical parameters (37.50 ± 10.10 years; 20 males and 18 females). All participants provided an informed consent for participation in the study.

AT biopsy samples of various localization (visceral – greater omentum (GO), mesentery of the small intestine (MES), subcutaneous AT (SAT) and liver samples) were obtained during bariatric surgery in obese patients with/without T2DM and during routine laparoscopic operations: right- or left-side inguinal hernia, femoral, diaphragmatic and ventral hernias, nephroptosis in apparently healthy donors (control group).

Total RNA from liver biopsies was isolated using the ExtractRNA reagent (Evrogen, Moscow, Russia). Reverse transcription was performed using the MMLV RT kit (Evrogen, Moscow, Russia).

The level of serum chemerin was determined using an enzyme-linked immunosorbent assay (BIO Vendor, Czech Republic). Statistical data processing was carried out using the IBM SPSS Statistics 20 software (Statistical Package for the Social Sciences), by plotting data with Graphpad Prism 9.0 software.

Results and discussion

Visceral vs subcutaneous AT adipocytes have a high lipolysis rate [14] and synthesize more molecules with a proinflammatory profile. Differences in cellular composition, innervation and blood supply suggest opposite roles of fat stores in the pathogenesis of obesity. Excessive accumulation of TG in adipocytes leads to their hypertrophy and subsequent AT dysfunction and inflammation. When adipocytes are unable to store fatty acids, their excessive amount enters the systemic circulation to be deposited in the liver, muscles, heart, leading to ectopic lipid accumulation [10]. Most obese people develop NAFLD. FFAs are the main source of lipid accumulation in the liver. IR occurs as a result of the accumulated lipids both at the organ and body level.

Mitochondria play an important role in regulating whole body energy homeostasis and controlling insulin and glucose metabolism sensitivity. In mitochondria, fatty acid β -oxidation occurs, the pro-

cess of degradation of fatty acids. Mitochondria exert high plasticity, which allows them to provide the cell with energy demands. Mitochondria under stress conditions activate mitochondrial quality control (MQC) processes (biogenesis, dynamics, autophagy/mitophagy). MQC maintains organelle homeostasis, structure and function [6]. Energy-sensitive mediators affect mitochondrial plasticity.

Xie Q. et al. (2015) demonstrated that chemerin is involved in regulating mitochondrial biogenesis and mitophagy in skeletal muscle [13]. Previously, our studies have shown that chemerin in patients with morbid obesity without T2DM has a protective effect on IR development by decreasing the number of mtDNA copies in visceral adipose tissue [9].

White AT is the main source of chemerin; its expression has tissue-specific features [12]. Earlier, we showed that the chemerin level depends on RARRES2 gene expression in GO of obese patients [12]. An increase in chemerin production is positively associated with increased BMI ($r = 0.25$, $p < 0.05$) in patients without T2DM, which is quite logical and consistent with other studies [5]. In obese patients with T2DM, the level of serum chemerin, on the contrary, decreased (Table 1). Horn P. et al. (2018) associated a low level of serum chemerin with impaired liver function [8]. Chemerin deficiency is associated with IR in the AT and liver. This leads to increased production of glucose in the liver and blood. Chemerin regulates the β -cell function and plays an essential role in tissue-dependent glucose homeostasis. It was found that the level of glucose and chemerin was positively associated ($r = -0.66$, $p < 0.05$) in the control group, whereas in group with obesity lacking type 2 diabetes it showed negative relation ($r = -0.47$, $p < 0.05$).

Our studies have shown that in patients with type 2 diabetes in the liver, hepatocyte dystrophy, karyolysis, intracellular cholestasis, lymphocytic infiltration, and liver capsules enlargement were detected [2].

Chemerin is secreted as an inactive precursor to be activated by proteases via C-terminal processing. Thus, different isoforms have distinct biological effects and activities. Moreover, it is extensively discussed about what receptors are more critical for the chimeric biological and pathophysiological effects.

Chemokine-like receptor 1 (CMKLR1 or ChemR23) is the main most examined molecule transducing chimer signals. Studies have shown that obesity increases the expression of mRNA CMKLR1 in AT that rises in obesity; this is associated with macrophage tissue recruitment. The G protein-coupled receptor 1 (GPR1) and Chemokine receptor-like 2 (CCRL2) receptors are less studied.

GPR1 is expressed on the AT stromal vascular cells. In the liver of obese patients, the level of mRNA expression of chemerin and CMKLR1 is positively associated with BMI and concomitant NAFLD [3].

Interestingly, CMKLR1 mRNA is expressed by hepatocytes, hepatic stellate cells, endothelial cells, and Kupffer cells. Another receptor, CCRL2, is expressed by various liver cells, except for hepatocytes, transmitting no signals [11].

Mitochondria are a primary source of energy production in cells. The mtDNA copy number is adapted to the energy requirement, physiological or pathological state. Increased FFAs, ROS, reactive nitrogen species, and lipid peroxidation products can damage mtDNA.

In obese patients without T2DM, mtDNA copy number in AT from diverse sites localization was comparable to the control. In contrast, in obese patients with T2DM, an increased mtDNA copy number in AT was recorded. In the liver of obese patients without T2DM, we found decreased amount of mtDNA copy number compared to the control, while in patients with T2DM, it was higher than control group and patients with T2DM. mtDNA copy number in healthy donor liver was increased compared to that in adipose tissue depots, which is necessary to maintain the liver metabolic processes (Figure 1).

A decrease in the amount of mtDNA copy number may be due to increased oxidative stress. "Mild" uncoupling of mitochondrial processes can stimulate their activity. Mitochondria send alarms to the nucleus to activate processes associated with cellular homeostasis. Along with excess of nutrients and IR, increased mtDNA copy number is necessary to support energy-consuming processes.

In obese patients without T2DM, negative relations were found between chemerin content and mtDNA copy number in the liver ($r = -0.59$, $p < 0.05$). Shen W. et al. (2003) showed that chemerin, through the ChemR23 receptor, increases ROS production and activates autophagy in endothelial cells. The activation of this process is considered as an adaptation to stressful conditions. Cultured endothelial cells treated with chemerin had upregulated gene expression responsible for mitochondrial biogenesis (*PGC-1 α*) [7]. Similar results were obtained by Xie Q. et al. (2015) on the C2C12 mouse myoblast cell line. The CMKLR1-dependent pathway of chemerin led to increased ROS production and decreased the number of mitochondria through the formation of autophagosomes [13].

On the contrary, in obese patients with T2DM, increased mtDNA copy number in GO negatively correlated with the chemerin level ($r = -0.44$, $p < 0.05$). It is possible that in patients with T2DM coupled to AT hypertrophy and increased inflammatory background in AT and liver, the production of chemerin decreases below the level when it is unable to exert its effects.

We investigated gene expression (*TFAM*, *SOD1*, *DRP1*) to characterize mitochondrial quality control (MQC) processes (biogenesis, mitochondrial division

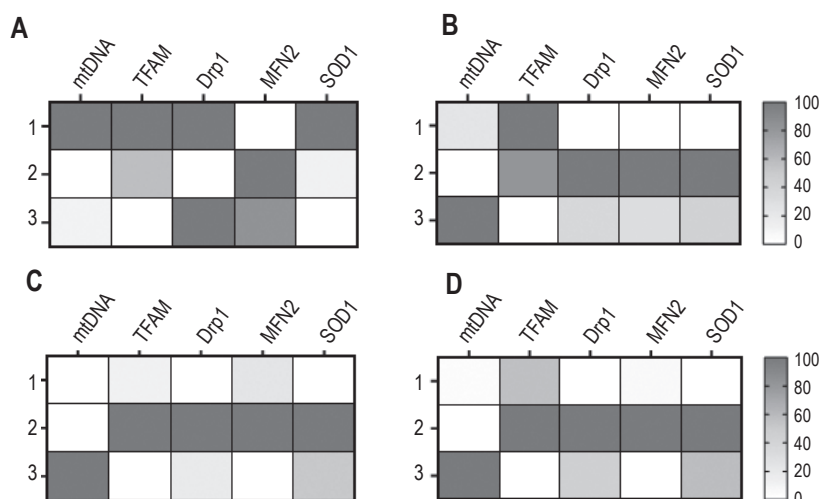


Figure 1. Heat map of the normalized expression levels of liver (A), greater omentum (B), mesentery of the small intestine (C) subcutaneous adipose tissue (D)

Note. 1, control group; 2, obesity without T2DM; 3, obesity with T2DM. Data are presented as a percentage.

TABLE 1. CLINICAL AND BIOCHEMICAL PARAMETERS OF THE STUDIED GROUPS

Indicators	Control group, n = 38	Obesity group without T2DM, n = 110	Obesity group with T2DM, n = 154
No.	1	2	3
BMI, kg/m²	22.20±8.40	41.80±9.20 $p_1 = 0.01^*$	45.10±7.10 $p_1 = 0.01^*$
Insulin, μU/ml	6.61 (5.00-12.48)	14.00 (8.35-23.46)	37.38 (28.60-73.76) $p_1 < 0.001^{**}$ $p_2 = 0.01^*$
Glucose, mmol/l	5.03 (4.67-5.91)	5.41 (4.84-6.15)	6.85 (5.58-8.80) $p_1 < 0.001^{**}$ $p_2 < 0.001^{**}$
Chemerin, ng/ml	141.00 (116.00-157.50)	173.00 (148.5-190.0) $p_1 < 0.001^{**}$	147.00 (133.8-173.5) $p_2 = 0.018^*$

and fusion) in various adipose tissue depots and liver biopsies of obese patients.

Mitochondrial biogenesis (the formation of new mitochondria) is the cooperation of two genomes (cellular and mitochondrial). Mitochondria contain core-encoded proteins (*PGC1 α* and β , *NFR-1*, 2) that regulate the transcription of mitochondrial genes.

Mitochondrial transcription factor A (*TFAM*) is a regulator of mtDNA transcription associated with changes in mitochondrial division/fusion and mtDNA replication [1].

In adipocytes of *Tfam*- knockout mice, the activity of proteins in complexes I, III, and IV of the respiratory electron transport chain was reduced, which led to the adipocyte death and AT inflammation.

Jin-Ho Koh et al. (2019) in mice with muscle-specific overexpression of human *TFAM* (hTFAM) showed that *TFAM* increases insulin sensitivity,

suppresses oxidative stress caused by a high-fat diet via enhanced antioxidant protection [4].

The expression level of *TFAM* increased in the SAT of patients without T2DM, which may point at increased mtDNA transcription under conditions of tissue inflammation. In GO and liver, the changes in gene expression were insignificant in study groups. We find no increased intensity of mitochondrial biogenesis in visceral AT and liver, but not in SAT (Figure 1).

ROS play an important role in the maintenance of mitochondrial dynamics. Mitochondrial dynamics are essential for maintaining a healthy mitochondrial population and depend on coordinated cycles of fusion and division. An imbalance in mitochondrial dynamics is associated with the pathophysiology of metabolic diseases such as obesity and T2DM, NAFLD.

Dynamin-related protein 1 (DRP1) regulates mitochondrial division and apoptosis. Mitophagy, a highly selective form of autophagy, utilizes damaged mitochondria. Disruption of mitophagy processes can lead to the accumulation of damaged mitochondrial components. In obese patients without T2DM, an increase in *DRP1* expression relative to control was revealed in all fat stores. On the contrary, in patients with diabetes, the *DRP1* expression was decreased. In liver of all obese patients with/without T2DM, the level of *DRP1* expression decreased compared to control. Strengthening the processes of division is associated with the degradation of mitochondria. In patients without T2DM, a decreased expression of the *DRP1* gene negatively correlated with increased concentration of chemerin ($r = -0.40$, $p < 0.05$) and steatosis ($r = -0.35$, $p < 0.05$) (Figure 1).

Chemerin can activate autophagy to remove damaged mitochondria. Potentially, liver biopsies from patients without T2DM had undisturbed balance between division and fusion. Fusion allows damaged mitochondria having oxidized lipids, proteins, and mutant mitochondrial DNA to mix with healthy ones that helps to restore mitochondrial function and maintain cellular homeostasis. However, we found no significant changes in *MFN2* expression.

Superoxide dismutase 1 (*SOD1*) regulates ROS levels that are produced by mitochondria in the respiratory chain. Changes in *SOD1* gene expression and its production may indicate the state of activity of the organ antioxidant system. The expression of *SOD1* gene was increased in the GO in obese patients with/without T2DM. An increase in *SOD1* expression in SAT was observed in patients without T2DM. *SOD1* expression decreased in MES and SAT in patients with T2DM relative to patients without T2DM. In liver biopsies, *SOD1* activity was reduced and negatively

correlated with detected steatosis ($r = -0.89$, $p < 0.05$). The multidirectional dynamics indicate a different effectiveness of the antioxidant system in liver and adipose tissue. The production of chemerin had a positive relationship with expressed *SOD1* ($r = 0.60$, $p < 0.05$) in GO patients without T2DM. In contrast, in patients with T2DM, negative relationships between chemerin and *SOD1* gene expression in MES were recorded ($r = -0.46$, $p < 0.05$). Thus, an increased level of chemerin in patients without T2DM can activate the antioxidant system in visceral AT, but not in liver (Figure 1).

Thus, an increased level of chemerin has a positive effect on mitochondrial quality control in patients without T2DM to maintain a balance between mitochondrial division and fusion in the liver. A decreased mtDNA copy number in liver may indicate activation of autophagy paralleled with high levels of chemerin, which leads to removal of damaged mitochondria. A decreased mtDNA copy number can be considered as an adaptation mechanism that protects against vicious cycle of ROS production. In visceral AT, chemerin can maintain the activity of the antioxidant system. In patients with T2DM, mitochondrial dysfunction is recorded, biogenesis processes, dynamics, antioxidant protection are suppressed along with elevated mtDNA copy number in all studied depots. The formation of inflammation and IR of AT and liver leads to decreased chemerin production. We hypothesize that stimulation of the production/administration of exogenous chemerin in T2DM can neutralize the effects of oxidative stress through activated autophagy. Further research is needed to find out how the ratios between different chemerin isoforms change during pathological processes and affect MQC processes in insulin-dependent tissues.

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ЦИТОКИНОВЫЙ ПРОФИЛЬ Th1/Th2/Th17 В ПЛАЗМЕ И ПОЛИМОРФИЗМ ГЕНОВ (*IL12B*, *IL13*, *IL31*, *IL33*) У БОЛЬНЫХ АСТМОЙ ДЕТЕЙ: МУЛЬТИПЛЕКСНЫЙ АНАЛИЗ

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Резюме. Изучение патогенеза бронхиальной астмы является актуальной проблемой в связи с ее высокой распространенностью и зачастую неконтролируемым развитием тяжелых форм, в том числе в детском возрасте. Первые признаки развития астмы обычно возникают в детстве, что приводит к ухудшению качества жизни пациента и ранней инвалидизации. Поскольку астма является генетически опосредованным процессом, предполагается, что тяжесть заболевания зависит от наличия определенного аллельного варианта в медиаторных (таких как цитокины) генах, участвующих в патогенезе БА. Целью данного исследования был поиск иммуногенетических маркеров тяжелого течения астмы у детей славянского происхождения, проживающих в г. Красноярск. Впервые с помощью метода мультиплексного анализа (xMAP) определены количественные показатели Th1/Th2/Th17-цитокинового профиля у больных бронхиальной астмой (БА) детей с различной степенью тяжести заболевания, в зависимости от полиморфизма генов цитокинов. Изменения цитокинового фона у больных БА укладываются в концепцию о том, что при тяжелых формах астмы возрастает доля нейтрофильного эндотипа заболевания, осуществляющего свои функции посредством Th1 и Th17-лимфоцитов. Кроме этого, получены данные цитокинового профиля в зависимости от наличия сопутствующих острых респираторных инфекций: показан дисбаланс уровня цитокинов, с тенденцией сохранения защитных функций иммунной системы у больных в состоянии ремиссии. Получено распределение в генах цитокинов: аллельные варианты *IL12B* rs321220*G, *IL13* rs1800925*C, *IL31* rs7977932*C и *IL33* rs7044343*T являются наиболее часто встречающимися в популяционной выборке г. Красноярска. Изучена вероятность ассоциации генотипов полиморфных генов цитокинов (*IL12B*, *IL13*, *IL31*, *IL33*) с состоянием иммунной системы при бронхиальной астме с различной степенью тяжести заболевания у детей: показана достоверная ассоциация генотипа *TT IL12B* rs321220 с низкой концентрацией IL-12B. Полученные данные можно использовать в комплексе с полученными нами ранее иммуногенетическими маркерами тяжелой степени и неконтролируемого течения астмы у детей с целью персонализированного прогноза характера заболевания.

Ключевые слова: бронхиальная астма, дети, цитокины, мультиплексный анализ, полиморфизм, тяжесть заболевания

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PLASMA Th1/Th2/Th17 CYTOKINE PROFILE AND CYTOKINE GENE POLYMORPHISMS (*IL12B*, *IL13*, *IL31*, *IL33*) IN ASTHMATIC CHILDREN: A MAGNETIC MULTIPLEX ASSAY

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Abstract. The study of the bronchial asthma pathogenesis is an urgent problem due to its high prevalence and often developing uncontrolled severe asthma, including in childhood. The first signs of asthma development tend to occur in childhood, which causes deterioration in the patient's quality of life and early disability. Since BA is a genetically mediated process, the severity of the disease is assumed to depend on the presence of a specific allelic variant in the mediator (e.g. cytokines) genes involved in the BA pathogenesis. The aim of this study was to search for immunogenetic markers of severe asthma in Slavs children living in Krasnoyarsk city. The quantitative indicators of the Th1/Th2/Th17-cytokine profile in children with bronchial asthma (BA) with varying disease severity, depending on the polymorphism of cytokine genes, using the method of multiplex analysis (xMAP), were first determined. Changes in the cytokine background in BA patients fit into the concept that a percentage of neutrophilic endotype, which performs its functions through Th1 and Th17-lymphocytes in severe asthma, increases. In addition, the cytokine profile data depending on concomitant acute respiratory infections were obtained. There was an imbalance when analyzing the cytokine plasma level, with a tendency to maintain the protective functions of the immune system among patients in remission. Distribution of cytokine genes was obtained: allelic variants of *IL12B* rs321220*G, *IL13* rs1800925*C, *IL31* rs7977932*C and *IL33* rs7044343*T are the most common in the population sampling from Krasnoyarsk. The probability of the genotype association of cytokine genes (*IL12B*, *IL13*, *IL31*, *IL33*) with the state of the immune system in bronchial asthma with varying disease severity in children was studied: a significant association of the *TT* genotype *IL12B* rs321220 with a low concentration of IL-12B was presented. Our data obtained can be used along with the previously obtained immunogenetic markers of severe and uncontrolled asthma in children for patient-specific prognosis of the disease nature.

Keywords: bronchial asthma, child, cytokines, multiplex assay, polymorphism, disease severity

Introduction

Bronchial asthma (BA) is the most severe disease of the bronchopulmonary system associated with chronic inflammation, mucus hypersecretion, hyper-reactivity and remodeling of the airways. There is a need for a detailed study of the developing severe forms and the search for methods to achieve asthma control due to its high prevalence. The first signs of asthma development tend to occur in childhood, which causes deterioration in the patient's quality of life and early disability. Bronchial asthma is a multifactorial disease (MD), i.e. its development appears to depend on both a variety of environmental factors and a genetic component. An unfavorable genetic background for this disorder is triggered by interacting with specific environmental factors and manifested in developing the pathological phenotype. Since BA is a genetically mediated process, the severity of the disease is assumed to depend on the presence of a specific allelic variant in the mediator genes involved in the BA pathogenesis.

The research on medical pathogenetics has been started in the early 1990s, when allelic polymorphism and the significance of individual allelic gene variants in predisposition to various diseases, their influence on the nature, severity and outcome of the disease were established [3]. The most common method of genetic MD studies is the candidate gene approach based on the analysis of a limited number of specific genes, with a choice being based on the identified or intended role of their expression products in the disease development [15]. Genetic analysis is mostly based on comparing frequencies of alleles and genotypes of certain genes in ill and healthy persons coupled to the studied pathology of among unrelated individuals (case-control study design).

Cytokines are important targets for the diagnostics of a wide range of human diseases, and their study is important for better understanding a mechanism of immunological changes observed in patients. Moreover, cytokines play an essential role in all stages of triggering and maintaining allergic inflammation, therefore, the study of their activity and regulation is

of great importance for understanding the molecular basis behind BA pathogenesis. The accumulated data indicate that Th2-cells and their cytokines such as IL-4, IL-5 and IL-13 play a critical role in the development and activation of allergic inflammation in asthma. Th1-lymphocytes, with specific cytokines (IL-2, IL-3, IFN γ , TNF α), regulate the “cellular” antigen-specific immune response, NK-cell function, phagocytic cells, and also participate in regulating IgM and IgG2 gene expression in B-lymphocytes. Cytokines produced by Th2-lymphocytes support IgE production and some other immunoglobulins (humoral immune response), and also participate in developing allergic inflammation by activating mast cells and eosinophils [12]. Recent studies have expanded the Th1/Th2-paradigm and have drawn attention to Th17-cells representing the third CD4⁺T-cell lineage, as they are responsible for a number of pathological processes [18].

To determine the cytokine profile in various biological fluids should be used for evaluating the inflammation activity, the polarization of immune response, the therapy effectiveness, and the disease prognosis. In recent years, the multiplex analysis has become widespread, including various formats for assessing cytokines in biological fluids using flow cytometry: xMAP (Multi-Analyte Profiling). This type of assay is based on binding to monoclonal antibodies fixed on the surface of microbeads conferring high sensitivity and specificity. Because xMAP technology is a method for determining metabolites in a small sample quantity, it makes it optimal for studying the patients in childhood. There is evidence on differences in production of certain cytokines among populations in different ethnic groups [1]. This results from different distribution of cytokine gene alleles in racial groups. In different populations, accurate reproduction of the obtained reliable association between individual polymorphisms and certain medical condition is rarely achieved, suggesting the specificity and population characteristics of the frequency distribution of polymorphic variants of the analyzed genes. Hence, when studying polymorphic gene variants, it is necessary to take into account human population characteristics.

Although BA symptoms in most patients with mild disease are well controlled with current treatment methods, around 10% of patients with severe asthma show poor control and increased risk of developing severe asthma despite a large baseline therapy [6]. Since the spectrum of genes for BA susceptibility cannot be considered as fully identified, the analysis of hereditary factors is a promising area for allergology, dermatology, and immunogenetics. A comprehensive analysis of cytokine genetic polymorphism and level of relevant protein products, in case of their participation in the BA pathogenesis related to disease

severity, seems promising for developing a personalized approach to determine disease susceptibility and severity. Preclinical risk diagnostics for developing multifactorial diseases including asthma, can help to lower cost of expensive treatment as well as to preserve human working capacity preventing disability.

Materials and methods

A total of 546 individuals were involved in the study: 317 bronchial asthma (mean age 13.6 $\pm$ 2.5 years) and 229 healthy individuals from the city of Krasnoyarsk. The control group included children (n = 127) and adults with no history of asthma and allergies (n = 102). No statistically significant differences were revealed between the control groups of different ages, which allowed to randomize individuals of different ages into one control group. Children with asthma were divided into groups depending on the severity of the disease: mild asthma (n = 133), moderate asthma (n = 102), severe asthma (n = 82). The diagnostics and severity of the disease were determined in accordance with the recommendations of the GINA working group. All examined patients met the general inclusion criteria for the study: BA diagnosis, severe /moderate course, absence of acute upper respiratory tract infection (URTI) and other acute diseases upon examination, as well as Slavic descent for the last three generations. Inclusion criteria for patients to be included in the control group: apparently healthy individuals, negative allergic history, total IgE level < 100 IU/ml, Slavic descent.

All study participants or their parents provided written informed consent prior to the study. The protocol for examining ill and healthy subjects complied with ethical standards and was approved by the Biomedical Ethics Committee of the Research Institute of Medical Problems of the North, Krasnoyarsk Scientific Center, Siberian Branch, Russian Academy of Sciences.

The research materials were presented by patient serum and DNA extracted from the peripheral blood by the sorbent method using the DIAtom DNAprep100 kit (OOO Isogen, Russia). Genotyping of polymorphisms *IL12B* (rs321220), *IL13* (rs1800925), *IL31* (rs7977932) and *IL33* (rs7044343) was performed using the real-time polymerase chain reaction (RT-PCR) as well as specific oligonucleotide primers and fluorescently labeled probes (TagMan) (DNA-synthesis, Russia).

Plasma cytokine concentrations were determined with magnetic microsphere test systems MILLIPLEX MAP Human TH17 (Merck, USA) using multiplex analysis by xMAP technology (Luminex, USA) according to the manufacturer's instructions. Data registration and analysis were performed with a Luminex MAGPIX device (Luminex, USA).

Differences at the $p < 0.05$ level were considered as statistically significant. The allele and genotype frequencies between groups were compared by using χ -square test and online calculator. An odds ratio (OR) with a 95% confidence interval (CI) was performed to assess a relationship between genetic markers and pathology phenotypes. Statistical significance of differences in quantitative traits was analyzed using the Mann–Whitney test. Data are expressed as median and interquartile range.

Results and discussion

In multiplex analysis, the values of the cytokine concentration produced by many immune cell types in pediatric bronchial asthma with varying severity and different status of disease exacerbation ($n = 106$) were obtained. We analyzed the concentration of 17 cytokines; significant differences are shown in the Tables.

The development of allergic reactions is known to be affected by a complex interplay in cytokine network with multiple reciprocal relationships [12]. A number of mediator genes and their interaction with environmental factors, hereditary predisposition, response of respiratory and immune systems enable to distinguish acute URTI BA-phenotypes. The most common is neutrophilic asthma (up to 81.8%), especially its severe form. Identifying BA-phenotypes/endotypes provides deeper insights into the essence of disease and is necessary to develop individualized therapy approach. However, currently, no consensus on the criteria for distinguishing phenotypes has been achieved due to the lack of specific biomarkers for the majority of phenotypes.

Our study on serum cytokine profile analyzed the data obtained from children with varying severity of bronchial asthma. Our data on BA patients with diverse cytokine background fit into the concept that a percentage of neutrophilic endotype executing functions through Th1- and Th17-lymphocytes in severe asthma, increases. The level of cytokines produced by various types of immunocompetent cells (Th1, Th2, Treg, Th17) has been shown to be significantly lower for severe BA patients, i.e decreased expression numerous pro- and anti-inflammatory cytokines associated with disease severity ($p < 0.05$). It suggests increased suppression due to inflammatory processes in the bronchopulmonary system and decreased protective functions as well as transition of the disease to uncontrolled course (Table 1).

One of the main factors in regulating proliferative response of B-lymphocytes and immunoglobulin isotype switching inducing expression of IgG and IgE is provided by IL-4, a growth factor for T-lymphocytes and mast cells, a key factor for the CD4⁺T-cell differentiation into type Th2-cells that affects their functioning [16]. Along with other cytokines such as

G-CSF and IL-6, IL-4 can stimulate growth of mast cell and myeloid and erythroid cell precursors. There has been noted that the level of IL-4 in the peripheral blood serum and plasma is increased in patients with asthma and related comorbidities [17]. Nevertheless, the systemic serum amount of IL-4 in BA patients has been observed in numerous studies to reflect disease clinical course, so that its imbalanced level was observed during disease exacerbation [9, 17]. The IL-4 and IL-6 concentration in patients with severe asthma observed in our study was 0.11 and 0.02 pg/ml, respectively, which were significantly lower than in mild asthma ($p < 0.05$).

Neutrophilic inflammation, characterized by an aggressive course, pronounced tissue destruction, low response to corticosteroids, is typical for children with bronchial asthma [7]. This phenotype is associated with the active neutrophilic products such as neutrophil elastase, α 1-antitrypsin, IL-8, IL-17, as well as Th17-cells for their maintenance [5]. Th17-cells differs from Th1, Th2 and Treg-cells characterized by the IL-17 family cytokine production. The IL-17 family has been recently described and includes IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (known as IL-25), and IL-17F, according to the order of discovery [11]. The ability of IL-17A and IL-17F to induce neutrophil migration suggests that they are involved in the pathogenesis of severe BA mainly characterized by neutrophilic airway inflammation [10]. The association between IL-17A and IL-17F and airway inflammation has also been confirmed experimentally in mouse asthma models. The role of these two cytokines has also been shown in development of eosinophilic inflammation. In fact, mice deficient in IL-17 receptors or IL-17 show reduced potential for eosinophils to migrate into the respiratory tract due to low expression of Th2 cytokines such as IL-4 and IL-5 acting as eosinophil chemokines [14]. Thus, the identified functions of IL-17 suggest that the airways may be affected by this potent proinflammatory cytokine in different directions. Understanding the effector functions of Th17-cells during inflammation in the bronchopulmonary system may be a key to unequivocal understanding of the BA pathogenesis [4, 8].

BA exacerbation is known to be aggravated by concomitant acute respiratory infections. The patients without exacerbation (remission) (group 1), patients with concomitant acute URTI (group 2) and patients without concomitant acute URTI (group 3) were involved in the study. An imbalanced cytokine profile between groups of patients was found that tended to maintain immune protective functions among patients in remission. The IL-17A and IL-17F concentration in patients with severe vs. mild BA obtained in the study was lowered (data not shown

TABLE 1. CYTOKINE PLASMA LEVEL IN BRONCHIAL ASTHMA PATIENTS WITH DIFFERENT SEVERITY, Me ($Q_{0.25}$ - $Q_{0.75}$), pg/ml

Cytokine	Mild asthma (1), n = 58	Moderate asthma (2), n = 37	Severe asthma (3), n = 11	p
IFN γ	9.69 (6.92-18.06)	9.69 (6.01-18.98)	4.02 (1.05-7.84)	$p_{1,3} = 0.02$ $p_{2,3} = 0.04$
IL-10	12.05 (6.92-16.79)	13.04 (6.12-17.49)	4.66 (2.99-9.79)	$p_{1,3} = 0.01$ $p_{2,3} = 0.01$
IL-9	20.81 (14.10-35.85)	25.98 (7.17-42.91)	12.03 (0-15.46)	$p_{1,3} = 0.01$
IL-1 β	15.27 (11.02-18.42)	20.68 (15.94-23.82)	18.98 (14.97-28.34)	$p_{1,2} = 0.01$ $p_{1,3} = 0.04$
IL-33	50.93 (36.74-72.87)	65.42 (51.57-73.49)	57.84 (26.78-76.57)	$p_{1,2} = 0.05$
IL-4	0.24 (0.14-0.29)	0.20 (0.11-0.32)	0.11 (0.06-0.19)	$p_{1,3} = 0.01$ $p_{2,3} = 0.02$
IL-6	26.02 (8.43-43.46)	25.75 (9.01-48.97)	0.02 (0.01-16.42)	$p_{1,3} = 0.01$ $p_{2,3} = 0.03$
IL-27	0.83 (0.62-1.13)	1.05 (0.85-1.34)	1.08 (0.96-1.42)	$p_{1,2} < 0.01$ $p_{1,3} = 0.03$
IL-28A	0.88 (0.63-1.43)	1.08 (0.56-1.46)	0.20 (0.00-1.27)	$p_{2,3} = 0.05$

in Table 1, $p > 0.05$). Regarding disease remission, the concentration of such cytokines was significantly higher than in exacerbations with or without concomitant acute URTI (Table 2).

It was noted above, that the level of protein expression and production was genetically determined and depended on specific polymorphic gene variants. The concentration level of the studied cytokines depending on the genotype of coding cytokine genes exemplified by *IL12B*, *IL13*, *IL31* and *IL33* was comprehensively analyzed in our study.

Based on the distribution frequency of cytokine gene polymorphisms in children with BA, features in the allelic variants distribution of the genes *IL12B* (rs321220), *IL13* (rs1800925), *IL31* (rs7977932), *IL33* (rs7044343) specific to Caucasians were shown. Thus, according to the website <http://www.ensembl.org>, the global Caucasian population seems to carry prevalent alleles *IL12B**G (90%), *IL13* *C (75%), *IL31**C (84%) и *IL33**T (63%). In our sampling of Slavic subjects from the Eastern Siberia were found to carry *IL12B**G (76%), *IL13* *C (75%), *IL31**C (88%) and *IL33**T (60%), respectively. While analyzing the distribution frequency of the *IL12B* (rs321220) polymorphism, a significant difference in the genotype frequencies between BA patients and control group was demonstrated. Statistically different data on the genotype distribution between the groups with moderate BA and control (OR 0.63 (0.41-0.97),

$p = 0.035$) were obtained. The frequency of the GG genotype in the moderate asthma was higher than in the control group (71.6% versus 57.2%), whereas the frequency of the GT and TT genotypes – higher in the control group than in moderate asthma (5.2% versus 4.9%; 37.6% versus 23.5%, respectively). Comparing the distribution frequency of the *IL13* rs1800925 polymorphism detected significant difference in the genotype frequencies between BA patients and control group. The variant allele T * was found to be more common in patients, regardless of disease severity compared to the control ($p = 0.01$). The frequency of CT and TT genotypes in BA patients is significantly higher than in the control group. Moreover, the frequency of TT genotype associated with increased IL-13 concentration was higher but at least 2-fold than in control group of children with severe BA (13.4% versus 5.7%, OR 1.67, $p = 0.01$). Thus, this genotype serves as a BA genetic marker predisposing to the pathologic behavior, i.e. the development of severe BA. At the same time, the CC genotype rs1800925 protects against the BA development as well as severe asthma.

While analyzing IL-12B plasma level in children with bronchial asthma, depending on the genotype, significant differences were revealed. In particular, a significant association between the TT genotype *IL12B* rs3212220 and low IL-12B concentration was found. Importantly, no published data are available

TABLE 2. CYTOKINE PLASMA LEVEL IN BRONCHIAL ASTHMA PATIENTS WITH DIFFERENT TYPE OF EXACERBATION, Me (Q_{0.25}-Q_{0.75}), pg/ml

Cytokine	Remission (1), n = 67	Exacerbation with acute URTI (2), n = 15	Exacerbation without acute URTI (3), n = 22	p
IL-17F	0.03 (0.00-0.06)	0.01 (0.00-0.02)	0.01 (0.00-0.04)	p _{1,2} = 0.04
IFN γ	11.55 (6.92-18.98)	7.38 (3.38-16.67)	7.84 (2.55-10.62)	p _{1,3} = 0.05
IL-17A	10.74 (6.66-13.94)	8.69 (3.62-15.31)	5.77 (3.41-10.51)	p _{1,3} = 0.01
IL-1b	15.55 (11.22-23.27)	20.68 (17.28-23.27)	16.81 (14.39-23.82)	p _{1,2} = 0.05
IL-6	25.21 (3.75-43.46)	16.42 (0.01-41.43)	27.36 (0.03-60.72)	p _{1,2} < 0.01
IL-27	0.83 (0.64-1.05)	1.08 (0.87-1.40)	1.23 (0.99-1.43)	p _{1,2} = 0.02 p _{1,3} < 0.01

on the association of this polymorphism with the expression level. The IL-12B plasma level in the *TT* genotype patients was 3.96 (2.98-8.42) pg/ml compared to the alternative homozygote *GG* – 12.73 (8.42-17.11) pg/ml and the heterozygote *GT* – 10.44 (6.42-15.04) (p < 0.01). The association of this polymorphism with the level of intra- and extracellular expression of the pro-inflammatory Th1-cytokine (IL-12B) can be assumed to determine the BA development. Another previously studied *IL12B* polymorphism (rs3212227) affects the expression level, i.e. allelic variant C is associated with decreased production of IL-12B [13].

Analysis of the associative relationships of the genotypes *IL13* (rs1800925), *IL31* (rs7977932) and *IL33* (rs7044343) showed no significant differences in the concentration level of the corresponding cytokines. It is noteworthy that the level of IL-13 in the plasma of BA patients in carrying the *CT* and *TT* vs. *CC* genotypes of *IL13* (rs1800925) is higher. According to the literature mentioned above, the allelic variant T of this SNP is associated with increased production of IL-13, airway hyperresponsiveness most often occurring in severe disease [2].

Conclusion

The immunological profile of multifactorial diseases such as bronchial asthma is a complex network of cytokines, chemokines and growth factors. As a result, it is worth studying asthma by using multiplex analysis as a modern tool for determining metabolite concentration, even in trace amounts that prominently differs from the ELISA.

Polymorphic cytokine genes are one of the most studied genetic markers, as the products of these genes are regulators of the main immune and inflammatory processes in the body. Determining the hereditary factors of BA along with the assessing cytokine level in biological fluids will allow to study disease immunopathogenesis and develop some personalized approaches to determining the disease severity and the appropriate therapy.

In the study, an integrated approach was applied to studying both the polymorphic cytokine genes produced by immune cells and concentration of expressed protein products involved in the BA pathogenesis in children with varying disease severity.

The data obtained for the first time provide insight into the variant distribution in the cytokine genes *IL12B*, *IL13*, *IL31*, *IL33*, produced by different immune cells in patients with bronchial asthma and in the population sampling of the Krasnoyarsk residents. According to the data, the allele and genotype frequencies for the studied polymorphisms in the Slavic subjects of the Eastern Siberia correspond to those in found other Caucasoid populations worldwide. Allelic variants of *IL12B*G*, *IL13*C*, *IL31*C* and *IL33*T* are the most common in the population sampling of the city of Krasnoyarsk.

The genetic markers of susceptibility to severe bronchial asthma in children have been identified: allelic variant of *G*IL12B rs321220* and variant allele of *T*IL13 rs1800925*. According to the literature, the products encoded by these genes are involved in allergic asthmatic airway inflammation. Therefore, the genetic polymorphism analysis enables to establish the pathogenetic role in emerging specific clinical BA

course in the Eastern Siberia population. The human populational characteristics should be taken into account. In different populations, an accurate reproduction of the obtained reliable associations between individual polymorphisms and a certain pathological state is rarely achieved, suggesting the specificity and populational features in frequency distribution of polymorphic variants for analyzed genes. In addition, there is evidence about differences in produced some cytokines among various ethnic populations.

When analyzing the cytokine profile in BA patients, complex data fitting into the concept that severe asthma is coupled to a percentage of neutrophilic endotype that exerts its functions through Th1 and Th17-lymphocytes. The level of cytokines produced by various immunocompetent cells is significantly lower for severe BA patients, i.e. a decreased expression for several pro- and anti-inflammatory cytokines correlates with the disease severity ($p < 0.05$).

Achieving control of severe asthma is a challenge and requires a multifaceted approach, taking into

account the phenotypic characteristics of severe bronchial asthma. Severe asthma is a common problem and significantly reduces the quality of life for patients and their families, leading to frequent and severe exacerbations, decreased lung function, and limited professional and social activity.

To sum up, we can conclude that prognosis criteria for the development of severe bronchial asthma is presented as a combination of genotypes for pro- and anti-inflammatory cytokines obtained in the current study and earlier, as well as the level of concentration of immune response mediators combined with clinical characteristics. To identify general and specific immunogenetic markers it opens up a potential for populational screening, as well as patients with multifactorial diseases, including bronchial asthma, which allows to find out risk factors for developing pathology before clinical manifestations as well as to predict a probability for developing severe and uncontrolled disease and apply personalized approach to treatment strategy of patients.

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РОЛЬ ПОЛИМОРФИЗМА ГЕНОВ TLR2, TLR4 В ФОРМИРОВАНИИ МИКРОСОСУДИСТЫХ ОСЛОЖНЕНИЙ У ПОДРОСТКОВ С САХАРНЫМ ДИАБЕТОМ 1-ГО ТИПА

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Резюме. Отсроченные осложнения сахарного диабета 1-го типа (СД1) у детей и подростков являются важной проблемой современной медицины. В последние годы активно обсуждается роль иммунных механизмов, в частности хронического воспаления в развитии как СД1, так и его микрососудистых осложнений. Активация Toll-like рецептора 2 (TLR2) и Toll-like рецептора 4 (TLR4) приводит к индукции синтеза провоспалительных цитокинов, хемокинов, молекул адгезии, участвующих в формировании диабетических микрососудистых осложнений. При этом наличие генетического полиморфизма TLR2 и TLR4 изменяет иммунное реагирование в ответ на их стимуляцию эндогенными лигандами, что может повышать риск возникновения диабетических микроангиопатий. Цель исследования – изучить распределение частот генотипов и аллелей генов TLR2 и TLR4 и определить содержание TNF α , IL-1, VCAM-1, фракталин, эндотелин-1 у подростков с СД1 и микрососудистыми осложнениями. Обследовано 139 подростков с СД1 от 14 до 18 лет и 56 здоровых подростков. Пациенты с СД1 распределены на две группы: I группа – пациенты с декомпенсацией СД1, (HbA1C > 9,0%), (n = 64); II группа – пациенты с удовлетворительным гликемическим контролем СД1 (HbA1C $\leq$ 9,0%), (n = 75). В зависимости от наличия осложнений выделены 4 подгруппы: Ia (n = 49), IIa (n = 38) – подростки с микрососудистыми нарушениями: Ib (n = 15), IIb (n = 37) – с неосложненным течением заболевания. Определение аллельных вариантов генов TLR проводили с использованием тест-систем ГосНИИ генетика (Москва). Определение содержания цитокинов в сыворотке крови осуществлялось методом твердофазного иммуноферментного анализа BIOSCIENCE. Обработка данных проводилась с использованием пакетов прикладных программ Statistica 6.0. При изучении распределения полиморфизмов TLR2 (Arg753Gln) и TLR4 (Thr399Ile) не было выявлено достоверных различий между исследуемыми подгруппами и контролем. Отмечено, что вариант Asp299Gly в Ia и IIa подгруппах (с осложненным течением) встречался достоверно реже, чем в Ib, IIb подгруппах и контролем. Установлено, что наличие аллеля Gly в гене TLR4 влияет на уровень экспрессии TNF α и VCAM-1 в сыворотке крови и может считаться протективным по развитию микрососудистых осложнений.

Ключевые слова: сахарный диабет 1-го типа, подростки, микрососудистые осложнения, полиморфизм генов TLR-рецепторов, цитокины

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ROLE OF TLR2, TLR4 GENE POLYMORPHISM IN DEVELOPING MICROVASCULAR COMPLICATIONS IN ADOLESCENTS WITH TYPE 1 DIABETES MELLITUS

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Abstract. Long-term complications of type 1 diabetes mellitus (T1DM) in children and adolescents are an important problem in modern medicine. Recently, the role of immune mechanisms, in particular, chronic inflammation, in the development of both T1DM and its microvascular complications has been actively discussed. Activation of Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) leads to hyperproduction of proinflammatory cytokines, chemokines, adhesion molecules involved in the formation of diabetic microvascular complications. At the same time, TLR2 and TLR4 gene polymorphism alters the immune susceptibility to the endogenous ligands, which may increase the risk of diabetic microangiopathies. The aim of this study is to evaluate the frequency of genotypes and alleles of TLR2 and TLR4 genes distribution and to determine the content of TNF α , IL-1, VCAM-1, fractalkine, endothelin-1 in adolescents with T1DM with microvascular complications. We examined 139 adolescents with T1DM from 14 to 18 years old and 56 healthy teenagers. Patients with T1DM were divided into two groups: Group I – patients with poor glycemic control (HbA1C > 9.0%), (n = 64); Group II – patients with satisfactory glycemic control of T1DM (HbA1C $\leq$ 9.0%), (n = 75), including adolescents with optimal (HbA1C < 7.5%) and suboptimal glycemic control (7.5% $\leq$ HbA1C $\leq$ 9.0%) (ISPAD clinical practice consensus guidelines 2014). According to the presence of microvascular complications, the groups were subdivided into subgroups: Ia (n = 49), IIa (n = 38) – adolescents with verified microvascular disorders: diabetic retinopathy, nephropathy and neuropathy; Ib (n = 15), IIb (n = 37) – without microvascular complications. Allelic variants of TLR genes were determined using test systems GosNII genetics (Moscow). The content of cytokines in blood serum was carried out by the method of enzyme-linked immunosorbent assay “BIOSCIENCE”. Data were analyzed using software packages Statistica version 6.0. The assessment of TLR2 (Arg753Gln) and TLR4 (Thr399Ile) polymorphism distribution did not reveal significant differences between the observed subgroups and the control. In Ia and IIa subgroups (with complications) Asp299Gly variant was noted to be significantly less common when compared to subgroups Ib, IIb and controls. The presence of Gly allele in TLR4 gene was found to disrupt the expression of TNF α and VCAM-1 and can be considered protective for the development of microvascular complications.

Keywords: type 1 diabetes mellitus, adolescents, microvascular complications, TLR receptors gene polymorphism, cytokines

Introduction

Long-term microvascular complications affecting both the quality of life and life expectancy of patients with type 1 diabetes mellitus (T1DM) are the primary issues in modern medicine. It should be noted that the incidence of microvascular complications 2-5 years after the onset of T1DM in adolescents is higher than predicted compared to other age groups [10]. Optimal metabolic control during childhood and adolescence is critical for the prevention of prematurely formed vascular complications in T1DM [4]. Many studies report risk factors such as arterial hypertension, microalbuminuria, dyslipidemia, aggravating existing disorders [2]. At the same time, a significant part of adolescents with T1DM, despite having close to

the target glycemic profile, show the initial signs of microvascular complications soon after the onset of the underlying disease, which suggests the presence of other concomitant factors, such as environmental, hormonal, immunological, genetic factors, creating the prerequisites for their early formation.

Current studies highlight an important role of vascular inflammation in development of diabetic nephro-, neuro-, retinopathy. Identification of the primary links in cascade of proinflammatory reactions in the vascular wall is of particular interest for developing new diagnosing strategies and early etiopathogenetic treatment, as well as identifying risk groups for microangiopathies. The most promising approach is to examine innate immunity receptors – Toll-like receptors (TLRs) and their genetic poly-

morphism. It is assumed that TLRs recognizing not only exogenous ligands (bacterial, viral, and other infectious agents), but also endogenous pathogens (acute phase proteins, oxidized lipoproteins), formed under the influence of metabolic changes existing in T1DM, cause an immune-inflammatory process and activate genes of adaptive response [5]. TLR2 and TLR4 are the main components of innate immunity, that stimulate subsequent gene expression of cytokines and other signal peptides. Moreover, TLR4 dysregulation contributes to multiple pathophysiological changes responsible for impaired glucose homeostasis, breaking the vicious circle of metabolic and immune abnormalities [1].

In the literature, an increased risk of diabetic nephropathy and retinopathy in T1DM patients with Arg753Gln TLR2 variant is reported, and the Asp299Gly polymorphism of the TLR4 gene is currently considered to be responsible for the development of retinopathy due to inflammation-induced angiogenesis [14, 16]. Thus, the study of TLR2 and TLR4 gene polymorphisms can be useful in predicting microvascular complications in patients with diabetes mellitus. At the same time, there are few studies that assessed modulating influence of different genetic variants of TLR2 and TLR4 on early manifestation of specific diabetic complications in adolescents with T1DM with different glycemic control.

Purpose of the work is to investigate the frequency of genotypes and alleles of the TLR2 and TLR4 genes distribution and to determine level of TNF α , IL-1, VCAM-1, fractalkin, endothelin-1 in adolescents with T1DM with microvascular complications.

Materials and methods

139 adolescents aged 14 to 18 years old, treated in the pediatric endocrinology department of Rostov State Medical University during the years 2012–2015 were examined. Adolescents with clinical and laboratory signs of acute inflammatory diseases or exacerbation of chronic infection at the time of the study were excluded. The control group consisted of 56 healthy adolescents with normal parameters of weight and height, without disorders of carbohydrate metabolism, without burdened by diabetes mellitus heredity.

All participants and their parents signed informed consent to be enrolled to the study. The study protocol was approved by the ethics committee.

The adolescents with T1DM underwent a number of tests to assess the metabolic control, early diagnosis of diabetic complications and determine immunological and genetic parameters. Screening for diabetic complications included examination by a neurologist, ophthalmologist according to the

standard technique, ophthalmoscopy to detect fundus microcirculation disorders.

To verify diabetic nephropathy, we studied twice the level of microalbumin in the sediment of the morning portion of urine, serum urea and creatinine. In addition, the levels of glycosylated hemoglobin (HbA1c) were determined in the examined patients by latex inhibition of agglutination (Randox, Great Britain).

The diagnostics of specific complications of type 1 diabetes and assessment of quality of glycemic control by HbA1c level was carried out according to 2014 ISPAD clinical practice consensus guidelines [6].

All patients with T1DM were divided into two groups: Group I – patients with poor glycemic control (HbA1c > 9.0%), (n = 64); Group II – patients with satisfactory glycemic control of T1DM (HbA1c $\leq$ 9.0%), (n = 75), including adolescents with optimal (HbA1c < 7.5%) and suboptimal glycemic control (7.5% $\leq$ HbA1c $\leq$ 9.0%). According to the detected microvascular complications, the groups were subdivided into subgroups: Ia (n = 49), IIa (n = 38) – adolescents with verified microvascular disorders: diabetic retinopathy, nephropathy and neuropathy; Ib (n = 15), IIb (n = 37) – without microvascular complications.

Determination of allelic variants genes TLR-2, TLR-4 was carried out using PCR method with subsequent restriction analysis by using a commercial test system for molecular genetic analysis GosNII genetics (Moscow).

The level of serum TNF α , IL-1, VCAM-1, fractalkin, endothelin-1 was determined by the method of enzyme-linked immunosorbent assay using “BIOSCIENCE” reagents.

To form the database and conduct a statistical study of empirical data, we used the capabilities of the Excel 2003 spreadsheet processor and application packages (Megastat and Statistica 6.0). To determine the statistical validity of the differences between the studied groups, the Mann–Whitney test was used for independent groups with the maximum allowable level of type I error equal to $p = 0.05$. The statistical significance of the differences between the compared groups or subgroups for frequencies of genotypes and alleles of the studied genes was assessed using the Fisher test or the chi-square by using the standard formula, taking into account Yates' correction for paired comparisons.

Results and discussion

The clinical characteristics of the patients did not reveal differences in anthropometric parameters, gender, the prevalence of comorbidity among adolescents with T1DM in the studied subgroups. While

verifying the specific complications of T1DM, it was found that 77.6% of adolescents with poor glycemic control and half patients with satisfactory glycemic control of T1DM had microvascular complications.

In the observed group of adolescents with T1DM, most common were peripheral neuropathy (I group – 43.8%, II group – 33.3%), early stages of diabetic retinopathy (mild to moderate non-proliferative retinopathy) (I group – 43.8%, II group – 30.7%) and nephropathy (i.e. microalbuminuria) (I group – 20.3%, II group – 9.3%). At the same time, patients

in I and II groups did not statistically differ in the prevalence of microangiopathies.

There were no significant differences in distribution of TLR2 (Arg753Gln) and TLR4 (Thr399Ile) gene polymorphisms between control and T1DM subgroups with and without complications (Table 1).

However, studying the distribution of TLR4 (Asp299Gly) gene polymorphisms, the frequency of the A/G genotype in subgroups Ia and IIa compared to subgroups Ib and IIb was lower by 4.1 and 2.3 times, respectively, ($p < 0.05$), and lower than in the

TABLE 1. FREQUENCY DISTRIBUTION OF GENOTYPES AND ALLELES OF TLR2 AND TLR4 GENES IN ADOLESCENTS WITH TYPE 1 DIABETES MELLITUS

			Clinical groups				Control (n = 56)
			I (n = 64)		II (n = 75)		
			Ia (n = 49)	Ib (n = 15)	Ila (n = 38)	Ilb (n = 37)	
TLR2 (Arg753Gln)	Genotype	Arg/Arg	42 (85.7%)	14 (93.3%)	35 (92%)	32 (86.5%)	53 (94.6%)
		Arg/Gln	7 (14.3%)	1 (6.7%)	3 (7.9%)	5 (13.5%)	3 (5.4%)
		Gln/Gln	0	0	0	0	0
	Alleles	Arg	91 (92.9%)	29 (96.7%)	73 (96.1%)	69 (93.3%)	109 (97.3%)
		Gln	7 (7.1%)	1 (3.3%)	3 (3.9%)	5 (6.8%)	3 (2.7%)
TLR4 (Thr399Ile)	Genotype	Thr/Thr	37 (75.5%)	13 (86.7%)	30 (78.9%)	32 (86.5%)	48 (85.7%)
		Thr/Ile	12 (24.5%)	2 (13.3%)	8 (21.1%)	5 (13.5%)	8 (14.3%)
		Ile/Ile	0	0	0	0	0
	Alleles	Thr	86 (87.8%)	28 (93.3%)	68 (89.5%)	69 (93.2%)	104 (92.9%)
		Ile	12 (12.2%)	2 (6.7%)	8 (10.5%)	5 (6.8%)	8 (7.1%)
TLR 4 (Asp299Gly)	Genotype	Asp /Asp	45 (91.8%)*▲	10 (66.7%)	34 (89.5%)*Δ	26 (70.3%)	35 (62.5%)
		Asp /Gly	4 (8.2%)*▲	5 (33.3%)	4 (10.5%)*	9 (24.3%)	16 (28.6%)
		Gly/Gly	0	0	0	2 (5.4%)	5 (8.9%)
	Alleles	Asp	94 (95.9%)*▲	25 (83.3%)	72 (94.7%)*Δ	61 (82.4%)	86 (76.8%)
		Gly	4 (4.1%)*▲	5 (16.7%)	4 (5.3%)*Δ	13 (17.6%)	26 (23.2%)

Note. Difference from the control: *, $p < 0.05$; difference from Ib subgroup: ▲, $p < 0.05$; difference from IIb subgroup: Δ, $p < 0.05$.

TABLE 2. CYTOKINE PRODUCTION IN ADOLESCENTS WITH TYPE 1 DIABETES MELLITUS WITH TLR-4 ASP299GLY GENE POLYMORPHISM

pg/ml	Clinical groups			
	Ia		IIa	
	Patients with genotype Asp/Asp	Patients with genotype Asp/Gly	Patients with genotype Asp/Asp	Patients with genotype Asp/Gly
TNFα	293,2* (255.3-300.4)	176,5 (150.2-185.9)	8,5 (6.4-50.9)	6,1 (6.4-50.9)
IL-1	21,1 (17.5-27.6)	14,7 (13.6-17.2)	1,8 (0.9-7.3)	1,5 (0.9-7.3)
VCAM-1	357,2* (320.4-370.3)	275,1 (230.4-300.1)	298,4 (237.7-418.4)	321,1 (237.7-418.4)
Fractalkine	0,89 (0.55-0.94)	0,75 (0.72-0.88)	0,9 (0.8-1.0)	1,1 (0.8-1.0)
Endothelin-1	1,87 (1.77-1.95)	1,51 (1.44-1.88)	0,9 (0.5-1.1)	0,8 (0.5-1.1)

Note. Difference from the patients with genotype Asp/Gly: *, $p < 0.05$.

control by 3.5 and 2.7 times, respectively, ($p < 0.05$) was noted.

Thus, according to the distribution of TLR4 Asp299Gly gene polymorphism, subgroups with microvascular disorders, both with poor and satisfactory glycemic control, were distinguished. In their genotype, the frequency of the Gly allele was significantly lower (subgroup Ia – 4.1%; subgroup IIa – 5.3% and 23.2%).

It is noteworthy that homozygous Gly299Gly TLR4 gene was found only among healthy individuals and patients with T1DM without complications. From our data, it appears that the absence of the Gly allele in the genotype increases the risk of complications (Ia/Ib – OR = 4.7; IIa/IIb – OR = 3.14) both in T1DM groups with poor and satisfactory glycemic control.

Our data are consistent with the results on contribution of polymorphic variants Asp299Gly and Thr399Ile to changes in TLR4 functioning [10] showing that presence of Asp299Gly polymorphism in cells interacting with endogenous and exogenous ligands results in inhibition of developing proinflammatory regulatory peptides compared to those without analogous mutations.

A number of studies revealed [18] an increase in IL-1 β and TNF α levels creating conditions for enabling inflammatory component of specific diabetic complications at the microcirculatory level in tissues of different locations.

Since the polymorphism in TLR2 and TLR4 genes is associated with changes in serum cytokine con-

centration and correlates with the degree of capillary wall damage, it is of great interest to assess the effect of Asp299Gly polymorphism on the level of cytokine production in adolescents with poor and satisfactory glycemic control. The data obtained presented in Table 2 demonstrated the relationship between the Asp/Gly TLR4 genotype and parameters of body nonspecific defense.

In the group with poor glycemic control the level of TNF α and VCAM-1 in patients with the Gly allele of the TLR4 gene in a heterozygous state were found to be significantly lower than in individuals with T1DM without this polymorphism ($p < 0.05$). Meanwhile, there were no significant differences in the level of the studied cytokines and adhesion molecules between adolescents with Asp299Gly and Asp299Asp variants in satisfactory control group, that confirms the hypothesis that the hyperglycemic environment in T1DM is a key trigger activating TLR4, responsible for the initiation of inflammatory damage to target organs. At the same time, the presence of Asp299Gly polymorphism TLR4 gene is currently considered to be a protective genotype downregulating the proinflammatory pathological cascade and the risk of diabetic microangiopathies [18].

Our data complement published data showing that the Asp299Gly TLR4 gene polymorphism disrupts the TLR4 signaling pathway, modulating the production of TNF α and adhesion molecules, in particular VCAM-1 [3].

In previous studies [7, 16], we showed that the main inflammatory mediator TNF α in hyperglycemia

and dyslipidemia environment triggers a sequence of biological events, that culminate in formation of retinopathy and nephropathy [8]. Moreover, experiments carried out in vitro have established that TNF α -mediated apoptosis of endothelial cells underlies vascular complications of T1DM [12]. At the stages preceding overt structural organ changes, dysfunction of nephrons and retinal capillaries is initiated by constant leukocyte infiltration and firm attachment to endothelial cells as a result of the stimulating effect of TNF α on the expression of chemokines, adhesive molecules, both on endothelial cells and leukocytes. It results in production of multiple angiogenic, inflammatory and fibrogenic factors that contribute to the rupture of intercellular connections, damage and apoptotic death of the endothelium and retinal pericytes [13]. Long before the onset of overt complications, the accumulation of

monocytes causes leukostasis in the retinal [7, 13] and kidneys capillaries [11], which obstructs blood flow and increases damage.

Thus, the presence of the Asp299Gln polymorphism in the TLR 4 gene affects the serum TNF α and VCAM-1 levels and increases the risk of microvascular complications in patients with different glycemic control.

The study of genetic associations and interactions not only for TLRs, but also TLR-dependent signaling molecules, can elucidate their role in the formation of diabetic microvascular complications in a new way, as well as explain the differences in the timing of manifestation and the severity of their clinical symptoms. This approach allows to use the obtained individual characteristics to justify a personalized approach to patients.

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КОЭКСПРЕССИЯ МЕМБРАНОСВЯЗАННЫХ РЕЦЕПТОРОВ К ФАКТОРУ НЕКРОЗА ОПУХОЛИ АЛЬФА НА ОСНОВНЫХ ПОПУЛЯЦИЯХ ИММУНОКОМПЕТЕНТНЫХ КЛЕТОК ЗДОРОВЫХ И БОЛЬНЫХ РЕВМАТОИДНЫМ АРТРИТОМ И БРОНХИАЛЬНОЙ АСТМОЙ

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Резюме. TNF α — плеiotропный цитокин, относящийся к важным воспалительным медиаторам ряда заболеваний и реализующий свои биологические функции через два его различных рецептора, TNFR1 и TNFR2. Важное значение в реализации ответа клетки на действие TNF α имеет изменение соотношения данных типов рецепторов, способствующее смещению баланса между проапоптотическими и пролиферативными сигнальными путями. Протекающие в организме патологические процессы могут изменять уровень экспрессии TNFR1 и TNFR2 на клетках, вовлеченных в развитие заболевания.

Цель исследования — изучение уровня коэкспрессии рецепторов 1-го и 2-го типа к TNF α на основных субпопуляциях клеток периферической крови у больных ревматоидным артритом и бронхиальной астмой.

Для оценки уровня коэкспрессии рецепторов 1-го и 2-го типа к TNF α использовалась цельная кровь условно-здоровых доноров больных ревматоидным артритом и бронхиальной астмой. Оценка фенотипических характеристик проводилась методом проточной цитометрии. Для одновременного определения количества рецепторов TNF α типов 1 и 2 на различных субпопуляциях проводили двойное мечение парных образцов. После цитометрического анализа подсчитывали количество рецепторов 1-го и 2-го типа, а также процент каждой фракции.

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Наиболее выраженными изменениями в соотношении процента клеток, экспрессирующих TNFR1 и TNFR2, отличалась популяция В-лимфоцитов. Для популяции Т-лимфоцитов характерны были различия по проценту клеток, экспрессирующих только TNFR1, у больных РА и БА по сравнению со здоровыми донорами и между собой. Для моноцитов характерен больший процент дубль отрицательных клеток при БА и РА по сравнению со здоровыми донорами. Полученные данные свидетельствуют о различии в профиле коэкспрессии рецепторов 1-го и 2-го типа к TNF α при РА и БА как по сравнению со здоровыми донорами, так и между данными заболеваниями.

Основные популяции иммунных клеток активно вовлечены в патогенез как ревматоидного артрита, так и бронхиальной астмы и полученные результаты могут свидетельствовать о возможностях этих клеток по-разному отвечать на действие TNF α при изменении соотношения процента и количества рецепторов на их поверхности.

Ключевые слова: клеточная иммунология, иммунная регуляция, коэкспрессия, рецепторы фактора некроза опухоли, ревматоидный артрит, бронхиальная астма

CO-EXPRESSION OF MEMBRANE-BOUND TUMOR NECROSIS FACTOR- α RECEPTORS IN MAJOR SUBPOPULATIONS OF IMMUNOCOMPETENT CELLS IN HEALTHY INDIVIDUALS AND PATIENTS WITH RHEUMATOID ARTHRITIS AS WELL AS BRONCHIAL ASTHMA

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Abstract. A pleiotropic cytokine TNF α is an important inflammatory mediator of a number of diseases; its biological functions are fulfilled through two different receptors, TNFR1 and TNFR2. Changes in the ratio between these types of receptors shifting the balance between the pro-apoptotic and proliferation signaling pathways play a crucial role in eliciting the cell response to TNF α . The pathological processes in the body can alter the levels of TNFR1 and TNFR2 expression on the cells involved in disease development. Therefore, this study was aimed at investigating the level of co-expression of type 1 and 2 TNF α receptors in the major subpopulations of peripheral blood cells in patients with rheumatoid arthritis (RA) and bronchial asthma (BA). The greatest changes in the percentage of cells expressing TNFR1 and TNFR2 were revealed for the B-lymphocyte subpopulation. For the T-lymphocyte subpopulation, there were some differences in the percentage of cells expressing exclusively TNFR1 in RA and BA patients compared with those in healthy subjects, as well as between the RA and BA groups. A higher percentage of double-negative monocytes was observed in patients with BA and RA compared to healthy subjects. These findings indicate that the co-expression profile of TNFR1 and TNFR2 receptors in patients with RA and BA differ within these groups as well as compared to that in healthy subjects. These immune cell populations are actively involved in the pathogenesis of both rheumatoid arthritis and bronchial asthma, so the results may indicate that these cells might show different responses to TNF α as the percentage and the number of receptors on their surface vary.

Keywords: cellular immunology, immune regulation, co-expression, tumor necrosis factor receptors, rheumatoid arthritis, bronchial asthma

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Introduction

TNF α is a pleiotropic cytokine playing a crucial role in maintaining immune homeostasis and contributing to disease development [1]. This cytokine is one of the important inflammatory mediators in a number of diseases such as autoimmune, cardiovascular, and allergic diseases, cancer, etc. [6]

It is believed that the biological functions of TNF α are fulfilled through its two different receptors, TNFR1 and TNFR2 [5, 6]. Along with the cytokine TNF α per se, these receptors significantly contribute to the development of immune-mediated diseases [7, 10]. Changed ratio between the different receptor types shifting the balance between the pro-apoptotic and proliferation signaling pathways play a crucial role in eliciting the cell cytokine response. It is noteworthy that the pathological processes occurring in the body can alter the expression levels of different receptor types, which may be one of the mechanisms explaining why immunocompetent cells exhibit different responses. This was supported by a number of publications showing that the major immune cell subsets differ in their expression and co-expression levels in healthy subjects and in patients with immune-mediated diseases being also associated with inflammation severity [2, 8, 9].

Co-expression of different types of TNF α receptors has been studied only for few diseases, so it seems rather promising to broaden the range of investigated pathologies and compare data both with those for healthy subjects and between the groups of patients with diverse diseases. The findings obtained in this study will contribute both to understanding the foundations of the cytokine network function and elaborating novel approaches to the diagnostics and prediction of the course of immunopathological conditions due to identified changes compared to healthy subjects. Therefore, the aim of this study was to investigate the co-expression level of type 1 and type 2 TNF α receptors on the major peripheral blood cell subsets in patients with rheumatoid arthritis (RA) and bronchial asthma (BA).

Materials and methods

The co-expression levels of type 1 and 2 TNF α receptors were assessed using whole blood samples from otherwise healthy subjects with rheumatoid arthritis and bronchial asthma receiving treatment

at the Clinic of Immunopathology of the Research Institute of Fundamental and Clinical Immunology.

The study involved 46 apparently healthy subjects aged 18–77 years (median (IQR): 35.6 (30–54) years), including 16 (34.8%) males and 30 (65.2%) females; 64 patients with rheumatoid arthritis aged 22–83 years (median (IQR): 55 (45–65) years), predominantly including females (54 (85.4%)) and 10 males; as well as 22 patients with bronchial asthma aged 22–70 years (median (IQR): 46 (32–49) years), including 16 (72.7%) females and 6 males.

Fasting venous blood samples were collected from the median cubital vein under sterile conditions into 6 ml vacuum tubes containing K3-EDTA anticoagulant (EDTA tripotassium salt, Vacuette K3-EDTA, Greiner Bio-One GmbH, Austria)

Sample preparation was performed by using BD FACS Lysing Solution (Catalog # 349202; BD, USA) according to the manufacturer's protocol.

Flow cytometry

The phenotypic traits were assessed by flow cytometry on a FACSVerse cytofluorometer (BD, USA) using anti-human CD3 APC/Cy7, anti-human CD19 PE/Cy7, anti-human CD14 PerCP, anti-human TNFR1-PE, anti-human TNFR2-PE, anti-human TNFR1-APC, and anti-human TNFR2-APC monoclonal antibodies (R&D Systems, Minneapolis, MN). The data on fluorescence intensity were processed and calculated by using the FACS Diva software (BD, USA).

In order to simultaneously quantify type 1 and 2 TNF α receptors on different cell subpopulations, sample pairs were double-labeled (TNFR1⁺TNFR2⁻, TNFR1⁺TNFR2⁺, TNFR1⁻TNFR2⁺, and TNFR1⁻TNFR2⁻). Each sample containing a certain quantity of rhTNF (or a control sample without TNF) was split and placed into two tubes to be stained with TNFR1-PE and TNFR2-APC or TNFR2-PE and TNFR1-APC.

After the cytometric analysis, the quantity of TNFR1 (for the TNFR1⁺TNFR2⁻ and TNFR1⁺TNFR2⁺ fractions) was counted in the tubes containing TNFR1-PE and TNFR2-APC. The quantity of TNFR2 (for the TNFR1⁻TNFR2⁺ and TNFR1⁺TNFR2⁺ fractions) was counted in the tubes containing TNFR2-PE and TNFR1-APC. The percentage of each fraction was determined as the mean value for two samples.

Statistics

Statistical analysis of the data was performed using the STATISTICA 7.0 software (StatSoft, USA). The data are presented as the median values normalized to the mean values. Independent samples were compared and statistical significance of the differences was determined using the Kruskal–Wallis non-parametric ANOVA test by ranks with multiple comparisons of

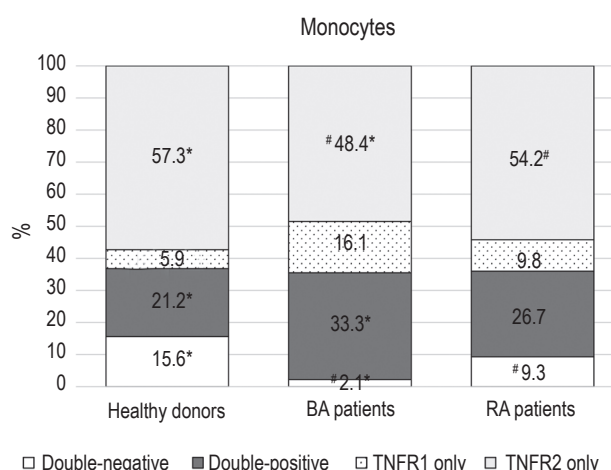


Figure 1. Percentage of cells carrying the receptors among monocytes in healthy donors and patients with BA and RA
Note. The data are presented as the medians normalized to the mean values. *, healthy subjects vs BA patients ($p < 0.05$); #, patients with RA and BA ($p < 0.05$).

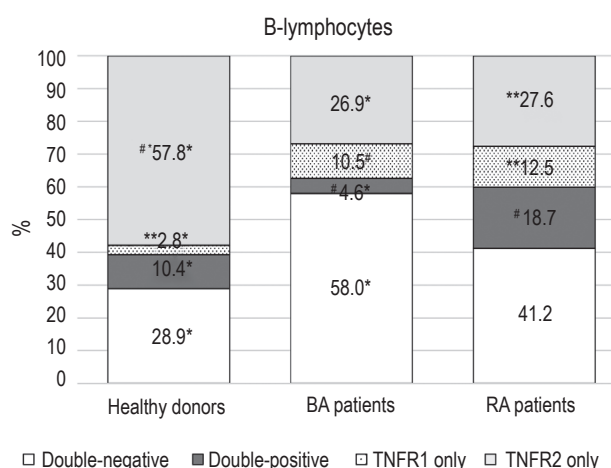


Figure 2. Percentage of cells carrying the receptors among B-lymphocytes in healthy donors and patients with BA and RA

Note. The data are presented as the medians normalized to the mean values. *, healthy subjects vs BA patients ($p < 0.05$); **, healthy subjects vs RA patients ($p < 0.05$); #, patients with BA and RA ($p < 0.05$).

the medians (when comparing identical parameters for different subpopulations.)

Results and discussion

Earlier studies focused on expression of TNFR1 and TNFR2 on the major mononuclear cell subsets (such as the total monocyte pool, total B-cell and T-cell pools), revealed that expression levels of receptors for immunomodulatory cytokines differ in healthy subjects and in patients with rheumatoid arthritis, atopic dermatitis, and pulmonary tuberculosis [3, 4], thus indicating that not only the para-

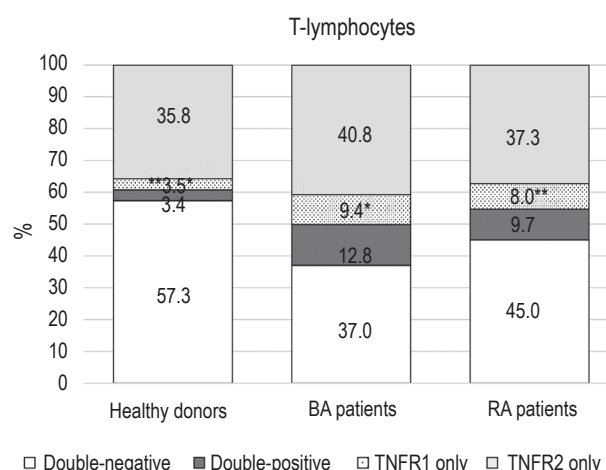


Figure 3. Percentage of cells carrying the receptors among T-lymphocytes in healthy donors and patients with BA and RA

Note. The data are presented as the medians normalized to the mean values. *, healthy subjects vs BA patients ($p < 0.05$); **, healthy subjects vs RA patients ($p < 0.05$).

meters of produced mediators and soluble receptors are changed during inflammatory diseases, but expression of membrane-bound receptors on the surface of immunocompetent cells is affected as well. In order to deeper understand a role played by the ratio between TNFR1 and TNFR2, we compared the percentage of cells carrying receptors on the major subsets of immunocompetent cells in healthy subjects and in patients with RA and BA.

It was found that the main subpopulations (monocytes, B-lymphocytes, the total T-lymphocyte pool, and the regulatory T-cells) in patients with BA and RA significantly differed both in terms of co-expressed TNFR1 and TNFR2 between the groups of patients with such diseases compared with healthy subjects.

In monocytes (Figure 1), significant difference in percentage of double-negative cells were revealed between patients with BA and healthy subjects (2.1 vs 15.6; $p < 0.0001$), as well as between patients with RA or BA (9.3 vs 2.1; $p = 0.0094$).

Healthy subjects and patients with BA significantly differed in percentage of double-positive cells (21.2 vs 33.3; $p = 0.0011$). The percentage of cells carrying solely TNFR1 differed in healthy subjects and in patients with BA (57.3 vs 48.4; $p < 0.0001$) as well as patients with BA and RA (48.4 vs 54.2; $p = 0.0006$).

Differences in the percentage of B-lymphocytes (Figure 2) are typically observed between healthy subjects and patients with BA and RA. The percentage of double-negative cells was higher in patients with BA (50.8 vs 28.8; $p < 0.0001$); in contrast, the percentage of double-positive cells was higher in healthy subjects (10.4 vs 4.6; $p = 0.0069$). The percentage of cells

expressing solely TNFR2 was significantly higher in healthy subjects compared to that in patients with BA (57.8 vs 26.9; $p < 0.0001$) and RA (57.8 vs 27.6; $p < 0.0018$). Groups of patients with BA and RA differed in percentage of double-positive cells (4.6 vs 18.7; $p = 0.0005$). The percentage of cells expressing solely TNFR1 differed between healthy subjects and patients with BA (2.8 vs 10.5; $p = 0.0006$) and RA (2.8 vs 12.5; $p < 0.0001$).

For the T-lymphocyte subpopulation (Figure 3), the significant changes involved only variation in the percentage of cells expressing solely TNFR1 between healthy subjects and patients with BA (3.5 vs 9.4; $p < 0.0001$) and RA (3.5 vs 8.0; $p = 0.0012$).

Conclusion

The study showed that the peak changes in the number of cells carrying TNF α receptors were observed in the B-lymphocyte subpopulation: the sig-

nificant difference in distribution of receptor-carrying cells in the group of BA patients compared to healthy subjects was observed. This cell subpopulation is actively involved in the pathogenesis of both diseases. The findings indicate that these cells might differ in their response to TNF α as the percentage and number of receptors vary. Differences in the percentage of cells expressing solely TNFR1 in patients with RA and BA compared to healthy subjects and between the groups of RA and BA patients were typical to T-lymphocyte subpopulation. A higher percentage of double-negative cells in patients with BA and RA compared to that in healthy subjects was revealed for monocytes.

These findings demonstrate that TNFR1 and TNFR2 are redistributed within the major subpopulations of immunocompetent cells in patients with rheumatoid arthritis and bronchial asthma compared to those in healthy subjects, as well as within the groups of patients having BA and RA.

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COVID-19: НЕКОТОРЫЕ СООБРАЖЕНИЯ О СТАНДАРТНЫХ АЛГОРИТМАХ ЛЕЧЕНИЯ

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Резюме. В данной статье проведен ретроспективный анализ историй болезни с целью выработки индивидуализированного подхода к лечению COVID-19. Поставлены вопросы о целесообразности всеобщего проведения иммуносупрессивной терапии, о необходимости назначения глюкокортикостероидов только с учетом цитокинового профиля больных, взаимосвязи глюкокортикостероидной терапии и нарушений углеводного обмена, как одних из наиболее часто встречающихся осложнений фармакотерапии COVID-19 у изученных нами больных. Неоправданная иммуносупрессия в стадии активной борьбы с инфекционным агентом как фактор хронизации процесса и снижения реактивности организма, а также нейтрализация иммуносупрессивной терапией мобилизованных на борьбу с возбудителем факторов защиты врожденного неспецифического иммунитета. Важность учета вероятности тромбообразования с рассмотрением показателей коагулограммы и маркеров тромбообразования (D-димер, продукты деградации фибрина) не исключает риск геморрагических осложнений при назначении высоких доз антикоагулянтов. Антибактериальная терапия при неосложненных вирусных инфекциях не является ни этиологическим, ни патогенетическим вариантом лечения, однако зачастую приводит к появлению резистентных штаммов микроорганизмов в результате мутационной изменчивости и эволюционных адаптаций бактерий, что усложняет выбор эффективного антибактериального препарата. Антитромботическая, глюкокортикостероидная и антибактериальная терапия должны быть клинически и лабораторно оправданы в каждом отдельно взятом случае. В статье описывается безопасность назначения противовоспалительных средств в лечении COVID-19 как противовоспалительных. В обзоре рассмотрены все вышеперечисленные аспекты терапии COVID-19 с ссылкой на зарубежные исследования, описаны варианты патофизиологического развития инфекции. Учитывая масштаб коронавирусной инфекции и тяжелые социально-экономические последствия пандемии, изучение основных этиопатогенетических аспектов, корреляции проводимого лечения с главными звеньями патогенеза, эффективности различных вариантов фармакотерапии COVID-19, поиск наиболее безопасной в отдаленной перспективе терапии — основная задача медицинского сообщества, так как вирусы имеют множество стратегий обхода защитных механизмов иммунной системы. Гарантом эффективности проводимого лечения является воздействие на основные патогенетические звенья, соответственно, проводимая терапия должна быть основана не только на клинических данных, но и подкреплена изменениями лабораторных показателей и соотноситься со степенью тяжести

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рентгенологической картины. Отмечаем необходимость разработки систематизированных данных о коронавирусе, изменениях, которые возбудитель индуцирует в организме. Это позволит выработать инновационные и эффективные терапевтические стратегии для лечения коронавирусной инфекции.

Ключевые слова: коронавирусная инфекция, пандемия, инфекционное заболевание COVID-19, цитокиновый шторм, острый респираторный дистресс-синдром, патогенетическая терапия

COVID-19: OBSERVATIONS ON STANDARD TREATMENT ALGORITHMS

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Abstract. In this article, a retrospective analysis of the medical histories was carried out in order to develop an individualized approach to treating COVID-19. Questions were raised about the advisability of the universal immunosuppressive therapy, the need to prescribe glucocorticosteroids only taking into account the cytokine profile of patients, the relationship of glucocorticosteroid therapy and carbohydrate metabolism disorders, as one of the most common complications of pharmacotherapy COVID-19 in the patients we studied. Unjustified immunosuppression in the stage of active fight against the infectious agent as a factor of chronization of the process and decrease of the body reactivity, as well as neutralization by immunosuppressive therapy of factors of protection of innate non-specific immunity mobilized to fight the causative agent. The importance of taking into account the probability of thrombosis with consideration of coagulogram indices and thrombosis markers (D-dimer, fibrin degradation products) does not exclude the risk of the hemorrhagic complications when prescribing high doses of anticoagulants. Antibacterial therapy for uncomplicated viral infections is neither an etiologic nor a pathogenetic treatment option. In addition, it leads to the emergence of resistant strains of microorganisms as a result of mutational variability and evolutionary adaptations of bacteria, which greatly complicates the choice of an effective antibacterial drug in some cases. Antithrombotic, glucocorticosteroid and antibacterial therapy should be clinically and laboratory justified in each individual case. The article focuses on the safety of prescribing antipodagric agents in the treatment of COVID-19 as anti-inflammatory. The review considers all the above-mentioned aspects of COVID-19 therapy with reference to foreign studies, describes options for the pathophysiological development of infection. We note the need to develop systematic data on coronavirus, changes that the pathogen induces in the body. This will allow the development of innovative and effective therapeutic strategies for the treatment of the new coronavirus infection.

Keywords: coronavirus infection, pandemic, infectious disease COVID-19, immunity, cytokine storm, acute respiratory distress syndrome, pathogenetic therapy

Introduction

The causative agent of the infectious disease COVID-19, the SARS-CoV-2 virus, spreads through the respiratory tract after infection, provoking the release of cytokines and the body immune response with decreased number of peripheral blood lymphocytes particularly T-lymphocytes. The initial stage of infection is bound to the penetration of SARS-CoV-2 into target cells that have type II angiotensin-converting enzyme receptors (ACE2). Cellular transmembrane serine protease type 2 (TSP2) promotes the binding of the virus to ACE-2, activating its S-protein, which is necessary for the penetration of

SARS-CoV-2 into the cell. According to the current understanding, ACE2 and TSP2 are expressed on the surface of various cells of the respiratory organs, esophagus, intestines, heart, adrenal glands, urinary bladder, brain (hypothalamus) and pituitary gland, as well as endothelium and macrophages. A large number of lymphocytes are consumed to fight the virus, which reduces the protective capabilities of the immune system and often leads to exacerbation of various chronic diseases. For most patients, severity of COVID-19 syndromes ranges from mild to moderate, but in some cases (about 20%) SARS-CoV-2 it causes intense inflammation, known as the “cytokine

storm”, which can lead to fatal pneumonia and acute respiratory distress-syndrome (ARDS). At the same time, the profiles of the “cytokine storm” may differ in various patients. Inflammatory cytokines are a type of signaling molecule that is secreted by immune cells such as helper T-cells (Th) and macrophages.

In case of COVID-19, severe illness was characterized by lymphopenia with decreased levels of CD4⁺, CD8⁺T-lymphocytes, B-lymphocytes and NK-cells, leukocytosis due to an increase in the number of neutrophilic lymphocytes against the background of a decrease in the content of monocytes, eosinophils and basophils. At the same time, the patients had high levels of the main proinflammatory cytokines in the peripheral blood plasma: tumor necrosis factor alpha (TNF- α), interferon gamma (IFN γ), IL-1, IL-2, IL-6, IL-8, IL-12, IL-18, which cause the “cytokine storm”, which in turn leads to a hyperinflammatory reaction in the lungs, multiple organ failure and death of patients [8]. Cytokine release syndrome is a potentially life-threatening host systemic inflammatory response to SARS-CoV-2. In this case, the increase in the level of interleukin 6 (IL-6), which correlates with respiratory failure, ARDS and subsequent complications, is of the greatest importance. In addition, new data has surfaced on the participation of mitochondria in activating immune response and the chronicity of inflammation. Damage of organ tissues and cells causes the release of mitochondrial proteins (n-FP, cardiolipin, etc.) and mitochondrial DNA (mtDNA). These products of degradation contribute to the formation of damage-associated molecular patterns (DAMP) that stimulate the innate immune response, by activating Toll-like receptors (TLR), NOD-like receptors (NLRP) and cGAS-STING signaling pathway. As a result, the production of proinflammatory cytokines, type I interferons and chemokines increases [6]. Thus, mitochondrial dysfunction aggravates the pathophysiological situation. In addition, novel coronavirus infection is coupled to the significantly increased D-dimer level, with a wide range of triggered damage to the cell membrane and endothelium, disrupted processes of coagulation and fibrinolysis, plasma being released from the blood vessels, and the progression of these symptoms provokes blood clotting.

At this stage of the COVID-19 treatment, the main goal of the medical community is to reduce the level of complications caused by the release of cytokines and clotting disorders. Glucocorticosteroid drugs (GCS) and monoclonal antibodies (mAb) used for this purpose, are pathophysiologically justified only in the case where characteristic changes in the leukocyte formula and cytokine profile are revealed in the patient laboratory data. This is due to the main

pharmacological drug effects. So, administration of GCS also correlates with disorders of carbohydrate, fat, protein, water-salt metabolism, calcium metabolism, and side effects of GCS targeting the cardiovascular system, musculoskeletal system, hematopoiesis, gastrointestinal tract. The prescription of GCS is associated with high stress for all organ systems and, therefore, requires a careful investigation of any clinical case for each individual patient.

According to the data published by Chinese researchers from Wuhan, one of the major markers associated with increased mortality risk is a high level of D-dimer and fibrinogen degradation products [7]. Thus, early anticoagulant and antiplatelet therapy to prevent the development of acute respiratory distress syndrome and chronic disseminated intravascular coagulation, to protect erythrocyte membranes and endothelium, in patients with a high risk of adverse outcomes, and antithrombotic therapy in maximum doses in patients with D-dimer concentration higher than 1000 ng/ml is associated with decreased frequency of deaths caused by intra-erythrocyte and microcirculatory disorders, intravascular coagulation disorders, erythrocyte hemolysis, microclot formation in the pulmonary vessels and intra-alveolar fibrin formation. However, both GCS therapy and antithrombotic therapy should have a strictly individualized approach, taking into account the side effects of the treatment.

Some hospitals in Russia began to use colchicine, a drug registered in the Russian Federation (P N014955/01) for the treatment of acute gouty attacks to prevent their recurrence. The anti-gout effect of colchicine is associated with a decrease in the migration of leukocytes to the site of inflammation and inhibited phagocytosis of uric acid salt microcrystals. It also prevents neutrophil degranulation, and has a pronounced antimitotic effect, completely or partially suppressing cell division in the anaphase and metaphase stages [2]. Currently, colchicine is not included in the Russian, American, Canadian, Australian, Italian [1, 3, 4] guidelines for the treatment of COVID-19. A retrospective analysis of a study of 14,520 people tested for SARS-CoV-2 in Israel showed no difference in the frequency of colchicine use among infected and uninfected individuals, which casts doubt on the prophylactic efficacy of this drug [5].

Materials and methods

A retrospective analysis of 80 case histories of patients who received treatment at the Hospital of Especially Dangerous Infections of the Kabardino-Balkaria Republican Children's Clinical Hospital, aged 18 to 86 years, from October to December 2020, was carried out. The following indicators were analyzed: the level of serum interleukin-6 measured

by electrochemiluminescence immunoassay (ECLIA) in the venous blood samples as the biomaterial, the INR value ("international normalized ratio") using appropriate formulas, taking into account patient prothrombin time and the thromboplastin ISI (international sensitivity index), ferritin level by immunoturbidimetry (a quantitative measurement of concentration of specific proteins by changing the turbidity of the solution during the antigen-antibody reaction, this method is characterized by high sensitivity), the level of C-reactive protein (CRP) by the method of precipitation in a capillary, during which we observe a haze formed due to complexing between soluble molecular antigen and antibodies. All patients underwent MPR (multiplanar reconstruction) CT scans of the chest cavity organs to identify the degree of damage to the lung tissue using the Siemens "Somatom perspective" 64 apparatus.

Results and discussion

Medical history analysis showed that 77 patients who underwent glucocorticosteroid therapy had no indication for this, because the level of serum interleukin-6 did not exceed 7 pg/ml, which does not correspond to the level observed in cytokine storm syndrome. The prescription of glucocorticosteroid therapy without changes in the cytokine profile could lead to severe immunosuppression and inability to mount proper immune response to invading virus, which led to aggravated patient condition and the appearance of complications. It should also be noted that taking glucocorticosteroids at high doses (16-24 mg/day) leads to hyperglycemia up to 30 mmol/L or more, which increases the risk of hyperglycemic coma. In confirmation of our results, in the Interim Guidelines of the Ministry of Health of the Russian Federation on the prevention, diagnosis and treatment of the new coronavirus infection (covid-19) (Version 10 (02/08/2021), we find that "it is not recommended to use GCS for the treatment of mild and moderate severity of COVID-19". Only in 3 patients, interleukin-6 level exceeded the reference values 82 pg/ml, 102 pg/ml, 168 pg/ml) so that it justified administered glucocorticosteroid therapy. This category of patients also showed significantly increased serum CRP and ferritin, severe lymphopenia (6% of lymphocytes), and increased ESR (erythrocyte sedimentation rate). Analysis of the chest CT examination data revealed more than 50% of lung damage (CT-3).

According to the recommendations of the Ministry of Health of the Russian Federation, the prescription of low molecular weight heparin (LMWH) at prophylactic doses is indicated for all hospitalized patients and should be continued at least until discharge. However, the choice of the heparin dose

below the intermediate or therapeutic dose is based on the content of D-dimer and detected additional risk factors for venous thromboembolic complications, as well as severe manifestations of COVID-19. The analysis of medical histories revealed that D-dimer was not detected in 76 patients after receiving high doses of anticoagulants, due to the lack of reagents in the laboratory at the time of examination. However, hospitalized patients with moderate COVID-19 require to measure the D-dimer once every two days, in severe patients – daily. Exceptional urgent analysis of the above indicators is conducted for patients in case of deteriorated condition. In these patients, the INR value was determined, which was normal in all patients. This increased the risk of bleeding, as patients received high doses of heparin (5,000 U 5-8 times a day) without D-dimer control.

Antibiotic therapy was received by 76 patients, of which 73 patients showed no signs of bacterial inflammation according to the general blood test, thereby accounting for inappropriate prescription of antibiotics. This is confirmed by the recommendations of the Ministry of Health of the Russian Federation, which states that "COVID-19, like any other viral infection is not an indication for the use of antibiotics". Antibiotic therapy is prescribed only if there are convincing signs of bacterial infection (increased PCT of more than 0.5 ng/ml, the appearance of purulent sputum, leukocytosis more than $12 \times 10^9/L$ (in the absence of previous use of glucocorticoids), an increase in the number of stab neutrophils by more than 10%). Bacterial infections rarely complicate the course of COVID-19. Therefore, the vast majority of patients with COVID-19, especially with mild to moderate course, do not require antibiotic therapy». The analysis of the data obtained showed that the patients received ceftriaxone 2 g per day, meropenem, ciprofloxacin 200 mg 2 times per day, buckperazone 2 g per day with no signs of bacterial infection.

The ferritin level in 45 out of 50 women was within the reference values (10-120 µg/l); similar indicators were also observed in male patients, where in 28 out of 30 patients the indicator was also within the reference values (20-250 µg/l), and that is also not an indicator of severe inflammatory process in the upper and lower respiratory tract, which requires administration of a glucocorticosteroid and antibacterial therapy.

The only justification for applying glucocorticosteroid therapy can be considered in case of increased serum CRP in patients, however, high doses of glucocorticosteroids were prescribed to patients with both mildly increased CRP (6.6-7.1 mg/L) and high CRP (80 mg/L) and more). No correlation was found between the degree of increased serum CRP and the dose of glucocorticosteroids administered.

Colchicine was taken by 36 patients with moderate to severe symptoms at a dose of 1 g with an interval of 12 hours, then 1 g per day until the 3rd day of stable normothermia. The same patients received glucocorticosteroid, antibacterial and antithrombotic therapy, complicating drawing specific conclusions about the effectiveness of colchicine in the treatment of patients. Taking into account the recommendations of the British Medicines Agency, the following dose adjustments are necessary: reduction of the colchicine dose by 4 times while used together with ritonavir, ketoconazole, clarithromycin, cyclosporine; 2 times – while used with diltiazem and verapamil. These guidelines appear to be particularly relevant for patients with COVID-19.

Conclusion

The retrospective analysis of medical histories showed that in most cases the prescription of high doses of anticoagulants, glucocorticosteroid and antibacterial drugs is not justified, since the analysis of laboratory parameters revealed no direct indications

for applying such an aggressive therapy. The lack of data from clinical studies confirming the efficacy and safety of colchicine in COVID-19 and allowing it to be recommended for widespread practice requires a collegial decision on the medical board for its prescription, with consideration of all safety aspects of its use (clinical and hematological control, monitoring of potential interactions with other drugs). We consider it advisable to proceed by a case-by-case approach to the treatment of each individual patient, taking into account the recommendations of the Ministry of Health of the Russian Federation for the prevention, diagnosis and treatment of a new coronavirus infection (COVID-19).

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СОВЕРШЕНСТВОВАНИЕ СПЕЦИФИЧЕСКОЙ ПРОФИЛАКТИКИ ХОЛЕРЫ С ПОМОЩЬЮ ИММУНОМОДУЛЯТОРОВ

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Резюме. Известно, что совместное использование вакцин, цитокинов и различных иммуномодулирующих препаратов способствует развитию полноценного иммунного ответа. Такой подход позволяет усилить иммуногенность современных вакцин и направить развитие иммунных реакций по гуморальному или клеточному типу в зависимости от свойств возбудителя той или иной болезни. Совершенствование профилактических средств за счет их сочетанного применения с цитокинами и иммуномодуляторами увеличивает напряженность иммунитета, повышает уровень продукции специфических иммуноглобулинов, протективность антигенов, а также снижает проявление побочных реакций, приводящих к поствакцинальным осложнениям.

Иммуномодуляторы уже успешно используются в препаратах, предназначенных для терапии и профилактики хронической герпетической инфекции и противогриппозной вакцине. Многочисленные экспериментальные и клинические данные свидетельствуют о положительном эффекте применения иммуномодулирующих препаратов при вакцинопрофилактике различных вирусных и бактериальных инфекций, в том числе и особо опасных.

Совершенствование специфической профилактики холеры может быть достигнуто за счет иммуномодулирующих средств, способных стимулировать формирование местного и системного иммунного ответа.

Нами проведена сравнительная оценка целесообразности сочетанного применения вакцины холерной бивалентной химической (ФКУЗ РосНИПЧИ «Микроб» Роспотребнадзора) и иммуномодуляторов с целью повышения эффективности вакцинопрофилактики холеры.

Так как холерная вакцина вызывает активацию гуморального иммунного ответа, оценивали выработку специфических иммуноглобулинов в организме привитых экспериментальных животных и влияние иммуномодуляторов на этот процесс в разные сроки поствакцинального периода.

Способность иммуномодуляторов повышать протективную активность холерной вакцины изучали, заражая животных вирулентным штаммом холеры через месяц и семь месяцев после вакцинации.

Выявлено, что иммуномодуляторы повышают иммуногенность и протективность антигенов, входящих в состав противохолерной вакцины. Использование всех иммунопрепаратов увеличивает продукцию специфических иммуноглобулинов в сыворотке вакцинированных экспериментальных

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животных. Показано, что в первый месяц после вакцинации наиболее эффективно стимулировал образование антител полиоксидоний, однако липоид способствовал более длительному сохранению противохолерных иммуноглобулинов в сыворотке вакцинированных кроликов. Сочетанное применение вакцины и липоида предотвращало развитие холеры у всех взятых в эксперимент животных, в том числе вакцинированных сниженной дозой. В отдаленные сроки поствакцинального периода этот иммуномодулятор повышал протективность противохолерной вакцины в три раза. Полиоксидоний и деринат также увеличивали защитный эффект противохолерной вакцины, но несколько уступали в этом липоиду. Совместное применение противохолерной вакцины и иммуномодуляторов, особенно липоида, может быть использовано для совершенствования специфической профилактики холеры.

Ключевые слова: профилактика, холера, иммуногенность, протективность, вакцина, иммуномодуляторы

IMPROVEMENT OF SPECIFIC CHOLERA PREVENTION USING IMMUNOMODULATORS

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Abstract. It is known that the combined use of vaccines, cytokines and various immunomodulatory drugs contributes to the development of a full-fledged immune response. This approach makes it possible to enhance the immunogenicity of modern vaccines and to direct the development of immune responses according to the humoral or cellular type, depending on the properties of the pathogen of a particular disease. The improvement of preventive drugs due to their combined use with cytokines and immunomodulators increases the intensity of immunity, increases the level of production of specific immunoglobulins, the protectivity of antigens, and also reduces the manifestation of adverse reactions leading to post-vaccination complications.

Immunomodulators are already successfully used in drugs intended for the treatment and prevention of chronic herpes infections and influenza vaccines. Numerous experimental and clinical data indicate a positive effect of the use of immunomodulatory drugs in the vaccination of various viral and bacterial infections, including particularly dangerous ones.

Improving the specific prevention of cholera can be achieved through immunomodulatory agents that can stimulate the formation of a local and systemic immune response.

We conducted a comparative assessment of the feasibility of the combined use of the cholera bivalent chemical vaccine (the Federal Government Health Institution Anti plague Research Institute "Microbe") and immunomodulators in order to increase the effectiveness of cholera vaccination.

Since the cholera vaccine causes the activation of the humoral immune response, the production of specific immunoglobulins in the body of vaccinated experimental animals and the effect of immunomodulators on this process at different times of the post-vaccination period were evaluated.

The ability of immunomodulators to increase the protective activity of the cholera vaccine was studied by infecting animals with a virulent strain of cholera one month and seven months after vaccination.

It was found that immunomodulators increase the immunogenicity and protectivity of antigens that are part of the anti-cholera vaccine. The use of all immunopreparations increases the production of specific immunoglobulins in the serum of vaccinated experimental animals. It was shown that in the first month after vaccination, polyoxidonium most effectively stimulated the formation of antibodies, but lycopide contributed to a longer retention of anti-cholera immunoglobulins in the serum of vaccinated rabbits. The combined use of the vaccine and lycopide prevented the development of cholera in all animals taken in the experiment, including those vaccinated with a reduced dose. In the long-term post-vaccination period, this immunomodulator increased the protectiveness of the anti-cholera vaccine by three times. Polyoxidonium and derinate also increased the protective effect of the cholera vaccine, but were slightly inferior to lycopide. The combined use of cholera vaccine and immunomodulators, especially lycopide, can be used to improve specific cholera prevention.

Keywords: cholera, cholera vaccine, immunomodulators, immunotherapy, protectivity, immunogenicity

Introduction

One of the approaches to improving the strategy for combating infectious diseases of any etiology is the development of schemes for the integrated use of vaccines and various groups of immunobiological drugs – immunomodulators, cytokines, etc., which can stimulate the formation of post-vaccination immunity [14]. An active search is underway for drugs that can, firstly, enhance the immunogenic effect of modern vaccines, especially in individuals with secondary immunodeficiency, and secondly, direct the development of an immune response via humoral or cellular type, depending on the properties of the pathogen [13]. Improvement of prophylactic agents due to their combined use with cytokine preparations and various immunomodulators can reduce the manifestation of systemic and local reactions, increase the intensity of immunity, the level of production of specific immunoglobulins, and increase the antigen immunogenicity and protectivity. In practical medicine, several vaccines that contain immunomodulators are already used. Polyoxidonium is included in the polyvalent vaccine for the treatment and prevention of chronic herpetic infection caused by herpes simplex viruses of groups 1 and 2 (HSV-1 and HSV-2), and in subunit influenza vaccine “Grippol”. Another multivaccine against HSV-1 and HSV-2 called Vitaherpavac contains sodium hyaluronate [6, 11].

In addition, it has been shown that the inclusion of cytokines and various immunomodulators in the vaccination regimen ensures the development of a full-fledged immune response to various antiviral vaccines and increases their immunological safety [1, 2, 3, 4, 5, 8]. Positive results of using immunomodulators to improve the specific prevention of particularly dangerous infections have been obtained [9, 10, 12].

The effectiveness of specific cholera prevention can be also enhanced by immunomodulatory agents that can stimulate the formation of both local and systemic immunity.

The aim of the study was to experimentally investigate the effect of immunomodulators on immunogenic and protective activity of the cholera bivalent chemical vaccine (the Federal Government Health Institution Anti plague Research Institute “Microbe”) in order to assess the feasibility of their use in specific cholera prevention.

Materials and methods

Taking into account that drugs that can be effective for improving specific cholera prevention should have a complex immunomodulatory effect on both systemic and local immunity, we chose the following immunomodulators: Polyoxidonium (azoximer bromide) (NPO Petrovax Pharm, Russia), which stimulates

antibody production, phagocytosis, restores immune responses in secondary immunodeficiency conditions, increases the body’s resistance to local and generalized infections, has the ability to control the immune response of the immune system, anti-inflammatory effect, etc.; Derinat (sodium deoxyribonucleate) (CJSC “FP “Technomedservis”, Russia) increases resistance to viral, fungal, bacterial infections, and also stimulates reparative processes on the mucous membranes: Lycopide (glucosaminylmuramyldipeptide) (CJSC “Peptek”, Russia) – a synthetic analog of the structural fragment of the (peptidoglycan) of bacterial cell wall is an activator innate and acquired immunity, enhances the body defense against viral, bacterial and fungal infections, exerts an adjuvant effect in developing immunological reactions.

Experiments were conducted on 45 adult rabbits (weighing 1.8-2.0 kg) purchased from the nursery of the Rostov-on-Don Plague Control Research Institute.

Before oral immunization, the animals were orally inoculated with 5% solution of baking soda (2 ml each) to reduce damaging effect of gastric juice on the cholera vaccine. The vaccination dose of cholera bivalent chemical vaccine and immunopreparations was calculated according to the weight of experimental animals, based on the human doses recommended by the manufacturer. Immunomodulators were administered to rabbits once simultaneously with the vaccine: Polyoxidonium at 0.17 mg; Derinate at 2.0 mg; Lycopide at 0.285 mg.

The total pool of rabbit serum anti-cholera antibodies was determined by solid-phase enzyme-linked immunosorbent assay in three replicates.

The potential of immunomodulators to increase protective activity of the cholera vaccine was evaluated by challenging animals with the virulent strain *Vibrio cholerae* O1 569B one month and seven months after vaccination. We used a model of a bandaged loop of the adult rabbit small intestine, which allows us to obtain a pathogenetic picture of the disease in animals similar to that in humans [7]. The development of the disease in rabbits was assessed by collecting fluid in the experimental bandaged loops of the small intestine, determining the coefficient of the loop stretching (K) by the formula: $K = \text{volume of liquid} / \text{length}$ the presence/severity of edema of the mucous and submucosal membranes, hemorrhages pointing at enteropathogenic effect ($K > 1$ indicates the development of a cholero-genic effect), as well as the presence / severity of edema of the mucous and submucosal membranes, hemorrhages indicating an enteropathogenic effect.

Statistical data analysis was performed by using the Microsoft Excel software. The magnitude of the confidence intervals (L) of the arithmetic mean (M) for the 95% confidence level (P) were determined.

Results and discussion

Given that the cholera vaccine causes the production of specific antibodies in vaccinated rabbits, we evaluated an effect of immunomodulators on this process after vaccination. It was found that in the first week of the post-vaccination period, all animals in experimental groups had similar serum level of produced anti-cholera immunoglobulins (titer value – 1:625). By day 14, the number of specific Ig in rabbits vaccinated and treated with immunomodulators increased significantly (titer value – 1:3125), compared with that in monovaccinated animals (titre value – 1:625). On day 21, the group of vaccinated animals showed decreased serum antibody production (titer value – 1:125). A decrease in the number of anti-cholera immunoglobulins on day 21 (titer value – 1:625) was also observed in vaccinated rabbits treated with Derinat and Lycopide. Only animals primed with Polyoxidonium had titer of specific antibodies that remained unchanged (titer value – 1:3125).

While studying the duration of sustained serum specific immunoglobulin level in vaccinated rabbits, it was found that seven months after the onset of the experiment, the titer of anti-cholera antibodies in the group of immunized animals decreased to 1:64. Vaccinated rabbits treated with Derinate and Polyoxidonium had the titer at 1:128, whereas after treating with Lycopide during vaccination retained immunoglobulins at fairly high titer 1:256.

While assessing the pathoanatomical picture in the bandaged loops of the small intestine in all intact (control) infected rabbits, it was revealed that the experimental loops were strongly stretched and filled with cloudy contents ($K=1.14\pm0.06$), hemorrhages and mucosal edema were recorded, which indicated the development of cholero-genic and enteropathogenic effects.

The results of challenging vaccinated animals one month after vaccination showed the presence of a rather durable post-vaccination immunity: 75% of rabbits lacked both cholero-genic and enteropathogenic effects. Only 25% of rabbits showed signs of developing disease in the form of a cholero-genic effect ($K=1.1\pm0.08$).

In all groups of vaccinated animals treated with immunomodulators, no pathomorphological changes in the experimental loops of the small intestine were detected during infection one month after the onset, i.e., enteropathogenic and cholero-genic effects were absent in 100% of cases, which indicated a positive

effect of immunopreparations on the protectivity of the cholera vaccine.

Seven months in the post-vaccination period, the intensity of cholera immunity in vaccinated rabbits decreased. The protective effect of the vaccine was recorded only in 25% of animals. Cholero-genic ($K=1.12\pm0.08$) and mild enteropathogenic effects were observed in 75% of rabbits.

While assessing the ability of immunomodulators to increase the protective activity of the cholera vaccine in the long-term period after vaccination, it was found that the effect of Polyoxidonium, Lycopide, and Derinate was manifested to varying degrees. No signs of developing disease were registered in 75% of animals treated with Lycopide ($K=0.12\pm0.06$), 25% of rabbits from this group developed only a cholero-genic effect ($K=1.05\pm0.02$). The pathomorphological pattern characteristic of cholera was observed in 50% of animals from the groups with Polyoxidonium and Derinate ($K=1.04\pm0.02$ and $K=1.06\pm0.04$, respectively), whereas vaccinated animals rabbits treated with Derinat also had a weak enteropathogenic effect. Half of the animals from these groups, showed no cholero-genic ($K=0.15\pm0.09$ and $K=0.14\pm0.07$, respectively) and enteropathogenic effects.

The results of experiments assessing a potential to reduce the recommended dose of the cholera vaccine combined with immunomodulators showed that after infection of rabbits one month after vaccination, signs of disease development were not detected only in experimental animals treated with Lycopide ($K=0.19\pm0.02$). Polyoxidonium and Derinate prevented the development of cholera in 50% of rabbits vaccinated with a reduced dose of the vaccine. It should be noted that in other animals from these groups, cholero-genic ($K=1.08\pm0.02$ and $K=1.06\pm0.04$, respectively) and mild enteropathogenic effects were recorded.

In animals vaccinated with half a dose of the vaccine, a pathogenetic picture characteristic of cholera was observed: the development of cholero-genic ($K=1.12\pm0.02$) and enteropathogenic effects. This was evidenced by the presence of edema and hemorrhages in the experimental bandaged loops of the small intestine, which were stretched and filled with reddish, moderately turbid contents.

Thus, we found that the use of immunomodulators enhances the immunogenic and protective properties of the antigens included in the chemical cholera bivalent vaccine. The use of immunopreparations increases the production of serum specific immunoglobulins of vaccinated experimental animals. Moreover, in the first month of the post-vaccination period, Polyoxidonium most effectively abrogated this

process. Seven months after vaccination, a sufficiently high anti-cholera antibody titer was maintained in animals treated with Lycopide. The most effective method was to use this immunomodulator while vaccinated animals were infected with a virulent cholera strain. Lycopide prevented the development of infection in the small intestine in all adult rabbits, including those vaccinated with a reduced dose. Also, the combined use of Lycopide and the vaccine tripled

its protectivity in the long-term post-vaccination period. Polyoxidonium and Derinate stimulated the protective ability of the cholera vaccine, but they were somewhat inferior to Lycopide. Considering the data noted above, it can be concluded that increased immunogenic activity of the cholera vaccine combined with immunomodulators, especially Lycopide, may be one of the approaches to improving specific cholera prevention.

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КЛИНИЧЕСКАЯ И ИММУНОЛОГИЧЕСКАЯ ЭФФЕКТИВНОСТЬ ЛЕЧЕНИЯ АМБРОЗИЙНОГО ПОЛЛИНОЗА В САМАРСКОЙ ОБЛАСТИ

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Резюме. Аллергический ринит и бронхиальная астма — широко распространенные респираторные аллергические заболевания. На некоторых территориях РФ доминирующей причиной поллиноза является амброзия. Целью исследования было оценить клиническую и иммунологическую эффективность АСИТ аллергеном *Ambrosia artemisiifolia* у пациентов, сенсибилизированных *Ambrosia trifida* в Самарской области. Пациентам с подтвержденной сенсибилизацией к амброзии трехраздельной была проведена иммунотерапия аллергоидом амброзии полыннolistной предсезонно. После лечения у пациентов было отмечено снижение степени выраженности симптомов аллергического ринита по ВАШ ($p = 0,00001$), снижение потребности в медикаментозных средствах ($p = 0,0003$), а также потребности в ГКС на фоне проводимой терапии с 34,6% до 0% ($p = 0,00001$). В 8% случаев результат лечения был хороший, в 69% — удовлетворительный, в 23% — неудовлетворительный. В группе контроля не было отмечено изменений степени выраженности симптомов ($p = 0,858$). Также в группе контроля потребность в лекарственных препаратах осталась без изменений и 14,3% пациентов продолжали использовать ГКС.

После АСИТ наблюдалось снижение уровня IL-4 ($p = 0,002$), и снижение соотношения IL-4/IL-10 ($p = 0,0063$); при этом изменения уровня остальных цитокинов (IL-10; IFN γ) оказались статистически незначимыми ($p > 0,05$). До лечения уровни IL-4/IL-10 в обеих группах были сопоставимы, а после лечения различия стали статистически значимы ($p = 0,031$). Мы не получили статистически-значимого изменения уровня IgG4 *Amb a 1*, IgG4 *Amb trifida*. Зависимости (корреляции) между уровнем отдельных цитокинов и результатами лечения получено не было. В результате проведенной АСИТ были получены положительные клинические и иммунологические результаты. У большинства пациентов заболевание приобрело контролируемое течение. При этом отсутствие отличных и низкое число хороших результатов АСИТ, вероятно, связано с внутривидовыми аллергенными свойствами амброзии.

Ключевые слова: амброзия, иммунотерапия, поллиноз, трифиды, цитокины, IL-4, *Amb a 1*

CLINICAL AND IMMUNOLOGICAL EFFECTIVENESS OF TREATMENT OF RAGWEED POLLINOSIS IN THE SAMARA REGION

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Abstract. Allergic rhinitis and bronchial asthma are widespread respiratory allergic diseases. In some territories of the Russian Federation, the dominant cause of pollinosis is ragweed. The aim of the study was to

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evaluate the clinical and immunological efficacy of ASIT with the allergen *Ambrosia artemisiifolia* in patients sensitized to *Ambrosia trifida* in the Samara region. Patients with proven sensitization to *Ambrosia trifida* were held immunotherapy with *Ambrosia artemisiifolia* allergoid pre-season. After treatment, patients had a decrease in the severity of symptoms of allergic rhinitis according to VAS ($p = 0.00001$), a decrease in the need for medications ($p = 0.0003$), as well as the need for corticosteroids against the background of therapy from 34.6% to 0% ($p = 0.00001$). In 8% of cases, the result of treatment was good, in 69% satisfactory, in 23% unsatisfactory. In the control group, there were no changes in the severity of symptoms ($p = 0.858$). Also, in the control group, the need for medications remained unchanged and 14.3% of patients continued to use corticosteroids.

After ASIT, there was a decrease in the level of IL-4 ($p = 0.002$), and a decrease in the ratio of IL-4/IL-10 ($p = 0.0063$); at the same time, changes in the level of other cytokines (IL-10; IFN γ) were statistically insignificant ($p > 0.05$). Before treatment, the levels of IL-4/IL-10 in both groups were comparable, and after treatment, the differences became statistically significant ($p = 0.031$). We did not get a statistically significant change in the level of IgG4 *Amb a 1* or IgG4 *Amb trifida*. There was no correlation between the level of individual cytokines and the results of treatment. As a result of the conducted ASIT, positive clinical and immunological results were obtained. In most patients, the disease has acquired a controlled course. At the same time, the lack of excellent and low number of good results of ASIT is probably due to the intraspecific allergenic properties of ragweed.

Keywords: ragweed, immunotherapy, pollinosis, *trifida*, cytokines, IL-4, *Amb a 1*

Introduction

Allergic rhinitis (AR) and allergic bronchial asthma (BA) are chronic diseases of the respiratory tract, characterized by constant minimal inflammatory reactions due to exposure to allergens.

According to epidemiological studies, the adult BA prevalence in the Russian Federation is 6.9%, and AR – 6.5% [5].

In the Omsk region, 40% of all visits to an allergist are associated with symptoms of hay fever. In the Moscow region, percentage of hay fever in the structure of allergic diseases is 17.5% [8]. In the Dagestan, 40% of patients with hay fever revealed sensitization to weed allergens, among which wormwood and ragweed predominate. In the Stavropol region, up to 40% of the population suffers from hay fever, with ragweed being the dominant taxon of weed pollen [13].

For the treatment of hay fever and other respiratory allergic diseases, the method of allergen-specific immunotherapy (ASIT) has been used for many years. ASIT is the only method capable of curbing allergic inflammation and retarding transition of AR to BA, which implies improved quality of patients' life.

The aim of the study was to evaluate the clinical and immunological efficacy of ASIT with the ragweed (*Ambrosia artemisiifolia*) allergen in patients sensitized with *Ambrosia trifida* in the Samara region.

Materials and methods

There were enrolled patients ($n = 41$) with manifestations of pollinosis and confirmed sensitization to ragweed tripartite, in the absence of the main allergic component of ragweed *Ambrosia Amb a 1*. All patients were randomized into the study group ($n = 26$) and the control group ($n = 15$).

In some patients with hay fever in both groups they had concomitant manifestations of respiratory allergy in the form of year-round rhinitis (in 30.7% in the study group and 20% in the control group) and a combination with bronchial asthma (in 15.5% and 13.3% in the study and control groups, respectively). The average age of patients in the study and control group was 28.52 ± 12.07 and 32.8 ± 13.2 years, respectively. In the study group, the proportion of males and females was 42.3% and 57.7%, respectively; in the control group 20% and 80%, respectively.

The patients of the study group were treated with the ragweed allergen. Patients in the control group received symptomatic treatment.

Immunotherapy was carried out with a standardized ragweed allergoid 10.000 PNU/ml (NPO Microgen, Russia) pre-season, starting from a dilution of 1:10 000 0.1 ml subcutaneous at gradually escalating dose according to the instructions of the drug. The average total dose was 10 593 PNU.

All patients before and after treatment were assessed for the following parameters: IL-4, IL-10, IFN γ , IgE to *Amb. trifida*, allergic component *Amb a 1*, IgG4 *Amb a 1*, IgG-trifida. An enzyme-linked immunosorbent assay (ELISA) was used to determine serum cytokines and IgG4. The study of the level of specific IgE was carried out on Phadia 250 equipment using the ImmunoCap ISAC diagnostic test system (Sweden).

The scale for assessing nasal symptoms, taking into account the need for drug therapy, was analyzed according to the generally accepted method. The severity of AR symptoms was also assessed using the VAS: results from 0 to 50 indicate a controlled course of AR, from 50 and above an uncontrolled course of

AR [3, 9]. The results are presented as the median Me ($Q_{0.25}$ – $Q_{0.75}$).

Evaluation of immunotherapy effectiveness was carried out according to the modified scheme of A.D. Ado, wherein an excellent effect – no symptoms of hay fever during the dusting season and no use of drug therapy, a good effect – occurrence of minor symptoms of hay fever and no / minimal drug therapy, a satisfactory effect a decreased severity of pollinosis symptoms and lowered need for drug therapy, an unsatisfactory effect the absence of positive effects of treatment (the presence of the usual symptoms of hay fever and the previous need for symptomatic drugs).

Statistical data processing was carried out using generally accepted methods of variation statistics. Methods of nonparametric statistics were used, the Mann–Whitney U-test (U), Wilcoxon's W-test (W) and the cross-tabulation method (χ^2) were used. The critical value of the significance level was taken equal to 5%. The data obtained were processed using the application package AtteStat, version 10.5.1, statistical formulas of the Microsoft Excel program, version 5.0.

Results and discussion

Prevalence of ragweed sensitization

According to the results of studies carried out earlier in the Samara region, 75% patients suffering from hay fever were sensitized to weeds (mono- or in combination with other groups of pollen allergens). More than half of such patients (53.8%) were sensitized to ragweed. At the same time, patients with negative skin tests to ragweed, had IgE to to *Ambrosia trifida* in 65.9% of cases, whereas in 13.6% of cases – a combination of IgE against two types of ragweed [15].

ASIT results (clinical effectiveness)

Patients with sensitization to *Ambrosia trifida* (n = 26) underwent pre-season ASIT with *Ambrosia artemisiifolia* allergoid according to the classical scheme.

During the ragweed dusting season, patients in the study group noted improved health condition: according to the VAS scale, the severity of AR symptoms before and after treatment was 70 (60–80) and 40 (30–50), respectively (T = 351; p = 0.00001). At the same time, in 8% of cases, the result of treatment was assessed as good, in the majority of patients (69%), the result was assessed as satisfactory, and in 23% of cases unsatisfactory results were obtained.

The need for drugs in patients receiving ASIT decreased from 2 (2–3) to 1 (1–2), (W = 120; p = 0.0003). The need for corticosteroids along with ongoing therapy (ASIT) in patients also decreased from 34.6% to 0% ($\chi^2 = 11$; p = 0.00001).

In the control group, no significant changes in the severity of symptoms within the study year vs. previous years were observed: the mean values on the VAS scale

were 65 (50–80) and 65 (55–80) in the current and last season of ragweed dusting, respectively (U = 88; p = 0.858). The need for drugs did not change and averaged 2 (1–2), (U = 98; p = 0.78), while 14.3% of patients continued to use glucocorticoids.

Despite the low percentage of good results and the absence of excellent ASIT results, we obtained positive treatment results, and in the majority of patients, the disease acquired a controlled course.

Effect of ASIT on cytokine levels (immunological efficacy)

We assessed the levels of serum cytokines (IL-4, IL-10) before and after treatment, and also diagnosed the level of IFN γ , which is an important marker of the Th1 immune response.

After the course of ASIT with the ragweed allergen in the active group, a significant decrease in the level of IL-4 from 36.19 to 20.19 pg/ml (W = 277; p = 0.002) was recorded; changes in the level of other cytokines (IL-10; IFN) were insignificant (p > 0.05) (Figure 1).

The level of IL-4 after treatment in the study group was also lower than that in the control group: 20.19 versus 51.79 pg/ml (W = 96.5; p = 0.0110).

The IL-4/IL-10 ratio after treatment in the study group decreased from 1.097 to 0.63 (W = 283; p = 0.0063), while in the control group it remained without significant changes (W = 35; p = 0.2719) (Figure 2). At the same time, before treatment, the levels of IL-4/IL-10 in both groups were comparable and reached 1.097 and 1.6 in the active group and control group (U = 232.5; p = 0.31), respectively, whereas after treatment the differences became statistically significant: 0.63 and 2.17, respectively (U = 258; p = 0.031).

The IL-4/IFN γ ratio in the study group did not change significantly after ASIT and reached 0.15 and 0.094 before and after immunotherapy, respectively (W = 254; p = 0.046). No relationship (correlation) between the level of individual cytokines (also their ratio) and the results of treatment was obtained.

Effect of ASIT on specific levels of IgE and IgG

After the treatment we found no significant change in the level of IgG4 *Amb a 1* (W = 90.5; p = 0.052), nor IgG4 *Amb trifida* (W = 131; p = 0.587). The IgE *Amb trifida* level in the study group did not change after treatment and was 14.4 vs 14.69 kUA/l before treatment (T = 150; p = 0.74).

It is believed that allergens of ragweed (mainly *Amb a1*) and *Ambrosia trifida* have strong crosslinkages and that one type of allergen is sufficient for the treatment of ragweed pollinosis [14]. The main component *Amb a 1* has a wide reactivity that exceeds and overlaps the levels of both isoallergens and allergens of related ragweed species. Many studies show a good efficacy of ragweed pollinosis specific immunotherapy (both sublingual and subcutaneous) with the ragweed allergen.

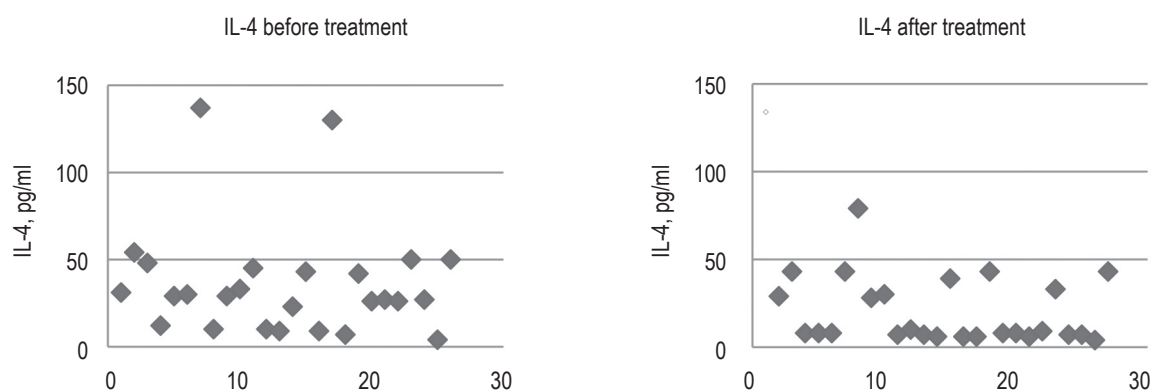


Figure 1. Levels of IL-4 in the study group before and after ASIT with ragweed allergen

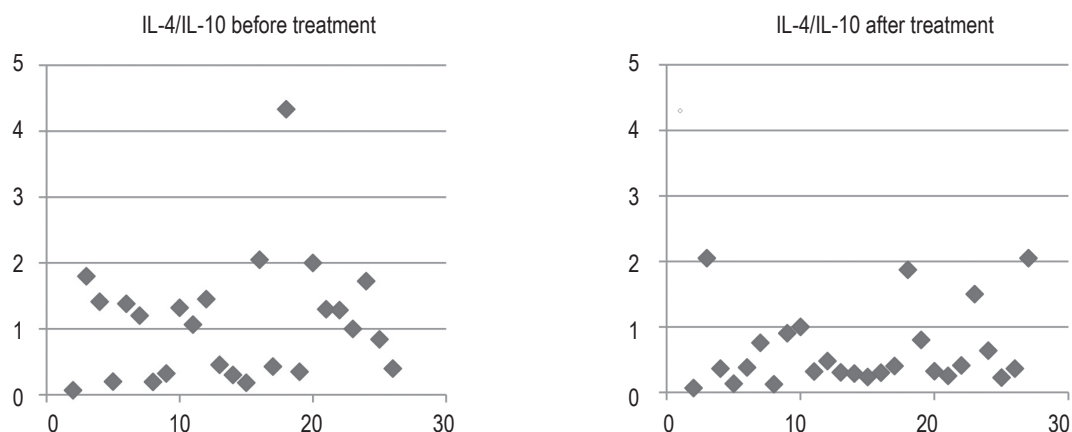


Figure 2. Levels of the IL-4/IL-10 ratio in the study group before and after treatment

However, a number of authors have shown that *Ambrosia artemisiifolia* and *Ambrosia trifida* are not allergenically equivalent species: about 50% of patients receiving specific immunotherapy with one type of ragweed showed little or no clinical response where another species of ragweed prevailed, while the results in these patients were excellent after allergen replacement [1].

To date, the International Union of Immunological Societies (IUIS) has identified 11 allergens of *Ambrosia artemisiifolia*, the main of which are *Amb a 1* and *Amb a 11*. Despite progress in this field, the authors have already paid attention to the fact that at the moment it is impossible to draw a conclusion about the clinical significance of all ragweed allergens, because allergenic activity for the majority of them have not been studied yet [4]. This is important both

for making a correct diagnosis and for improving the effectiveness of treatment.

It is known that ASIT is accompanied by decreased IL-4 levels, increased IL-10 and IFN γ concentration, production of specific IgG4, and decreased level of specific IgE. Currently, the search for immunological markers of the effectiveness of ASIT has been continued. A decrease in IL-4 with good results after ASIT has been noted in many studies [10]. An increase in IL-10 was also recorded by some studies [2]. The level of IFN γ may not change after ASIT, although there is evidence of its increase [11]. It was noted that IL-4/IL-10 or IL-4/IFN γ ratios often have better predictive value than isolated cytokine levels [12].

In our study, we observed a significant decrease in the level of IL-4 from 36.19 to 20.19 pg/ml ($W = 277$; $p = 0.002$); the changes in the level of other cytokines

(IL-10; IFN γ) were insignificant ($p > 0.05$), which is consistent with the data from multiple studies.

The IL-4/IL-10 ratio after treatment in the study group decreased significantly from 1.097 to 0.63 ($p = 0.0063$), while in the control group it remained unchanged ($p = 0.2719$).

Some authors point to a noticeable increase in IgG4 level, which is detected as early as within the first 2 months after the onset of ASIT. IgG, due to the competitive binding of IgE to the allergen, can prevent the activation of, basophils, and dendritic cells. IgG can suppress specific T-cell responses. Allergen-specific IgG4 antibodies against IgE persist after discontinuation of treatment and may provide long-term clinical tolerance [6].

At the same time, researchers often note no clear relationship between the concentrations of IgG antibodies and the clinical results of ASIT. And, perhaps, the clinical effect occurs before the noticeable changes in IgG antibodies [7]. We found that in the study group, there was a slight tendency to increased IgG4 *Amb a 1* and IgG4 *Amb trifida* ($p > 0.05$). The

IgE *Amb trifida* level in the study group did not change after treatment ($W = 150$; $p = 0.74$).

Conclusion

ASIT with *Ambrosia artemisiifolia* allergen in patients with sensitization to *Ambrosia trifida* allows to control the course of an allergic disease. The lack of excellent and low number of good ASIT results is likely due to the intraspecific ragweed allergenic properties. To increase the effectiveness of immunotherapy, the treatment of patients with ragweed pollinosis should be carried out by taking into account individual patient IgE profile and type of allergen typical to any geographic region.

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СРАВНИТЕЛЬНАЯ ЭФФЕКТИВНОСТЬ РАЗЛИЧНЫХ СХЕМ ИММУНОРЕАБИЛИТАЦИИ ПРИ БЕСПЛОДИИ ТРУБНО- ПЕРИТОНЕАЛЬНОГО ГЕНЕЗА

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Резюме. Целью исследования явилось сравнение эффективности различных схем фармакотерапии при бесплодии трубно-перитонеального генеза. Под постоянным наблюдением находились 96 пациенток, направленных в стационар для проведения диагностической лапароскопии по поводу бесплодия трубно-перитонеального генеза, разделенных поровну на 4 группы в зависимости от способов фармакологического лечения: 1-я группа получала базовую фармакотерапию (БФТ) после эндоскопического операционного вмешательства (антибактериальная, притивогрибковая, витаминотерапия). Пациентки 2-4-й групп, кроме БФТ, соответственно получали гепон, циклоферон или лавомакс. Контрольная группа была сформирована из 38 гинекологически здоровых женщин. Лабораторное обследование осуществляли в течение 24 часов после операции и на 30-е сутки после БФТ. В вагинально-цервикальном смыве и в плазме крови оценивали активность процессов перекисного окисления липидов, состояние антиоксидантной системы, выявляли уровень стабильных метаболитов оксида азота, неоптерина, С-реактивного белка, цитокинов (TNF α , IL-1 β , IL-8, IFN γ , IL-18, G-CSF, IL-4, IL-10), иммуноглобулинов (IgM, IgG, IgA, sIgA), компонентов системы комплемента (C3, C4, C5, C5a), фагоцитарную и кислородзависимую активность полиморфноядерных лейкоцитов крови. Установлено, что применение в БФТ препаратов с иммуномодулирующей и антивирусной активностью по степени возрастания эффективности в коррекции иммунометаболических лабораторных параметров на системном и местном уровне при бесплодии трубно-перитонеального генеза представляет собой следующую последовательность: базовая фармакотерапия < базовая фармакотерапия + гепон < базовая фармакотерапия + циклоферон < базовая фармакотерапия + лавомакс.

Ключевые слова: бесплодие трубно-перитонеального генеза, фармакологическая коррекция иммунометаболических нарушений

COMPARATIVE EFFECTIVENESS OF VARIOUS SCHEMES OF IMMUNOREHABILITATION IN INFERTILITY OF TUBOPERITONEAL GENESIS

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Abstract. The study aimed to compare the effectiveness of various pharmacotherapy regimens for infertility of tubo-peritoneal genesis. Under constant supervision were 96 patients referred to the hospital

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for diagnostic laparoscopy for infertility of tubo-peritoneal genesis, divided equally into 4 groups depending on the pharmacological treatment methods: the 1st group received basic pharmacotherapy (BPT) after endoscopic surgery (antibacterial, antifungal, vitamin therapy). Patients of groups 2-4, in addition to BPT, received Hepon, Cycloferon or Lavomax, respectively. The control group consisted of 38 gynecologically healthy women. Laboratory examination was performed within 24 hours after the operation and on the 30th day after BPT. Vagino-cervical lavage and plasma were assayed for the activity of lipid peroxidation processes, the state of the antioxidant system, the level of stable nitric oxide metabolites, neopterin, C-reactive protein, cytokines (TNF α , IL-1 β , IL-8, IFN γ , IL-18, G-CSF, IL-4, IL-10), immunoglobulins (IgM, IgG, IgA, sIgA), components of the complement system (C3, C4, C5, C5a), phagocytic and oxygen-dependent activity of polymorphonuclear leukocytes. It was established that the use of immunomodulatory and antiviral activity medication with BPT according to the degree of increasing efficiency in the correction of immunometabolic laboratory parameters at the systemic and local level in infertility of tuboperitoneal genesis is as the following sequence: basic pharmacotherapy < basic pharmacotherapy + Hepon < basic pharmacotherapy + Cycloferon < basic pharmacotherapy + Lavomax.

Keywords: infertility of tubo-peritoneal genesis, pharmacological correction of immunometabolic disorders

Introduction

The restructuring of the etiological system of infectious and inflammatory diseases of the female reproductive system health that revealed in the last decade indicates the need to change opinions and positions on the influence of conventional-pathogenic bacterial flora in the genesis of this nosology. The realization of microflora pathological potential properties, as well as the majority of viral agents, occurs in the presence of an imbalance, primarily in the link of local immunity of a macroorganism. Microbial associations enhanced the pathogenic characteristics of each individual representative of this association, which in turn leads to increased resistance of microorganisms to external factors, antibiotic therapy is no exception [1, 2].

It is believed that the reason for the development of tubal infertility in 55-85% of cases is untimely therapy of pelvic inflammatory diseases (PID). In addition, the risk of developing an ectopic pregnancy increases by 7-10 times in patients of this category in the anamnesis. Now the pathology of organs located in the pelvis in women is determined by the duration and chronicity of this inflammatory process. This leads to the addition of components of the immune and endocrine systems to this inflammatory process. An integral part is the manifestation of local and somatic pathological symptoms. Most often, the triggering mechanism in the development of PID is the effect of a microbial fact, while the development of the inflammatory process, as well as its course and outcome are determined not only by the nature of the pathogen, but also by the state of the microorganism, environmental conditions, the presence of somatic pathology, including viral etiology, leading to prolonged persistence of viruses and, as a rule, immune changes occurring against this background. In the genesis of all inflammatory diseases of the female reproductive system, especially chronic diseases of the pelvic organs, there is a violation of local immune

mechanisms of anti-infection protection with the protective effect of the endometrium and endosalpinx. All this is a trigger factor for the involvement in this process of systemic mechanisms of stabilization of the immune and oxidative homeostasis of the body [6, 9].

In a chronic inflammatory process occurring in the pelvic organs, the highest incidence of adhesions is observed, including due to impaired immune status, which in essence is the cause of secondary infertility. Solving this problem often requires the use of radical invasive surgical correction techniques. The full restoration of the woman's reproductive function after such treatment, with an average and severe inflammatory adhesion process, varies from 15 to 35%. Diagnostic laparoscopy is the standard for the diagnosis of tuboperitoneal infertility, however, the history of the chronic infectious and inflammatory process of the reproductive spheres, the surgery itself, the presence of anesthetic benefits, antibiotic prophylaxis adversely affect the immune and metabolic status, which leads to inadequate rehabilitation of patients and requires adequate postoperative pharmacological correction [7].

Previous publications presented data on immunometabolic laboratory disorders at the systemic and local levels and methods for their pharmacological correction in infertility of tuboperitoneal genesis [3, 6]. Based on this, the aim of the study was the need to use various statistical methods to compare the effectiveness of the applied pharmacotherapy regimens for this pathology.

Materials and methods

For the period 2010-2018 Under constant supervision in the gynecological department of the Kursk Perinatal Center and City Clinical Hospital No. 4 of Kursk, there were 96 patients referred to the hospital for diagnostic laparoscopy for infertility of tuboperitoneal origin. In order to verify the tuboperitoneal genesis of infertility and exclude ot-

her forms of infertility, all patients underwent a basic clinical and laboratory examination including a family and obstetric-gynecological history, infectious screening (microscopic examination of smears from the vagina, cervical canal, and urethra; PCR diagnosis of Chlamydia and Herpes viral infection; screening for Syphilis (RW), Hepatitis B, C (HBsAg, anti HCV antibodies); determination of antibodies of class IgG, IgM to TORCH complex infections), assessment of ovulatory function (Ovulation "ClearPlan" test), and pelvic ultrasound.

The following parameters were determined by the inclusion of criteria:

- age of patients 18-40 years old;
- verified diagnosis – secondary infertility of tuboperitoneal genesis against the background of a chronic infectious and inflammatory process of bacterial-viral etiology, which caused immune disorders that require pharmacological correction;
- presence of bad obstetric and gynecological history (chronic inflammatory diseases of the pelvic organs, a history of infertility, spontaneous miscarriages, miscarriage, abortion);
- availability of indications for the use of medications used in this study;
- medical history up to 10 years;
- absence of severe extragenital pathology;
- negative results on sexually transmitted infections;
- invasive treatment method is applied for the first time;
- signed consent to participate.

Exclusion criteria:

- the general condition of the patient, not allowing to participate in the study;
- persons with a history of surgical treatment;
- the presence of a verified specific sexually transmitted infection;
- persons with uncompensated somatic pathology requiring continuous drug therapy;
- persons with a history of allergies
- patients who refused to participate in the study.

Randomization was formed according to age, concomitant pathology of the history of the disease.

All the women were divided into 4 groups of 24 patients each, depending on the pharmacological treatment methods: the 1st group received basic pharmacotherapy (BPT) after endoscopic surgery (antibacterial – cefotaxime, antifungal – metronidazole, vitamin therapy – B1, B6). Patients of groups 2-4, besides for BPT, respectively received Hepon (as a solution for oral administration in a volume of 10 mg once a day, for 30 days), Cycloferon (12.5% injection for 2 ml, intramuscularly, 1 time per day according to the basic scheme – on 1, 2, 4, 6, 8, 11, 14, 17, 20, 23, 26, 29 days; course dose – 2.5 g) or

Lavomax (in the form of tablets according to 125 mg in the first 2 days at 125 mg, then after 48 hours at 125 mg; course dose – 2.5 g).

The control group was formed in the same period of time, from persons of completely gynecologically healthy women, who came to an outpatient appointment for their annual medical examination and amounted to 38 people.

Laboratory examination was carried out within 24 hours after surgery and on the 30th day after BPT. The activity of lipid peroxidation processes was evaluated by the content of malondialdehyde and acyl hydroperoxides in the vaginal-cervical flushing and plasma, the state of the antioxidant system – by the general antioxidant activity, catalase activity, superoxide dismutase, concentrations of ceruloplasmin. In addition, the level of stable metabolites of nitric oxide, neopterin, C-reactive protein was revealed. All findings were carried out using commercial kits for Enzyme-Linked Immunosorbent Assay (ELISA).

In the blood plasma and vaginal-cervical flushing, cytokines (TNF α , IL-1 β , IL-8, IFN γ , IL-18, G-CSF, IL-4, IL-10), immunoglobulins of classes M, G, A (IgM, IgG, IgA), secretory immunoglobulin A (sIgA) was detected by ELISA using the Vector Best kit (Russia), the components of the complement system (C3, C4, C5, C5a) using the diagnostic kit "Cytokine Ltd" (Russia). The activity of the C1-inhibitor (C1-inh.) was determined by the chromogenic method with the ability to inhibit C1-esterase. Registration was carried out using a microplate photometer "Sunrise", Tecan (Austria).

The phagocytic activity of polymorphonuclear blood leukocytes after their isolation from the blood at a density gradient of Ficoll-Urografin ($d = 1,077$) was evaluated by determining the phagocytic index, phagocytic number and phagocytosis activity index by conventional methods. The activity of oxygen-dependent neutrophil-mediated systems was evaluated on a PD-303S Apel spectrophotometer (Japan) by the reduction reaction of nitro-blue tetrazolium (NBT-test) spontaneous and stimulated by zymosan (NBT-sp., NBT-st.) and a functional neutrophil reserve.

All clinical, instrumental and laboratory studies, pharmacological therapy was carried out with the voluntary informed consent of patients on the use of materials of medical and diagnostic measures related to this disease, exclusively for research purposes.

Statistical and mathematical processing of the research results was carried out according to the criteria of variational-statistical analysis with calculation of average values (M), arithmetic mean errors (m) using the Microsoft Excel 2010 software package. The significance of differences was evaluated by the

U-criterion. Only those differences that corresponded to $p < 0.05$ were considered statistically significant.

Results and discussion

Comparing the number of total differences in laboratory indicators of immune status at the systemic (peripheral blood plasma) and local (cervical-vaginal swab) levels with normal parameters (healthy women) with tuboperitoneal infertility, it was found that before treatment, such indicators were 100% of the studied. After the BPT, this percentage decreased to 93.3%, while only 6.7% returned to normal, 76.7% of the laboratory parameters studied were corrected, and 16.7% remained at the level of treatment initiation. The use of Hepon did not affect the total number of altered and normalized indices, but increased the number of corrected ones to 83.3% and reduced the number of laboratory parameters remaining at the beginning of BPT up to 10%. The use of Cycloferon in BPT reduced the number of parameters changed from the norm to 73.3%, changed the level of normalized and corrected indicators to 26.7% and 73.3%, respectively. After the introduction of Lavomax into the BPT, the corresponding indicators are as follows: 63.3%, 36.7% and 63.3%.

The total differences in metabolic indicators at the systemic and local levels compared with the norm amounted to 93.3%. BPT reduced their number to 80%, while it was respectively normalized, corrected and remained unchanged 13.3%, 66.7%, and 13.3%. The introduction of Hepon into BPT led to the following distribution of the studied parameters: altered from the norm – 73.3%, normalized 20.0% and corrected 73.3%. The use of Cycloferon: altered from the norm – 60.0%, normalized 33.3%, corrected – 60.0%. The use of Lavomax: altered from the norm – 40.0%, normalized 53.3%, corrected – 40.0%.

It was important to compare the changes in all studied laboratory parameters individually at the systemic and local levels. The total differences in immune and metabolic parameters at the systemic level compared with the norm amounted to 100%. BPT reduced their number to 93.5%, while it was respectively normalized, corrected and remained unchanged at 6.5%, 80.6% and 12.9%. The introduction of Hepon into the BPT led to the following distribution of the studied parameters: 90.3% changed from the norm, 9.7% normalized, 83.9% corrected, remained at the level of the BPT – 6.5%. The use of Cycloferon: altered from the norm – 74.2%, normalized 25.8%, corrected – 74.2%. The use of Lavomax: altered from the norm – 58.1%, normalized 41.9%, corrected – 58.1.0%.

The total differences in immune and metabolic indices at the local level compared with the norm amounted to 92.9%. BPT reduced their number to 84.6%, while it was respectively normalized, corrected

and remained unchanged at 15.4%, 61.5% and 23.1%. The introduction of Hepon into BPT changed only the level of corrected parameters – 76.9% and those remaining at the level before BPT – 7.7%. The use of Cycloferon: altered from the norm – 61.5%, normalized 38.5% and corrected – 61.5%. The use of Lavomax: changed from the norm – 53.8%, normalized 46.2%, corrected – 53.8%.

The total differences of all the studied laboratory immune and metabolic indicators at the systemic and local levels compared with the norm amounted to 97.8%. BPT reduced their number to 90.9%, while it was respectively normalized, corrected and remained unchanged 9.1%, 75.0% and 15.9%. The introduction of Hepon into BPT changed the number of parameters studied: differences from the norm – 88.6%, 11.4% normalized, 81.8% corrected, remained at the level of BPT – 6.8%. The use of Cycloferon: altered from the norm – 70.5%, normalized 29.5%, corrected – 70.5%. The use of Lavomax: altered from the norm – 56.8%, normalized 43.2%, corrected – 56.8%.

It was important to find out not only the general quantitative but also the changes in each of the studied laboratory parameters according to the degree of disorder [8, 9].

It was found that with infertility of tuboperitoneal genesis before surgery, 100% of the studied indicators of immune status were changed from the normal values at the systemic (blood plasma) and local (cervical-vaginal swab) levels, while it was found that I degree of disturbances had 16, 7%, and II and III, respectively, 30.0% and 53.3%. It should be noted that with the II-III degree of disturbed laboratory parameters requiring mandatory profile correction [8, 9], 56.7% were found. After BPT, 93.3% of the parameters of the immune status turned out to be altered, and accordingly, I, II and III, the degree of disorders was 20.0%, 23.3%, and 50.0%. After the inclusion of Hepon in the BPT, 93.3% of indicators remained impaired, respectively, I, II and III, the degree of impairment was 26.7%, 20.0%, and 46.7%. The use of Cycloferon reduced the number of violated laboratory parameters to 73.3%, respectively, according to the degree of violation from I to III: 30.0%, 10.0%, and 36.7%. The inclusion of Lavomax in the BPT was the most effective since 63.3% of laboratory parameters remained altered, and I, II, and III degrees of violations had 20.0%, 10.0%, and 30.0% of parameters, respectively, at the systemic and local levels.

In infertility of tube-peritoneal genesis before BPT, 93.3% of the studied metabolic status indicators changed at the systemic (blood plasma) and local (cervical-vaginal swab) levels from normal values, while it was found that I degree of disturbances was 26.7%, and II and III, respectively, 13.3% and 53.3%. After BPT, 80.0% of metabolic parameters turned out to be altered, and accordingly, I, II and III degree of

disturbances were 26.7%, 20.0%, and 33.3%. After the inclusion of Hepon in the BPT, 73.3% of the indicators remained impaired (respectively, I, II and III, the degree of violations was 20.0%, 40.0%, and 13.3%). The use of Cycloferon reduced the number of violated laboratory parameters to 60.0%, respectively, according to the degree of violation from I to III: 20.0%, 26.7%, and 13.3%. The inclusion of Lavomax in BPT was most effective, since 40.0% of metabolic indicators remained altered, and I, II, and III degrees of disorders had 13.3% respectively at the systemic and local levels.

The total differences of all the studied laboratory immune and metabolic indicators at the systemic and local levels compared with the norm amounted to 100% of the metabolic status at the systemic and local levels, while it was revealed that I degree of disturbances were 19.4%, and II and III, respectively 29.0% and 51.6%. After BPT, 93.5% of the immunometabolic parameters turned out to be altered, and accordingly, I, II and III, the degree of disorders was 29.0%, 16.1%, and 48.4%. After the inclusion of Hepon in the BPT, 90.3% of the indicators remained impaired (respectively, I, II and III, the degree of impairment was 32.3%, 22.6%, and 35.5%). The use of Cycloferon reduced the number of violated laboratory parameters to 74.2%, respectively, according to the degree of violation from I to III: 32.3%, 12.9%, and 32.3%. The inclusion of Lavomax in the BPT was most effective, since 58.1% of metabolic indicators remained altered, and I, II, and III degrees of disturbances were 22.6%, 12.9%, and 22.6%, respectively, at the systemic and local levels.

The total differences in immune and metabolic indices at the local level compared with the norm amounted to 92.9%, while it was revealed that I degree of disorders was 21.4%, and II and III, respectively, 14.3% and 57.1%. After BPT or after incorporation of Hepon into it, 84.6% of immunometabolic parameters turned out to be altered; respectively, I, II and III degree of disturbances were 7.1%, 35.7%, and 35.7%. The use of Cycloferon reduced the number of impaired immunometabolic laboratory parameters to 61.5%, respectively, according to the degree of impairment from I to III: 14.3%, 21.4%, and 21.4%. After the inclusion of Lavomax in the BPT, 53.8%

of the indicators remained altered, and I, II and III degree of violations were 7.1%, 7.1%, and 21.4%, respectively, at the systemic and local levels.

The total differences of all the studied laboratory immune and metabolic parameters at the systemic and local levels compared with the norm amounted to 97.8%, while it was found that I degree of disorders was 20.0%, and II and III, respectively, 24.4% and 53.3%. After BPT, 90.9% of the immunometabolic parameters turned out to be altered, and, accordingly, I, II, and III degrees of disturbances were 22.2%, 22.2%, and 44.4%. After the inclusion of Hepon in the BPT, 88.6% of the indicators remained impaired (I, II and III, respectively, had a degree of impairment in 24.4%, 26.7%, and 35.6%). The use of Cycloferon reduced the number of violated laboratory parameters to 70.5%, respectively, according to the degree of violation from I to III: 26.7%, 15.6%, and 28.9%. The introduction of Lavomax into BPT reduced to 56.8% immunometabolic indices altered from the norm, and I, II and III degree of disturbances were 17.8%, 11.1%, and 20.0%, respectively.

When assessing the proper corrective effects of Hepon, Cycloferon, and Lavomax in the correction of laboratory immunometabolic disorders in infertility of tuboperitoneal origin, the following was established.

The introduction of Hepon into BPT, in comparison with it, had a positive effect on improving laboratory parameters of the immune status (19 points), immunometabolic parameters at the systemic and at the combined (systemic and local) level (10 and 6 points, respectively).

The use of Lavomax in the postoperative BPT in patients with tuboperitoneal infertility in assessing their own corrective effects was most effective since the sum of correction indicators was higher compared to Hepon and Cycloferon in the study at all levels: in terms of immune status – 46 points, metabolic status parameters – 50 points, laboratory immunometabolic parameters at the system level – 45 points, immunometabolic indicators at the local level – 60 points and the total immune and metabolic performance at the systemic and local level – 53 points.

The use of Cycloferon in BPT compared with Hepon and Lavomax showed an intermediate result.

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ЦИРКУЛИРУЮЩИЕ БИОМАРКЕРЫ СИСТЕМНОГО ВОСПАЛИТЕЛЬНОГО ОТВЕТА В ОЦЕНКЕ ПОСТПЕРИКАРДИОТОМИЧЕСКОГО СИНДРОМА У ПАЦИЕНТОВ ПОСЛЕ КАРДИОХИРУРГИЧЕСКИХ ВМЕШАТЕЛЬСТВ

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Резюме. В настоящее время одним из частых осложнений, регистрируемых у 9-65% пациентов после кардиохирургического вмешательства, является постперикардотомический синдром (ПКТС). Несмотря на широкую распространенность, механизмы развития ПКТС до сих пор мало изучены. Особенный интерес представляет использование колхицина, являющегося мощным противовоспалительным препаратом. Предполагаемые механизмы действия данного препарата обусловлены способностью колхицина ингибировать мобилизацию сборки инфламасомы NLRP3, подавлять активацию каспазы-1 и, как следствие, предотвращать высвобождение провоспалительных цитокинов. На сегодняшний день имеются противоречивые данные об использовании колхицина и его влиянии на развитие ПКТС в рамках системного воспалительного ответа после кардиохирургических вмешательств. В связи с этим актуальным представлялось изучить динамику сывороточного содержания IL-6, IL-10, IL-1 β , TNF α у пациентов перед аортокоронарным шунтированием (Т1), через 6 ч (Т2), на 10-е сутки (Т3) после операции и оценить влияние колхицина на развитие постперикардотомического синдрома. Анализ цитокинового профиля показал, что в обеих группах через 6 ч после операции отмечался пик высвобождения в кровоток IL-10 с последующим снижением к 10-м суткам, но в группе 1 уровень IL-10 оказался выше исходных значений, чем в группе 2. В обеих группах содержание IL-6 многократно повышалось на Т2 и значимо снижалось к Т3, при этом уровни IL-6 в группе 2 оставались выше по сравнению с исходными значениями. Частота развития плеврита была ниже в группе пациентов, принимающих колхицин. Только в группе 1 показаны положительные корреляционные связи между IL-6 и TNF α . У пациентов без признаков плеврита уровень IL-10 напрямую ассоциировался с повышенным содержанием TNF α . Не было выявлено значимых межгрупповых отличий сывороточного уровня IL-1 β и TNF α , а также значимых изменений IL-1 β между этапами наблюдения. Анализ экспрессии TNF α выявил в группе 1 значимые отличия содержания TNF α между этапами Т1-Т3 и Т2-Т3 ($p < 0,05$). В обеих группах были установлены множественные позитивные ассоциации между изучаемыми показателями. Таким образом, полученные данные свидетельствуют

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о противовоспалительном эффекте колхицина у кардиохирургических больных. Клинически это выразалось в тенденции к меньшей частоте развития плевритов и сопровождалось повышенной экспрессией IL-10, обладающего противовоспалительным и иммуномодулирующим действием на фоне приема препарата в послеоперационном периоде.

Ключевые слова: колхицин, кардиохирургические вмешательства, постперикардотомический синдром, плеврит, системный воспалительный ответ

CIRCULATING BIOMARKERS OF SYSTEMIC INFLAMMATORY RESPONSE IN THE ASSESSMENT OF POSTPERICARDIOTOMY SYNDROME IN PATIENTS AFTER CARDIAC SURGERY

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Abstract. Postpericardiotomy syndrome (PCTS) is one of the most frequent cardiac surgery complications seen in 9-65% of patients. Despite its widespread occurrence, the mechanisms of the development of PCTS are still understudied. drug. The use of colchicine in cardiac surgery patients is of particular interest. Due to the ability of this drug the colchicine mechanisms of action are able to inhibit the mobilization of the NLRP3 inflammasome assembly, to suppress the activation of caspase-1. As a result, it can prevent the release of pro-inflammatory cytokines, namely IL-1 β and IL-18. There are conflicting data on the effect of colchicine on the PCTS progression within the systemic inflammatory response after cardiac surgery. In this regard, it was important to study the dynamics of serum levels of IL-6, IL-10, IL-1 β , and TNF α in patients before coronary artery bypass grafting (T1), 6 hours (T2), and 10 days (T3) after surgery, and to evaluate the effect of colchicine on the development of PCTS. The results of our research showed a significant increase of IL-10 in both groups 6 hours after surgery. However, on the 10th day, the increase in the level of IL-10, compared with the initial values, was higher in the 1st group – 2 times, compared with the 2nd group. In both groups, showed significant increase in serum concentration of IL-6 after 6 h surgery, with a subsequent decrease in the expression at the stage of T3, while the IL-6 levels in the 2nd group was statistically notably higher than T1. The incidence of pleurisy was lower in the group of patients taking colchicine. Only in the 1st group IL-6 levels were directly associated with IL-10. In patients with pleurisy, the level of released IL-10 and TNF α was significantly higher in the 2nd group. There were no significant intergroup differences in serum levels of IL-1 β and TNF α , as well as significant changes in IL-1 β between the stages of observation. Analysis of TNF α expression revealed significant differences in TNF α content in the 1st group between the T1-T3 and T2-T3 stages. In both groups, multiple positive associations were found between the studied indicators. Thus, data were obtained indicating the anti-inflammatory effect of colchicine in cardiac surgery patients. This was clinically expressed in a tendency to a lower incidence of pleurisy, and was accompanied by increased expression of IL-10, which has an anti-inflammatory and immunomodulatory effect against the background of the drug in the postoperative period.

Keywords: colchicine, cardiac surgery, postpericardiotomy syndrome, pleurisy, systemic inflammatory response

Introduction

Coronary artery disease (CAD) is a common disease of the cardiovascular system, accompanied by a high risk of vascular complications and death. Currently, coronary artery bypass grafting (CABG) is one of the most effective treatment methods for patients

with multifocal atherosclerosis of the coronary arteries and complicated CAD forms.

The CAD excess incidence, the number and complexity of CABG operations makes it necessary to improve the safety and effectiveness of surgical intervention, identify predictors of development, impro-

ve early diagnostics methods and develop new approaches to prevent cardiac surgical complications. Postpericardiotomy syndrome (PCTS) is one of the most frequent cardiac surgery complications observed in 9-65% of patients [13, 14]. Despite its widespread occurrence, the mechanisms of developing PCTS are still understudied.

During operations with artificial blood circulation, many aggressive factors are experienced, including surgical trauma, exposure to cardioplegia, hypothermia, ischemic/reperfusion injury, blood contact with bioactive surfaces of the circuit in the artificial blood circulation apparatus, which leads to the launched inflammatory reaction, including the activation of proteins and blood plasma cells, the release of pro-inflammatory molecules, enzymes and cytotoxic substances [9, 12, 15]. Extensive research was conducted for studying preventive treatment of patients with cardiac surgery. There were used drugs with anti-inflammatory effects that affect various stages of PCTS formation. At the moment, all drugs that were used for treatment of both PCTS and pericarditis are “off-label”, i.e. initially, they were not registered for treatment of such pathologies. In this regard, the use of colchicine in cardiac surgery patients is of particular interest as a powerful anti-inflammatory drug traditionally used for gout treating. Due to the ability of this drug, the colchicine mechanism of action is able to inhibit the mobilization of the NLRP3 inflammasome assembly and suppress the activation of caspase-1. As a result, it can prevent the release of pro-inflammatory cytokines, namely IL-1 β and IL-18. The drug has an antimitotic effect, suppresses (fully or partly) cell division in the anaphase and metaphase stages, prevents neutrophil degranulation and reduces migration of white blood cells to the focus of inflammation [4, 11]. It is known that by interfering with the microtubule polymerization, colchicine inhibits the chemotaxis of monocytes and neutrophils, which are found in large numbers in the lungs of patients with COVID-19 [1]. Currently, several randomized trials are ongoing to evaluate the feasibility of using colchicine in patients with COVID-19. Among them, COLCORONA – a large-cohort study involving patients with cardiac manifestation of COVID-19 and a smaller study COLHEART-19, which includes hospitalized patients. To this date, conflicting data have been obtained on the use of colchicine, including cardiac surgery patients. During the study [1], colchicine was used in hospitalized patients with severe COVID-19 to prevent a “cytokine storm”. The first COLORIT results emphasizing the effectiveness of colchicine (at a dose of 1 mg for 1-3 days, with the following treatment at a dose of 0.5 mg/day for 14 days) as a

proactive anti-inflammatory therapy in hospitalized patients with COVID-19 and viral pneumonia have been obtained [3]. The results of the first LoDoCo research involving 532 individuals demonstrated the efficacy, safety, and long-term tolerability of colchicine (0.5 mg once daily), as well as improved cardiovascular outcomes in patients with chronic CAD [7]. The COPPS study showed that colchicine significantly reduced the incidence of pericarditis for 12 months compared to placebo (12.2% vs 25.6%, $p = 0.002$) in cardiac surgery patients, however, in the COPPS-2 research, prescription of colchicine for 48 and 72 hours before surgery did not reduce the incidence of postoperative pericarditis compared to placebo [2]. In the POPE-2 study: colchicine prescription to patients after cardiac surgery did not reduce the volume of effusion in the pericardium [10]. The results of the COLCOT research showed that low doses of colchicine reduce the risk of recurrent cardiovascular events in patients with recent myocardial infarction [5]. At the same time, controversial data on the effect of colchicine on the PCTS progression during systemic inflammatory response after cardiac surgery were obtained.

Objective: to study the dynamics of inflammatory response markers in patients after coronary artery bypass grafting and evaluate an effect of colchicine on the developing postpericardiotomy syndrome.

Materials and methods

There were enrolled 84 patients (aged 62.5 ± 6.7 years) with a coronary artery disease diagnosis, angina pectoris (functional class II-III), with multi-vessel stenosing coronary atherosclerosis. All patients were hospitalized at the Department of Cardiovascular Surgery of the Research Institute of Cardiology for performing CABG under conditions of artificial blood circulation. The exclusion criteria were: reduced left ventricular ejection fraction ($\leq 35\%$), valvular pathology requiring surgical correction, hepatic insufficiency with increased level of hepatic transaminases by ≥ 1.5 times, renal insufficiency (increased creatinine levels of more than 130 mmol/L), persistent or persistent form of atrial fibrillation, hypersensitivity, neutropenia, and a history of alcoholism. The research protocol was approved by the local ethics committee.

Patients were divided into two groups: group 1 ($n = 45$), in order to prevent postoperative complications, colchicine was prescribed at a dose of 0.5 mg once per 4 hours before surgery, followed by 0.5 mg twice a day for 10 days after surgery; group 2 ($n = 39$) received standard treatment with nonsteroidal anti-inflammatory drugs (NSAID) after surgery.

The dynamics of biomarkers of the systemic inflammatory response were evaluated before CABG (T1), as well as 6 hours (T2), and 10 days (T3) after surgery. In all patients, the ulnar vein was punctured in the morning within the followup period. Blood samples were collected and incubated at room temperature for 30-45 minutes followed by centrifuge at 3000 rpm for 15 minutes at room temperature. The serum levels of interleukins 6 (IL-6), 10 (IL-10), 1- β (IL-1 β), and tumor necrosis factor (TNF α) were measured by multiplex immunoassay using MILLIPLEX[®] MAP KIT (Merck KGaA, Darmstadt) and FLEXMAP 3D System (Luminex[®] Corporation) diagnostic panels.

In the preoperative and postoperative periods, all patients underwent a set of laboratory tests, including measurement of C-reactive protein, white blood cell count, and specific enzymes (AST, ALT, creatinine). In the postoperative period, fluid accumulation in the pleural cavities and in the pericardium were daily monitored.

Statistical processing of the data obtained was performed by using the Statistica 10.0 application software package. Due to the data differed from the normal distribution, all quantitative parameters were described by using the median (Me) and interquartile range ($Q_{0.25}$ - $Q_{0.75}$). For pairwise comparison, the Kruskal-Wallis rank criterion was used. The statistical significance of the differences was evaluated by using the nonparametric Wilcoxon test. The Spearman rank correlation coefficient was calculated to assess

inter-parameter relationship. In all statistical analysis procedures, the critical significance level of p is assumed to be 0.05.

Results and discussion

According to the results of the routine tests and clinical data in the preoperative period, no signs of inflammation in patients from both groups were found. Table 1 shows the results of quantitative analysis of expressed cytokines before CABG (T1), as well as 6 hours (T2) and 10 days (T3) after surgery (Table 1).

Despite the fact that at the time of inclusion in the study for the main disease, types of surgical correction, duration of surgery and artificial blood circulation, as well as other comorbidities of the group, both groups were comparable, while analyzing the level of IL-10, significant differences were initially revealed in group 1 vs group 2, being markedly elevated by about 2-fold. Further, 6 hours after the operation, we noted its significant increase in both groups. However, on day 10 vs baseline, an increased level of IL-10 was higher in group 1 vs group 2 by 2-fold.

The results of our research also showed significantly increased serum concentration of IL-6 6 hrs after surgery that subsequently decreased at the stage of T3, with varying degrees of severity: in group 1 level of IL-6 did not differ from baseline values, but in group 2 was significantly higher than at T1 ($p = 0.0005$).

The results of the comparative analysis revealed no statistically significant intergroup difference in serum

TABLE 1. DYNAMICS OF INFLAMMATORY RESPONSE BIOMARKERS BEFORE CORONARY ARTERY BYPASS GRAFTING (T1), 6 HOURS (T2) AND 10 DAYS (T3) AFTER SURGERY, Me ($Q_{0.25}$ - $Q_{0.75}$)

	Group 1 (n = 44)			p	Group 2 (n = 39)			p	p between groups
	T1	T2	T3		T1	T2	T3		
IL-10, pg/ml	1.95 (1.18-4.52) ^{# **}	15.75 (9.86-59.49) ^{***}	3.90 (2.30-8.37) [#]	$p_{T1-T2} < 0.000$ $p_{T2-T3} < 0.000$ $p_{T1-T3} < 0.000$	1.01 (0.56-2.41) ^{# *}	14.93 (7.25-33.02) ^{***}	1.66 (0.75-3.30) [#]	$p_{T1-T2} < 0.000$ $p_{T2-T3} < 0.000$	$p_{T1} = 0.007$ $p_{T3} < 0.000$
IL-1β, pg/ml	0.39 (0.28-0.62)	0.49 (0.32-0.67)	0.42 (0.30-0.58)	N/D	0.46 (0.27-0.90)	0.46 (0.30-0.70)	0.45 (0.30-0.61)	N/D	N/D
IL-6, pg/ml	0.52 (0.04-3.47) [*]	61.54 (42.05-86.58) ^{***}	2.09 (0.82-5.74)	$p_{T1-T2} < 0.000$ $p_{T2-T3} < 0.000$	0.17 (0.10-0.70) ^{**}	46.45 (33.05-73.39) ^{***}	2.63 (1.13-5.33)	$p_{T1-T2} < 0.000$ $p_{T2-T3} < 0.000$ $p_{T1-T3} < 0.000$	N/D
TNFα, pg/ml	13.49 (7.19-19.18) ^{**}	12.42 (6.03-19.95) ^{***}	17.13 (8.38-23.20)	$p_{T1-T3} = 0.036$ $p_{T2-T3} = 0.036$	11.71 (7.63-14.55)	9.86 (4.15-14.60)	10.39 (6.42-19.52)	N/D	N/D
IL-10/TNFα	0.23 (0.10-0.41) ^{# **}	1.45 (0.76-6.74) ^{***}	0.33 (0.16-0.84) [#]	$p_{T1-T2} < 0.000$ $p_{T2-T3} < 0.000$ $p_{T1-T3} = 0.020$	0.13 (0.05-0.28) ^{# *}	2.07 (0.84-3.93) ^{***}	0.18 (0.09-0.36) [#]	$p_{T1-T2} < 0.000$ $p_{T2-T3} < 0.000$	$p_{T1} = 0.024$ $p_{T3} = 0.019$

Note. The difference ($p < 0.05$): *, between T1 – T2; **, between T1-T3; ***, between T2-T3. Intergroup statistically significant ($p < 0.05$) differences: #, $p < 0.05$; N/D, not detected.

TABLE 2. INFLAMMATION RESPONSE BIOMARKERS IN PATIENTS WITH PLEURISY

Pleurisy			
Cytokines	Group 1 (n = 10)	Group 2 (n = 14)	p
IL-10 (T1), pg/ml	2.17 (1.43-3.51)	0.66 (0.10-1.66)	p = 0.006
TNF α (T3), pg/ml	20.56 (13.05-23.06)	8.73 (7.62-13.59)	p = 0.004
Without pleurisy			
	Group 1 (n = 34)	Group 2 (n = 25)	
IL-10 (T3), pg/ml	3.68 (2.44-8.73)	1.71 (0.48-3.34)	p = 0.003
IL-10/TNF α (T3)	0.41 (0.16-0.97)	0.13 (0.09-0.32)	p = 0.009

levels of IL-1 β , as well as significant changes in IL-1 β between the stages of observation ($p > 0.05$).

Comparative analysis of TNF α expression showed no differences between the studied groups, but revealed the peculiarities in its dynamics between the stages of observation. Thus, in group 1, the peak of TNF α release was observed 10 days after surgery. Significant differences in the TNF α level were found between the T1-T3 and T2-T3 stages ($p < 0.05$). In group 2, the serum TNF α level did not differ between the stages.

Our study revealed significant inter-group differences in the IL-10/TNF α index: patients receiving colchicine had IL-10/TNF α ratio significantly higher at stages T1 and T3 vs group 2 ($p = 0.024$ and 0.019 , respectively). In both groups, a maximum of IL-10/TNF α ratio was observed 6 hours after surgery. 10 days after CABG, the magnitude of this parameter in group 1 remained markedly elevated compared to baseline level at T1 ($p = 0.02$).

In the group of patients receiving colchicine, the incidence of pleurisy was lower than in the group with NSAID (22.2% and 35.9%, respectively). The results of the comparative analysis showed that patients with pleurisy had the level of IL-10 and TNF α significantly elevated in group 1 (Table 2).

In both groups, multiple positive associations were found between the studied indicators. Thus, a high level of IL-10 released into the bloodstream was associated with increased amount of TNF α at all stages of the follow-up period. Similar correlations were shown for IL-6 and IL-1 β , as well as IL-10/TNF α and IL-6 at stage T1. Only in the group of patients receiving colchicine, the correlation analysis showed positive associations between IL-6 and TNF α . IL-6 levels were directly associated with IL-10 in group 1 at 6 hrs, whereas in group 2 – at day 10 after surgery. Patients without signs of pleurisy in both groups had the level of released IL-10 that was directly associated with increased level of TNF α , while this relationship was not found in patients with pleurisy.

Direct traumatization of the pericardial leaflets, ischemic (during anoxia) and reperfusion (after removing the clamp from the aorta) damage to cardiomyocytes, hypothermia, and contact of blood cell elements with the surface of the artificial circulatory apparatus are considered as triggers that contribute to development of systemic inflammation in the postoperative period [15]. In addition, in the first hours of the postoperative period, there is a high probability of bleeding into the pericardial cavity, which also serves as a trigger for the inflammatory process [12]. The action of damaging factors leads to the activation of immunocompetent cells, as well as release of inflammatory mediators into the systemic bloodstream, which contribute to developing local and systemic inflammatory reactions.

In our study, the analysis of the cytokine profile of cardiac surgery patients showed that in both groups, 6 hours after surgery, the release of IL-10 into the bloodstream, which has anti-inflammatory and immunosuppressive properties, controlling the severity and duration of the inflammatory process was at peak. In addition, 10 days after CABG, the level of IL-10 decreased, but in group 1, its increase to the baseline magnitude was higher than in group 2.

IL-1 β and TNF α are proinflammatory cytokines, which potentiate intensity of inflammatory process manifested by increased exudation and mobilization of neutrophils in the focus of inflammation. High levels of such cytokines immediately after exposure to the damaging factor are necessary for formation of proper inflammatory response, but their production should decline later. TNF α levels vs other cytokines rise early that reflects its role as an initiator in the inflammatory response. TNF α is a mediator of various pathologies and involved in the “cytokine storm” [14]. The results of our study revealed no significant inter-group differences in serum IL-1 β levels, as well as significant changes in IL-1 β between follow-up stages. Comparative analysis of TNF α expression also showed no differences between the

studied groups. It is recognized that chronic diseases are a significant factor that reduces the level of proinflammatory cytokines. The absence of marked changes in the dynamics of IL-1 β and TNF α in our study seem to be connected to the chronic nature of the underlying disease.

IL-6 acts as an indicator of the intensity in the inflammatory process. In cardiac surgery, IL-6 is produced mainly in the myocardium. Plasma levels of IL-6 correspond to the severity of tissue damage caused by surgical trauma and the severity of inflammatory response caused by artificial blood circulation. IL-6 also has a high predictive value for 30-day and total mortality in patients after cardiac surgery. In our study, the IL-6 level increased frequently 6 hours later and notably decreased by day 10 after surgery, while the levels of IL-6 in group 2 remained elevated compared to the baseline magnitude.

The serum IL-10/TNF α ratio is an indicator of inflammatory response and a prognostic sign of the severity and outcome of the infectious process [15]. In our study, the magnitude of IL-10/TNF α ratio in the group of patients receiving colchicine vs group 2 was markedly higher at the T1 and T3 stages. A maximum of IL-10/TNF α ratio was observed 6 hours after surgery in both groups. 10 days after CABG, its magnitude in group 1 remained significantly elevated compared to baseline level.

The incidence of pleurisy in our study was lower in the group of patients receiving colchicine, but did not significantly differ from that one in group 2. The results

of the comparative analysis showed that in patients with pleurisy, the level of released IL-10 and TNF α was significantly higher in the group 2. It is necessary to take into account that the biological effects of many cytokines have a high degree of redundancy, which creates wide opportunities to compensate for the insufficiency or deficiency of some mediators, as well as many cytokines are able to interact with components of the same receptor complexes.

Only in the group of patients receiving colchicine, the correlation analysis showed the positive association between IL-6 and TNF α . IL-6 levels were directly associated with IL-10 in group 1 at 6 hrs, whereas in group 2 – at day 10 after surgery. In patients without signs of pleurisy in both groups, the level of released IL-10 was directly associated with increased level of TNF α , while this relationship was not found in subjects with detected pleurisy.

Thus, the data obtained indicate that colchicine had anti-inflammatory effect in cardiac surgery patients clinically manifested as a tendency to lower incidence of pleurisy and accompanied by increased expression of IL-10 exerting anti-inflammatory and immunomodulatory effect compared to the postoperative period.

A comprehensive assessment of the dynamics for pro- and anti-inflammatory cytokines can be used for studying features of inflammatory response and preventing development of postoperative complications in cardiac surgery patients.

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ЭФФЕКТИВНОСТЬ ИММУНОМОДУЛЯТОРОВ И АНТИОКСИДАНТОВ В КОРРЕКЦИИ ПАРАМЕТРОВ ИММУННОГО СТАТУСА ПАЦИЕНТОК С АДЕНОМИОЗОМ

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Резюме. Целью исследования явилось изучение эффективности использования иммуномодуляторов и антиоксидантов в коррекции параметров иммунного статуса у пациенток с аденомиозом. Обследовано 70 пациенток, среди которых 57 женщинам (основная группа), у которых по результатам комплексного клинического, ультразвукового и гистероскопического обследования был выставлен диагноз аденомиоз. Контрольную группу составили 23 гинекологически здоровые женщины. После верификации диагноза все пациентки ОГ получали стандартное лечение (СЛ) (клинические рекомендации МЗ РФ от 2016 года). Среди пациенток ОГ 19 женщин получали только СЛ (1-я подгруппа). 38 обследованных ОГ дополнительно к СЛ получали различные комбинации антиоксиданта, иммуномодулятора и мембранопротектора и были разделены на две подгруппы. Во вторую подгруппу вошли 20 больных дополнительно к СЛ получавших Натрия рибонуклеат; Гипоксен и фосфолипиды. В третью подгруппу вошло 18 пациенток дополнительно получавших Инозин + Никотинамид + Рибофлавин + Янтарная кислота; Меглюмина акридонацетат, и Глицирризиновую кислоту + фосфолипиды. Проводился анализ цитокинового статуса и системы комплемента на момент поступления и к 15-му дню наблюдения. Установленные нами у пациенток с аденомиозом увеличение в системной циркуляции содержания хемокинов и провоспалительных цитокинов (TNF α , IL-8, IL-1 β , IL-6, IL-18) со снижением концентрации противовоспалительных цитокинов (IL-1ra, IL-4) отражает реакцию резидентных и рекрутированных клеток врожденного иммунитета на молекулярные паттерны, ассоциированные с повреждением. Выявленные изменения цитокинового статуса, активация системы комплемента, повышение кислородзависимой активности нейтрофилов периферической крови (повышенная продукция активных форм кислорода в результате респираторного взрыва) свидетельствует о наличии иммунного воспаления на системном уровне.

Недостаточная клинико-лабораторная эффективность СЛ в коррекции иммунных изменений обосновала использование в фармакологической терапии аденомиоза препаратов с иммуномодулирующими, антиоксидантными и мембранопротекторными свойствами, что было успешно использовано при лечении других заболеваний с аналогичными нарушениями. Не следует исключать и прямого положительного влияния использованных в работе препаратов на ангиогенез в очагах аденомиоза,

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контролируемый на локальном уровне ангиогенными факторами роста и цитокинами, секретируемыми перитонеальными макрофагами, клетками эндометриодных гетеротопий и эндотелия. Нами выявлены оптимальные сочетания иммуномодуляторов и антиоксидантов в коррекции иммунного статуса пациенток с аденомиозом. Проведенное исследование показывает эффективность коррекции нарушенных параметров иммунного статуса пациенток с аденомиозом при комбинации стандартного лечения с антиоксидантными и иммуномодулирующими средствами.

Ключевые слова: аденомиоз, иммунный статус, иммуномодуляторы, антиоксиданты, эффективность лечения

EFFICACY OF IMMUNOMODULATORS AND ANTIOXIDANTS IN THE CORRECTION OF IMPAIRED IMMUNE STATUS PARAMETERS IN PATIENTS WITH ADENOMYOSIS

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Abstract. The aim of the study was to study the effectiveness of the use of immunomodulators and antioxidants in the correction of immune status parameters in patients with adenomyosis. 70 patients were examined, including 57 women (the main group), who were diagnosed with adenomyosis according to the results of a comprehensive clinical, ultrasound and hysteroscopic examination. The control group consisted of 23 gynecologically healthy women. After verification of the diagnosis, all patients with hypertension received standard treatment (SL) (clinical recommendations of the Ministry of Health of the Russian Federation from 2016). Among the female patients, 19 women received only SL (1st subgroup). 38 exhaust gases examined, in addition to SL, received various combinations of antioxidant, immunomodulator, and membrane protector and were divided into two subgroups. The second subgroup included 20 patients in addition to the SL receiving sodium ribonucleate; Hypoxene and phospholipids. The third subgroup included 18 patients who additionally received Inosine + Nicotinamide + Riboflavin + Succinic acid; Meglumine acridone acetate, and glycyrrhizic acid + phospholipids. The analysis of the cytokine status and the compliment system was performed at the time of admission and by the 15th day of observation. Detected changes of the cytokine status, complement system activation, increased oxygen-dependent activity of neutrophils in the peripheral blood (increased production of active oxygen forms as a result of respiratory burst) confirm the presence of immune inflammation on the systemic level. Insufficient clinical-laboratory efficacy of ST in the correction of immune changes has justified the use of drugs with immunomodulating, antioxidant, and membrane protective properties in the pharmacological therapy of adenomyosis, which have been successfully used in the treatment of other diseases with similar disorders. Optimal combinations of immunomodulators and antioxidants in the correction of the immune status of patients with adenomyosis were revealed. The study performed demonstrates the efficacy of correcting immune status parameters in patients with adenomyosis when the standard treatment is combined with antioxidant and immunomodulating agents.

Keywords: adenomyosis, immune status, immunomodulators, antioxidants, treatment effectiveness

Introduction

According to the WHO definition, endometriosis is a dishormonal, immune-dependent, and genetic pathological process, wherein overgrowth of the benign tissue morphologically and functionally resembling endometrium occurs outside the uterine cavity [1].

There is every reason to believe that the endometrial tissue located in atypical places may acquire antigenic properties, which promotes development of body systemic immune reaction. Potential local immune “failures”, acting as specific pathogenetic links of endometriosis, include disorders of: 1) cellular pro-

liferation and localization control; 2) regulation of the apoptosis process; 3) neoangiogenesis mechanisms [3, 4, 7]. The aforementioned facts allow to suppose that adenomyosis conditions are accompanied not only by local, but also systemic changes characterized by the development of chronic systemic inflammatory response, impaired lipid peroxidation, antioxidant protection factors, which leads to the formation of endothelial dysfunction, impaired microrheology, hypoxia, and dysmetabolism [6, 12].

The purpose of the study was to assess efficacy of immunomodulators in correction of immune status parameters in patients with adenomyosis.

Materials and methods

70 patients that underwent examination at the SHI Lipetsk Regional Perinatal Center in 2014-2017 were investigated. The main group included 57 females diagnosed with adenomyosis according to the results of complex clinical, ultrasound, and hysteroscopic examination. 23 gynecologically healthy females were included into the control group.

Patients with exacerbated extragenital pathology, acute bacterial and viral infections, hematological diseases, gastric and duodenal peptic ulcers, necrotic ulcerative colitis, oncological diseases, as well as those receiving anticoagulants, antiaggregants, non-steroid anti-inflammatory drugs, were not included into the study.

Along with the common clinical and laboratory examination (the Order of the Ministry of Health of Russia dated 1 November 2012 No. 572n) on day 3-7 of the menstrual cycle, all patients underwent ultrasound examination of pelvic organs with subsequent hysteroscopic examination in MG patients. After diagnosis verification, all MG patients were administered with standard treatment (ST) [1], presuming oral Dienogest 2 mg/day for three months. Among MG patients, 19 females took only ST (Subgroup 1). 38 examined MG patients, in addition to ST, were administered with different combinations of antioxidant, immunomodulator, and membrane protector; all subjects were divided into two subgroups. The second subgroup included 20 patients, that (in addition to ST) were administered with Sodium ribonucleate (Ridostin) 1.0 i/m every 48 hours No. 5; oral Hypoxenum 1 tab. TID No. 30; and phospholipids (Essentiale Forte H) 2 capsules 3 TID during meals No. 10. The third subgroup included 18 patients that were additionally administered with Inosine + Nicotinamide + Riboflavin + Succinic acid (Cytoflavin) i/v drip 10 ml BID for 7 days; Meglumine acridoneacetate (Cycloferon) 3 tab. orally every 24 hours No. 10; and Glycyrrhizinic acid + Phospholipids (Phosphogliv) i/v 10 ml every 24 hours

No. 7. All drugs were administered according to the guidelines provided in the Federal Guidelines on the Use of Drug Products (formulary system) [2].

Laboratory blood tests were performed when patients were admitted into the inpatient department as well as on day 15. Results obtained in the control group were considered as conditional normal values. Cytokines (TNF α , IL-1 β , IL-6, IL-8, IFN γ , IL-2, IL-17, IL-18, G-CSF, IL-4, IL-10, IL-1ra) were detected by using the enzyme-linked immunosorbent assay with the use of Vektor-Best JSC kits (Russia); components of the complement system (C3, C3a, C4, C5, C5a) and factor H – with the diagnostic kit manufactured by Cytokine Ltd (Russia). C1-Inhibitor activity was determined using the chromogenic method based on assessing C1-esterase inhibition. Registration was performed by using the “Sunrise” microplate photometer, Tecan (Austria).

Phagocytic activity of polymorphonuclear leukocytes after their isolation from peripheral blood samples on the density gradient Ficoll-Urographin (d = 1.077) was evaluated, determining the phagocytic index (PI), phagocytic number (PN), and phagocytosis activity index (PAI) by using general methods. The activity of oxygen-dependent neutrophil systems was evaluated on the PD 303 Sapel spectrophotometer (Japan) according to the nitroblue tetrazolium test (NBT-test), spontaneous and zymosan-induced (NBT-sp., NBT-st.), neutrophil stimulation index and functional reserve (NSI, NFR).

All clinical, clinical-instrumental examinations, and pharmacological correction were performed after receiving the patient informed consent for use of biomaterials from therapeutic and diagnostic measures related to diseases for scientific-research purposes.

Statistical processing of study results was performed by using analysis of variance criteria with the calculation of means (M) and mean arithmetic error (m) by using the Microsoft Excel 2010 software package. Significance of differences was evaluated by using the U-criteria. Differences with $p < 0.05$ were considered significant.

Results and discussion

All patients were randomized by age (34.7 ± 2.6 years), body mass index (not more than 26 kg/m^2). Patients from the main group in 78% observations complained of dysmenorrhea, 72% – dyspareunia; 65% – chronic pelvic pain, 48% – inability to conceive within the year of regular sex life without using contraception methods. The age of menarche in groups did not significantly differ and was 13 ± 1.4 years ($p_{1-2} > 0.05$). The volume of menstrual blood loss in MG was $134 \pm 10.2 \text{ mm}$, which was 3 times larger

compared to CG (43.2 ± 2.3 ml) ($p_{1-2} < 0.05$). Ferritin values among CG patients were 84.6 ± 4.9 ng/ml, which was significantly higher compared to MG results (10.6 ± 1.2 ng/ml). The comparative analysis of contraceptive methods showed that 2/3 (64%) of CG patients underwent the hormonal method, which significantly exceeded the value of this parameter in MG patients (15%). Analysis of pregnancy and labor parity demonstrated that the overwhelming majority of CG patients (72%) had a history of one or more pregnancies, and in 78% cases the delivery was vaginal. 84% CG females did not have intrauterine manipulations. Every third MG patient (31.2%), had delivery via the cesarean section; 86% patients

underwent instrumental curettage of the uterine cavity due to pregnancy termination.

The ultrasound study results have shown that 94.6% gynecologically healthy females demonstrate the uniformity of myometrium structure, clear visualization of the basal layer, smooth endometrial contours that correspond to the first phase of the menstrual cycle. In 85% of MG patients, the following signs were detected: non-uniform thickness and structure of the basal layer, asymmetry of the anterior and posterior uterine walls, presence of increased and decreased echogenicity zones that cover more than 2/3 of the myometrium. In 15% observations, non-uniform and irregular endometrial contours were found in combination with multiple hypo- and

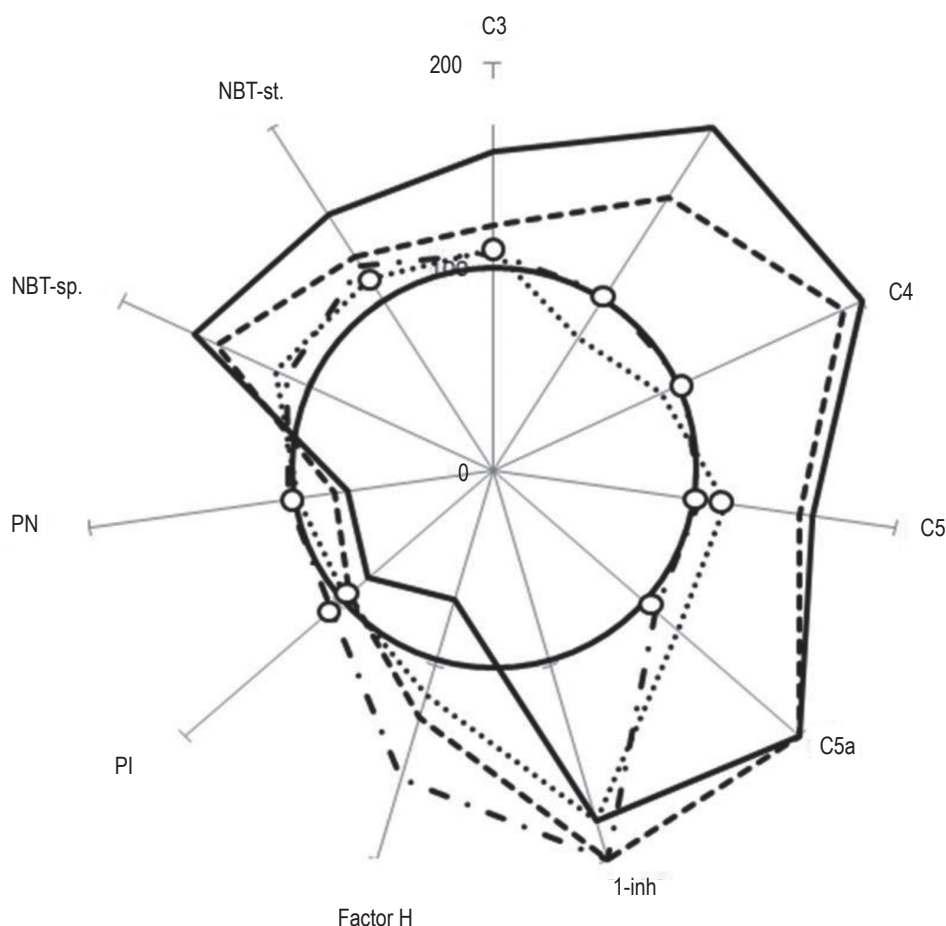


Figure 1. Complement system and functional metabolic activity of blood neutrophils in patients with adenomyosis before and after treatment

Note. 1. Circumference radius, parameters in healthy volunteer donors (CG);

2. — — —, parameters in patients with adenomyosis (MG);

3. ······, parameters in patients with adenomyosis after ST (Subgroup 1);

4. ······, parameters in patients with adenomyosis after ST + Ridostin + Hypoxene + Essentiale H (Subgroup 2);

5. — · —, parameters in patients with adenomyosis after ST + Cycloferon + Cytoflavin + Phosphogliv (Subgroup 3);

6. ○, $p < 0.05$ between parameters relative to MG.

TABLE 1. LEVELS OF BLOOD PLASMA CYTOKINES IN PATIENTS WITH ADENOMYOSIS BEFORE AND AFTER TREATMENT

Parameters	Units of measurement	1	2	3	4	5
		Healthy females	Patients with adenomyosis			
			Before treatment	Standard treatment	ST + Ridostin + Hypoxene + Essentiale H	ST + Cycloferon + Cytoflavin + Phosphogliv
TNF α	pg/ml	3.3 $\pm$ 0.2	8.40 $\pm$ 0.18* ¹	5.20 $\pm$ 0.21* ^{1, 2}	3.90 $\pm$ 0.22* ^{2, 3}	3.1 $\pm$ 0.2* ^{2, 3}
IL-1 β	pg/ml	1.90 $\pm$ 0.21	8.30 $\pm$ 0.24* ¹	6.3 $\pm$ 0.5* ^{1, 2}	4.90 $\pm$ 0.32* ¹⁻³	3.4 $\pm$ 0.4* ¹⁻⁴
IL-6	pg/ml	5.80 $\pm$ 0.21	22.90 $\pm$ 1.87* ¹	14.60 $\pm$ 2.17* ^{1, 2}	10.10 $\pm$ 1.6* ¹⁻³	4.70 $\pm$ 0.23* ¹⁻⁴
IL-8	pg/ml	5.20 $\pm$ 0.12	37.7 $\pm$ 2.1* ¹	24.8 $\pm$ 1.8* ^{1, 2}	14.3 $\pm$ 1.3* ¹⁻³	8.10 $\pm$ 0.87* ¹⁻⁴
IL-18	pg/ml	291.4 $\pm$ 22.5	409.9 $\pm$ 23.6* ¹	367.9 $\pm$ 21.4* ^{1, 2}	371.5 $\pm$ 19.1* ^{1, 2}	309.9 $\pm$ 18.5* ²⁻⁴
IL-4	pg/ml	10.00 $\pm$ 0.45	7.5 $\pm$ 0.6* ¹	7.5 $\pm$ 1.1* ¹	7.70 $\pm$ 0.45* ¹	15.45 $\pm$ 0.47* ¹⁻⁴
IL-10	pg/ml	3.70 $\pm$ 0.21	4.1 $\pm$ 0.2	6.60 $\pm$ 0.34* ^{1, 2}	8.40 $\pm$ 0.42* ¹⁻³	9.50 $\pm$ 0.75* ¹⁻³
IL-1ra	pg/ml	370.0 $\pm$ 17.9	43.9 $\pm$ 9.2* ¹	277.6 $\pm$ 15.0* ^{1, 2}	349.5 $\pm$ 33.1* ^{2, 3}	463.67 $\pm$ 14.90* ¹⁻⁴
IFN γ	pg/ml	2.4 $\pm$ 0.1	15.3 $\pm$ 1.4* ¹	13.3 $\pm$ 2.2* ¹	6.10 $\pm$ 0.42* ¹⁻³	5.90 $\pm$ 0.44* ¹⁻³
G-CSF	pg/ml	73.6 $\pm$ 1.8	112.5 $\pm$ 12.1* ¹	123.2 $\pm$ 5.9* ¹	119.5 $\pm$ 6.8* ¹	90.33 $\pm$ 3.11* ¹⁻⁴
IL-2	pg/ml	0.60 $\pm$ 0.56	6.20 $\pm$ 0.21* ¹	5.9 $\pm$ 0.8* ¹	4.1 $\pm$ 0.8* ^{1, 2}	2.60 $\pm$ 0.42* ¹⁻⁴

Note. *, p < 0.05.

hyperechogenic myometrial inclusions spreading to the depth of no more than 1/3 myometrium. Clinical and ultrasound signs in 92% observations were confirmed by the hysteroscopy data.

Blood plasma tests in patients with adenomyosis have confirmed the increased concentration of proinflammatory cytokines (TNF α , IL-1 β , IL-6, IL-8, IL-18) and decreased levels of anti-inflammatory (IL-4 and IL-1ra) cytokines. The levels of IFN γ , IL-2, and the G-CSF growth factor were higher than those in healthy donors; the concentration of anti-inflammatory cytokine IL-10 remained unchanged. After ST, the levels of all examined anti-inflammatory cytokines and IL-1ra were slightly closer to healthy donor parameters; the IL-10 concentration was increased as a compensatory measure, whereas levels of other cytokines remained unchanged. Introduction

of Ridostin, Hypoxene, and Essentiale Forte H (Subgroup 2) additionally normalized TNF α and IL-1ra concentrations, corrected IL-1 β , IL-6, IL-8, IFN γ , IL-2 levels to a larger extent, and even more increased the IL-10 level; the level of other cytokines remained unchanged. The inclusion of Cycloferon, Cytoflavin, and Phosphogliv into ST (Subgroup 3) compared to the prior drug combination additionally normalized the IL-18 concentrations, increasing in a compensatory manner the level of analyzed anti-inflammatory cytokines higher than control group values, and to a larger extent corrected IL-1 β , IL-6, IL-8, G-CSF, IL-2 levels (Table 1).

On hospital admission, the complement system activation (increased levels of all analyzed components) was observed in the blood plasma of the main group patients, with the opposite changes of its inhibitors

(C1-inhibitor increase, H factor decrease). ST adjusted the concentration of complement components closer to control parameters (except for C5), increasing the level of inhibitors higher than those in donors. The administration of Ridostin, Hypoxene, and Essential Forte H (Subgroup 2) normalized the levels of C3a, C4, and C5a complement components, strongly adjusting concentrations of C3 and C5 components to those in control group. The use of Cycloferon, Cytoflavin, and Phosphogliv (Subgroup 3) together with ST additionally normalized the C5 component concentration and significantly increased the level of complement system inhibitors compared to control values (Figure 1).

The analysis of functional-metabolic activity (FMA) of neutrophils in patients with adenomyosis has revealed decreased phagocytosis activity and intensity parameters (PI, PN, and PAI) as well as increased parameters of oxygen-dependent systems (NBT-sp., NBT-st., NFR). ST normalized two FMA parameters (PI and NFR) and corrected PAI; other parameters of neutrophil FMA remained unchanged. In the second subgroup, parameters of phagocytic (PN and PAI) and oxygen-dependent (NBT-st.) activity were additionally normalized. In the third subgroup, NBT-sp. also normalized, and PAI increased (Figure 1).

While performing a quantifiable comparison for the number of impaired immune status parameters, it has been demonstrated that before treatment onset, 97.9% of all parameters were changed compared to controls. After the standard treatment, 18.4% parameters became normalized, 34.7% were corrected to healthy donor magnitude, but not to their values, whereas 46.9% remained at the level found at treatment onset. After using Ridostin, Hypoxenum, and Essentiale Forte H as a part of ST, 44.9%, 40.8%, and 14.3% of parameters analyzed were normalized, corrected, and remained unchanged, respectively. After including Cycloferon, Cytoflavin, and Phosphogliv addition into ST, 69.4% immune status parameters were normalized, and 30.6% parameters were corrected.

The majority of studies devoted to the role of immune mechanisms in the pathogenesis of adenomyosis reveal the decreased activity of natural killer cells, increased activity of serum and peritoneal fluid proinflammatory cytokines, decreased ability of white blood cells for interferon production, increased amount of peritoneal macrophages as well as subsequent proinflammatory cytokine and chemokine secretion both in the peritoneal fluid and in endometroid implants [4, 7, 10, 11].

Increased levels of serum chemokines and proinflammatory cytokines (TNF α , IL-8, IL-1 β , IL-6, IL-18) along with decreased concentration of proinflammatory cytokines (IL-1ra, IL-4) in patients

with adenomyosis reflect the reaction of resident and recruited innate immune cells to damage-associated molecular patterns [13].

High IFN γ level (effector of the cellular immune response of inflammatory type) and prolonged preservation of the increased serum IFN γ /IL-4 ratio not decreasing after performed ST suggest about activation of IFN γ -produced NK-cells as a part of type 1 innate lymphoid cells (ILC1), which promote polarization of T-cell differentiation towards Type 1 T-helpers (Th1) followed by activating macrophages that express enzymes for reactive oxygen species formation, activation of NO-synthetase with NO formation. A significantly increased level of the colony-stimulating factor G-CSF and IL-2 which also does not decrease after the ST performed, may activate mature neutrophils and support growth of both mixed granulocytic-monocytic lineage colonies as well as separate granulocyte and monocyte/macrophage colonies, whereas IL-2 markedly able to stimulate activity of almost all cytotoxic cell clones activates monocytes and macrophages, thus increasing production and secretion of chemokines, proinflammatory cytokines, colony-stimulating factors.

Detected changes in the cytokine profile, complement system activation, increased neutrophilic oxygen-dependent activity in the peripheral blood (increased production of reactive oxygen species as a result of respiratory burst) confirm ongoing systemic immune inflammation.

Insufficient clinical-laboratory efficacy of ST in the correction of immune changes has justified the use of drugs with immunomodulating, antioxidant, and membrane protective properties in the pharmacological therapy of adenomyosis, which have been successfully used in the treatment of other diseases with similar disorders.

Pharmacological drugs Hypoxene and Cytoflavin that were used in the current study exert antioxidant and energy-correcting properties, minimize oxidative stress signs found in endometriosis, prevent development of free radical oxidation and activation of lipid peroxidation, thus stabilizing the phospholipid layer of damaged cell membranes, including immune cells. Substituting and embedding into cellular membranes, phosphatidyl cholines of the Essentiale H and Phosphogliv can restore membrane functional activity and intracellular metabolism. Ridostin and Cycloferon belong to immunomodulators, which function to correct immune system functioning manifested by enhancing of weakened immunity and inhibition of stimulated immunity. It is also possible that they may normalize complement system, cells producing cytokines and chemokines, function

of phagocytes, which are important suppliers of non-oxidated radicals. It is logical to presume that detection and correction of immune disorders can have an impact on severity of adenomyosis symptoms. A more significant efficacy of combining Cycloferon, Cytoflavin, and Phosphogliv might potentially be related to the presence of a trisodium glycyrrhizinic acid salt in Phosphogliv; this substance exhibits immunomodulating properties, primarily able to activate natural killers (NK-cells) and T-lymphocytes. Cytoflavin also contains succinic acid, inosine, vitamins PP and B2; these thoroughly selected and balanced components have self-potentiating metabolic and energy-correcting effects.

it might not be ruled out that the drugs in the study could exert direct positive impact on the angiogenesis in adenomyosis foci that is controlled at the local level

by angiogenic growth factors and cytokines secreted by peritoneal macrophages, cells of endometroid heterotopies and endothelium.

Thus, the study performed demonstrates the efficacy of correcting immune status parameters in patients with adenomyosis by combining the standard treatment with antioxidant and immunomodulating agents.

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ХАРАКТЕРИСТИКА ИММУНОКОМПЕТЕНТНЫХ КЛЕТОК У ДЕТЕЙ С АЛЛЕРГЕН-ИНДУЦИРОВАННЫМ ФЕНОТИПОМ БРОНХИАЛЬНОЙ АСТМЫ ПРИ ТЕРАПИИ ГЛЮКОЗАМИНИЛМУРАМИЛДИПЕПТИДОМ

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Резюме. Цель: изучение динамики количественных показателей и функциональной активности иммунокомпетентных клеток при применении глюкозаминилмурамилдипептида у детей с аллерген-индуцированным фенотипом бронхиальной астмы.

Проведен комплексный анализ показателей врожденного и адаптивного иммунитета у 60 детей с аллерген-индуцированной бронхиальной астмой (БА) средней тяжести клинического течения в возрасте 3-11 лет и у 30 здоровых сверстников. Верификация фенотипа БА проводилась в соответствии с международным согласительным документом PRACTALL (2008). Критерии исключения из исследования: тяжелое течение БА и иммунокорригирующая терапия в предшествующие 6 месяцев. В проспективном параллельном открытом исследовании изучали влияние глюкозаминилмурамилдипептида на характеристики иммунокомпетентных клеток в течение трех месяцев с разделением на две группы сравнения методом случайной выборки в зависимости от проводимой терапии. В венозной крови оценку клеток проводили на проточном цитофлуориметре COULTER EPICS XL фирмы Beckman Coulter Inc. Иммуноферментным методом определяли уровни цитокинов реактивами R&D Diagnostics Inc (США) и IgE-реагентами «Компания Алкор Био» (Санкт-Петербург), продукцию цитокинов исследовали реагентами «Вектор-Бест» (г. Новосибирск). Статистическая обработка с использованием программы Statistica 10 с критическим уровнем значимости $p < 0,05$, исследование связей коэффициентом ранговой корреляции Спирмена, многомерный корреляционный анализ с построением плеяд по В.П. Терентьеву (1959) и проверку нормальности распределения значений признака (Shapiro–Wilk). Объем выполненных исследований позволил оценить результаты с достоверностью 95-99%.

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Изменения в системе адаптивного реагирования у детей с аллерген-индуцированным фенотипом БА характеризовались усилением пролиферации, угнетением процессов негативной регуляции, активацией синтеза цитокинов Th2-профиля, усилением синтеза IgE.

Выявлены нарушения обеспеченности, функциональной активности иммунокомпетентных клеток и продукции цитокинов сохранялись при терапии ингаляционными глюкокортикоидными препаратами с усугублением снижения синтеза IFN γ .

Применение при БА у детей глюкозамурамилдипептида обеспечивало снижение спонтанной и митоген-индуцированной продукции IL-4 и коррекцию девиации структурно-функциональных характеристик иммунокомпетентных клеток.

Включение глюкозаминилмурамилдипептида в схемы лечения аллерген-индуцированного фенотипа астмы у детей обеспечило нормализацию показателей клеточного звена иммунитета, коррекции дисбаланса Th1/Th2-лимфоцитов, повышение активности Th1, адекватную спонтанную и индуцированную продукцию IFN γ клетками периферической крови

Ключевые слова: иммунокомпетентные клетки, глюкозаминилмурамилдипептид, аллерген-индуцированная бронхиальная астма, дети

CHARACTERISTICS OF IMMUNOCOMPETENT CELLS IN CHILDREN WITH ALLERGEN-INDUCED PHENOTYPE OF BRONCHIAL ASTHMA TREATED WITH GLUCOSEMINYLMURAMILDIPEPTIDE

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Abstract. Purpose: to study the changes in quantitative values and functional activity of immunocompetent cells on application of glucoseminylmuramildipeptide in children with allergen-induced phenotype of bronchial asthma.

We have performed an integrated assessment of parameters of innate and acquired immunity in 60 children at the age of 3-11 years old with allergen-induced bronchial asthma (BA) with mild clinical course of disease, and in 30 healthy children of the same age. BA phenotypes were verified in accordance with PRACTALL international consensus report (2008). Study exclusion criteria were: severe course of bronchial asthma and application of immunocorrecting therapy during preceding six months. We conducted a prospective parallel open study of the effect of glucoseminylmuramildipeptide on the parameters of immunocompetent cells during three months with division into two control groups by random sampling technique on the basis of therapy being performed. To analyze the venous blood cells, we used flow cytometer COULTER EPICS XL by Beckman Coulter Inc. Cytokine levels were determined using immunoenzyme method with reagents by R&D Diagnostics Inc (USA) and IgE – with reagents by Alkor Bio Company (St. Petersburg), production of cytokines – using reagents by Vektor-Best (Novosibirsk). Statistical processing of data was performed using Statistica 10 program with significance level $p < 0.05$, assessment of correlations by Spearman correlation analysis, multidimensional correlation analysis with V.P. Terentyev's method of correlation pleiades (1959) and testing for normal distribution of characteristic values (Shapiro–Wilk). The scope of our study permitted to evaluate its findings with accuracy 95-99%.

The changes of the adaptive response system in children with allergen-induced phenotype of BA were characterized by the intensified proliferation, suppression of negative regulation processes, activation of synthesis of Th2-profile cytokines, and intensified synthesis of IgE.

The identified impairments of availability, functional activity of immunocompetent cells and cytokine production were preserved in application of inhaled corticosteroids therapy, with further decreasing of IFN γ synthesis.

Application of glucosaminilmuramildipeptide in children with BA provided for reduction of spontaneous and mitogen-induced production of IL-4 and correction of deviated structural and functional characteristics of immunocompetent cells.

Incorporation of glucosaminilmuramildipeptide into therapeutic regimens for allergen-induced phenotype of BA in children promoted normalization of parameters of the cellular component of immune system, amelioration of Th1/Th2-imbalance, increase of Th1 activity, and adequate spontaneous and induced production of IFN γ by peripheral blood cells.

Keywords: immunocompetent cells, glucosaminilmuramildipeptide, allergen-induced bronchial asthma, children

Introduction

Immunological indicators reflect the body response to the action of physiological or pathological factors, immunological activation or exhaustion [2]. Pathogenic mechanism of allergic diseases involves imbalance of immune system functioning and impairments of quantitative availability and functional activity of immunoregulatory cells [1, 8].

Chronic nature of inflammation, heterogeneity of pathogenetic mechanisms and clinical forms of bronchial asthma, variability of response to anti-inflammatory and immunotropic therapy dictate the urgent need for optimization of therapeutic regimens in accordance with immune mechanisms underlying various phenotypes of the disease [6, 8, 12, 15].

The ability of immunocompetent cells to demonstrate effector properties, including synthesis of cytokines, secretory degranulation, release of channel-forming proteins and granzymes, is characterized by cells' maturity or lineage stage [10, 13].

Scope of activity of immunocompetent cells and cytokine profile regulates the type of inflammation and phenotype of the disease and influences the efficiency of anti-inflammatory therapy in patients with bronchial asthma [1, 4, 8].

Group of critical factors for the development of BA-phenotypes includes cytokine dysregulation and imbalance of cellular profile of immune response [4, 6].

Pathogenetic significance of immune and cytokine mechanisms in allergic inflammation determine the information value and importance of monitoring of immunological indicators in the course of immunotropic therapy for bronchial asthma [3, 7, 9].

Purpose: to study the changes in quantitative values and functional activity of immunocompetent cells on application of glucosaminilmuramildipeptide in children with allergen-induced phenotype of bronchial asthma.

The tasks of the study were to analyze the immune mechanisms of allergen-induced phenotype of bron-

chial asthma in children and to monitor the parameters of immunocompetent cells during incorporation of glucosaminilmuramildipeptide into therapeutic regimen.

The study was conducted in Federal State Budgetary Educational Institution of Higher Education "Pacific State Medical University" of the Ministry of Healthcare of the Russian Federation (President V.B. Shumatov), design of the study was approved by the Interdisciplinary Ethics Committee, and informed consents were signed by parents.

Materials and methods

The enrolled patients were 90 children at the age of 3-11 years old, of which 60 children had the verified diagnosis of allergen-induced phenotype of bronchial asthma (BA) with mild clinical course of disease, and 30 were healthy children of the same age.

Diagnosis and phenotype of BA were verified in the City Centre for Allergic and Respiratory Diseases of Regional State Budgetary Healthcare Institution "Vladivostok Clinical and Diagnostic Centre" (Chief Medical Officer A.A. Kabieva) in accordance with recommendations of the international consensus document PRACTALL (The European Pediatric Asthma Group Diagnosis and treatment of asthma in childhood: a PRACTALL consensus report) by European Academy of Allergy and Clinical Immunology and the American Academy of Allergy [4]. In verification of the diagnosis, hereditary history and case history were taken into account, and allergological assessment was performed (skin testing, determination of total and specific IgE in blood serum). The group of healthy children of the same age was under care of the Health Centre of Regional State Budgetary Healthcare Institution "Vladivostok Clinical and Diagnostic Centre".

Study exclusion criteria were: age below 3 or over 11 years old, acute respiratory disease, mild or severe course and/or exacerbation of bronchial asthma, presence and/or application of immunocorrecting drugs during preceding six months. Clinical laboratory

study was conducted in the Department of Clinical Laboratory Diagnostics, General and Clinical Immunology of Federal State Budgetary Educational Institution of Higher Education "Pacific State Medical University" of the Ministry of Healthcare of the Russian Federation and in the immunology laboratory of the Regional Clinical Centre for Prevention and Control of AIDS and Infectious Diseases of the Regional State Budgetary Healthcare Institution "Regional Clinical Hospital No. 2" (Head S.P. Kruglyak).

To analyze the immunological indicators, venous blood was used. To analyze leucocyte count, lymphocyte subpopulations and processes of activation in the peripheral blood cells, we used multiparameter flow cytometer COULTER EPICS XL by Beckman Coulter Inc, sample preparation stations Coulter Prep Plus and Coulter TO-prer with selection of panels of monoclonal antibodies with multicoloured combination of fluorochromes. For immunophenotyping, fluorescent particles Flow Count were used. We identified the lymphocyte populations and subpopulations, such as: T-cells, T-helpers, T-cytotoxic, regulatory index, B-cells, natural killers (NK-cells), cytolytic T-cells (NKT-cells) and activated T- and B-cells ($CD3^+CD19^-$, $CD3^+CD95^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+CD8^+$, $CD3^-CD19^+$, $CD3^-CD16^+$, $CD56^+$, $CD3^+CD16^+$, $CD56^+$, $CD3^+CD25^+$, $CD3^+HLA-DR^+$, $CD3^+HLA-DR^-$). The results were counted as a percentage of positive cells and in absolute values. Spontaneous and mitogen-induced production of IL-4 and IFN γ by peripheral blood cells was evaluated using CYTOKINE-STIMUL-BEST sets by Vektor-Best JSC (Novosibirsk). Total and specific IgE content was measured in IU/ml by enzyme linked immunosorbent assay using reagent kits by Alkor Bio Company Ltd. (St. Petersburg).

The object of study was the effect of glucose-minylmuramildipeptide on the parameters of immunocompetent cells in children with allergen-induced phenotype of bronchial asthma within the scope of prospective parallel open study with division into two control groups comparable in gender and age using random sampling technique on the basis of therapy. The patients from the first group ($n = 30$) were receiving basic anti-inflammatory therapy with inhaled glucocorticosteroids, and in the second group ($n = 30$) such basic therapy with inhaled glucocorticosteroids was combined with application of glucoseminylmuramildipeptide (Licopide – Peptek JSC, Russia) at a dose of 1 mg once daily for 10 days, three courses per month.

For statistical processing of numerical data, we used descriptive, parametric and non-parametric statistic

methods of "Statistica 10" program with calculation of: arithmetical mean (M), standard deviation (σ), standard error of the mean ($\pm m$), confidence interval (CI), indicator confidence factor (t) and differences (t and p) with significance level $p < 0.05$. Correlations (r – correlation coefficient) were analyzed using Spearman correlation analysis, multidimensional correlation analysis with V.P. Terentyev's method of correlation *pleiades* (1959) and testing for normal distribution of characteristic values (Shapiro–Wilk). The scope of our study permitted to evaluate its findings with accuracy 95-99%.

Results and discussion

The undertaken study of structural and functional characteristics of immunocompetent cells showed presence of differences in availability and functional activity of the parameters being studied in children with allergen-induced phenotype of bronchial asthma as compared to the same of healthy children of the same age. Absolute lymphocyte count and absolute T-lymphocyte count in children with BA is significantly lower than the same in healthy children ($2.59 \pm 0.18 \times 10^9/L$ against $3.37 \pm 0.18 \times 10^9/L$, at $p < 0.05$ and $1,996.7 \pm 88.95$ cells/ μL against $2,356.90 \pm 126.95$ cells/ μL , at $p < 0.05$); they have stronger expression of $CD3^+CD25^+$ activation markers than healthy children (203.5 ± 18.03 cells/ μL against 137.45 ± 4.67 cells/ μL , at $p < 0.01$) and low absolute B-lymphocyte count (433.5 ± 24.58 cells/ μL against 548.59 ± 41.31 cells/ μL , at $p < 0.05$).

The findings of the study are coherent with the reported information that chronic inflammatory process during bronchial asthma is accompanied with intensification of synthesis of biologically active substances which have an effect on the lymphocytes' receptor system, resulting in change of the level of their functional capacities [9, 13].

We registered the intensification of expression of T-lymphocyte activation marker $HLA-DR^+B$ in the group of children with allergen-induced phenotype of bronchial asthma ($2.20 \pm 0.20\%$ and 61.6 ± 6.11 cells/ μL against $1.10 \pm 0.24\%$ and 46.01 ± 11.96 cells/ μL in the group of healthy children of the same age, respectively at $p < 0.05$). Number of lymphocytes with apoptosis marker $CD3^+CD95^+$ on their surface is significantly lower than in control group ($2.62 \pm 0.34\%$ against $6.10 \pm 0.26\%$, respectively at $p < 0.01$). Children with BA also demonstrated high concentration of T-lymphocytes with early activation marker (CI $162.48-215.79$ cells/ μL), deficit of T-lymphocytes ($1,902.83 \pm 112.39$ cells/ μL), T-helpers (953.42 ± 58.12 cells/ μL) and B-lymphocytes (412.92 ± 34.35 cells/ μL). Also, we recorded in this group the following average positive correlations: between the

TABLE 1. DYNAMICS OF STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF IMMUNOCOMPETENT CELLS IN CHILDREN WITH VARIOUS TREATMENT PROGRAMS FOR ALLERGEN-INDUCED BA PHENOTYPE (M±M; p)

No.	Indicators/unit of measure	Therapy program				
		IGCS inhaled glucocorticosteroids		IGCS with glucosaminylmuramyl dipeptide		
		Before treatment	Three months of therapy	Before treatment	Three months of therapy	
1	T-lymphocytes CD3 ⁺ CD19 ⁺	%	74.56±0.62	73.15±0.80 p > 0.05	71.21±0.87	68.0±1.13 p < 0.05
		C/mkl	1996.70±88.95	1905.60±114.61 p > 0.05	1675.50±71.06	1503.10±89.23 p > 0.05
2	T-helpers CD3 ⁺ CD4 ⁺	%	39.91±0.73	39.29±0.83 p > 0.05	36.57±0.98	43.62±0.90 p < 0.001
		C/mkl	1061.10±46.46	1043.95±44.39 p > 0.05	956.85±36.43	1137.65±61.19 p < 0.05
3	T-cytotoxic CD3 ⁺ CD8 ⁺	%	27.085±0.710	27.35±0.67 p > 0.05	28.5±0.96	34.69±1.47 p < 0.001
		C/mkl	634.10±058.66	625.40±52.79 p > 0.05	718.90±49.22	916.70±61.29 p < 0.05
4	T-lympho- cytes activated CD3 ⁺ CD25 ⁺	%	7.46±0.33	7.20±0.32 p > 0.05	7.26±0.27	6.92±0.39 p > 0.05
		C/mkl	203.50±18.03	186.60±17.54 p > 0.05	164.65±9.06	159.95±14.97 p > 0.05
5	CD3 ⁺ /HLA-DR ⁺	%	2.20±0.20	2.02±0.21 p > 0.05	3.41±0.24	2.87±0.27 p > 0.05
		C/mkl	61.60±6.11	57.42±6.32 p > 0.05	95.31±6.67	72.00±5.72 p < 0.05
6	B-lymphocytes CD3 ⁺ CD19 ⁺	%	13.16±0.56	13.34±0.53 p > 0.05	14.87±0.64	12.93±0.77 p > 0.05
		C/mkl	433.50±24.58	438.15±23.54 p > 0.05	349.75±21.52	279.05±25.84 p < 0.05
7	Cytolytic NK-cells CD3 ⁺ /CD16 ⁺ /CD56 ⁺	%	9.28±0.35	8.95±0.33 p > 0.05	11.33±1.09	13.73±0.97 p > 0.05
		C/mkl	296.20±13.55	285.55±12.42 p > 0.05	274.10±31.75	318.73±0.77 p > 0.05
8	Cytolytic NKT- cells CD3 ⁺ /CD16 ⁺ CD56 ⁺	%	5.54±0.53	5.53±0.52 p > 0.05	6.09±0.42	7.75±0.65 p < 0.05
		C/mkl	179.15±28.56	172.00±25.71 p > 0.05	141.30±14.37	193.91±8.89 p < 0.05

Note. M±m is the average and average error, p is the confidence coefficient of differences in indicators in the study groups.

quantity of neutrophils and leucocytes; between the quantity of cytotoxic T-lymphocytes and activated B-lymphocytes; between the quantity of T-lymphocytes and T-helpers and the level of induced production of $\text{IFN}\gamma$, natural killers and activated T-lymphocytes. We also identified the reverse correlation between the quantity of T-lymphocytes, natural killers and T-lymphocytes with late activation markers.

The differences in functional activity of T-lymphocytes in children with allergen-induced phenotype of the disease and in the group of healthy children are illustrated with immune response imbalance, activation of antigen recognition regulation, induction of immune responsiveness, and proliferation and differentiation of lymphocytes.

Correlation analysis of structural and functional characteristics of immunocompetent cells and cytokine production with formation of pleiads in accordance with P. Terentyev's method revealed the differences in the scope, strength and direction of interrelations between the immune response links in the groups of healthy children and children with allergen-induced bronchial asthma.

One of mechanisms of BA pathogenesis is imbalance of cytokines of various T-helper profiles, in particular, $\text{IFN}\gamma$ and IL-4, which characterize the immune response polarization of Th1 and Th2 inflammation type [2, 7]. $\text{IFN}\gamma$ regulates the differentiation of naive CD4^+ -cells into Th1 and inhibits the proliferation of Th2 [7, 9]. Average levels of spontaneous production of $\text{IFN}\gamma$ and IL-4 in children with allergen-induced phenotype were equal to 8.07 ± 1.42 pg/ml and 5.04 ± 0.08 pg/ml, after mitogen induction – to 16.38 ± 1.77 pg/ml and 13.15 ± 1.21 pg/ml.

The identified stable changes in availability, functional activity of immunocompetent cells, in the adaptive response system, in proliferation with disturbance of negative regulation processes, persisting against the background of application of inhaled corticosteroids, justify the introduction of glucoseminylmuramildipeptide into therapeutic regimen for allergen-induced phenotype of BA in children.

The immunological efficiency of immunotropic drug was evaluated on the basis of monitoring of quantitative values and functional activity of immunocompetent cells before and after treatment for three months within the scope of parallel open prospective study with random division into subgroups in accordance with therapy being performed.

During the observation period, no amelioration of immune imbalance was identified in the children with allergen-induced BA undergoing therapy with inhaled corticosteroids.

While analyzing the changes of immunological indicators in children with allergen-induced BA during application of glucoseminylmuramildipeptide, we registered normalization of the quantity of eosinophiles, leucocytes, lymphocytes, neutrophil granulocytes and the number of T-helpers, T-cytotoxic lymphocytes and cytolytic NKT-cells, as well as decrease of absolute B-lymphocyte number. Introduction of glucoseminylmuramildipeptide into therapeutic regimen for BA provided for normalization of differential white blood cell count, T-lymphocytes availability and activation processes, and balance of T-cytotoxic and cytolytic NKT-cells (Table 1).

In application of the therapy with inhaled corticosteroids, no changes in spontaneous production of $\text{IFN}\gamma$ by peripheral blood cells were registered (from 7.75 ± 0.28 pg/ml to 7.15 ± 0.28 pg/ml at $p > 0.05$). We also registered suppression of induced production of $\text{IFN}\gamma$ (from 12.03 ± 0.67 pg/ml to 8.41 ± 0.40 pg/ml at $p > 0.05$); continued imbalance of spontaneous and induced production of IL-4 (from 4.94 ± 0.26 pg/ml to 4.88 ± 0.23 pg/ml at $p > 0.05$ and from 11.97 ± 0.64 pg/ml to 11.89 ± 0.52 pg/ml at $p > 0.05$). Therapy with glucoseminylmuramildipeptide provided for activation of spontaneous and mitogen-induced production of $\text{IFN}\gamma$ by peripheral blood cells (from 7.31 ± 0.34 pg/ml to 8.97 ± 0.47 pg/ml at $p < 0.01$ and from 11.37 ± 0.90 pg/ml and 15.34 ± 0.90 pg/ml at $p < 0.05$), normalization of IL-4 production ($p < 0.01$), correction of structural and functional impairments of immune response and adequate production of opposite cytokines.

As was earlier reported, the immunotropic effects of glucoseminylmuramildipeptide include intensification of macrophages' activity, activation of T-cells and B-cells, and in combination with basic anti-inflammatory therapy in patients with BA increase of allergen tolerance and stimulation of immunity are observed. One of significant immunotropic effects of glucoseminylmuramildipeptide as a part of therapy for asthma is changing of the profile of cytokines being synthesized from pro-inflammatory allergic one (IL-4, $\text{TNF}\alpha$, IL-5) to anti-inflammatory ($\text{IFN}\gamma$, IL-2), most probably, due to stimulation of Th1 as opposed to Th2 [14].

Study of immunopathogenetic peculiarities of inflammation in the realization of allergen-induced phenotype of bronchial asthma creates the necessary prerequisites for personalized selection of immunotropic therapy which allows increasing the level of disease control [3, 5, 7, 11].

In their studies, N.D. Titova et al. (2017) reported that introduction of glucoseminylmuramildipeptide into the regimen of basic anti-inflammatory therapy for BA resulted in activation of immune response,

amelioration of cytokine production imbalance and increase of allergen tolerance [14].

Conclusion

Our studies revealed the pathogenetic role of immune disorders in the realization of allergen-induced phenotype of bronchial asthma. The identified peculiarities of immunopathogenesis are important for evaluation of inflammatory reaction, determination of severity and prognosis of the disease and serve as additional criteria for customization of anti-inflammatory and immunotropic therapy.

Allergen-induced phenotype of BA is characterized with stable changes in the adaptive response system, in proliferation with disturbance of negative regulation processes.

The peculiarities of immunopathogenesis of allergen-induced BA in children justify the need for customization in the selection of immunotropic therapy with due regard to the immune mechanisms of realization of the disease phenotype and permit to recommend application of glucosaminylmuramildipeptide.

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ПЕРСОНАЛИЗИРОВАННАЯ ФАРМАКОЛОГИЧЕСКАЯ КОРРЕКЦИЯ ИММУННЫХ, МЕТАБОЛИЧЕСКИХ И НЕЙРОПСИХИЧЕСКИХ ПАРАМЕТРОВ ПРИ ХРОНИЧЕСКОЙ ИШЕМИИ МОЗГА I И II СТАДИИ НА ФОНЕ ГИПЕРТОНИЧЕСКОЙ БОЛЕЗНИ

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Резюме. Целью исследования была разработка персонализированной фармакологической коррекции иммунных, метаболических и нейропсихических нарушений при хронической ишемии мозга (ХИМ) I и II стадии. В исследование было включено 104 пациента, из которых 76 больных женского и 28 мужского пола, с ХИМ на фоне гипертонической болезни II степени, из которых 52 больных были с I стадией и 52 со II стадией в возрасте 50 ± 5 лет. Изучены клинические и лабораторные показатели у 22 здоровых доноров того же возраста, сформировавших контрольную группу. Рандомизация больных с ХИМ проводилась по полу, возрасту, способу лечения, сопутствующей патологии, длительности заболевания. Оценку клинико-лабораторных данных осуществляли в начале лечения и через 2 недели после его окончания. Определяли в плазме крови и эритроцитах сорбционную способность эритроцитов и сорбционную емкость гликокаликса (СЕГ), активность процессов перекисного окисления липидов, состояние антиоксидантной системы, выявляли уровень стабильных метаболитов оксида азота (SMNO), неоптерина, С-реактивного белка, цитокинов (TNF α , IL-1 β , IL-8, IFN γ , IL-18, G-CSF, IL-4, IL-10), иммуноглобулинов (IgM, IgG, IgA), компонентов системы комплемента (C3, C4, C5, C5a), фагоцитарную и кислородзависимую активность полиморфноядерных лейкоцитов крови. Установлено, что пациентам с ХИМ I с высокими значениями концентрации IL-8, IL-10, SMNO и низким показателем СЕГ, прием церетона и актовегина или цераксона и мексикора будет недостаточен для эффективной коррекции иммунометаболических нарушений, что требует дополнительного назначения иммуномодулятора. Пациентам с ХИМ II, имеющим более высокий уровень в плазме TNF α , IL-10 и низкие значения СЕГ, необходимо назначение цераксона, мексикора и глутоксима или цераксона, мексикора и полиоксидония с целью получения максимального клинико-лабораторного положительного эффекта.

Ключевые слова: хроническая ишемия мозга, персонализированная фармакологическая коррекция иммунометаболических нарушений

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PERSONALIZED PHARMACOLOGICAL CORRECTION OF IMMUNE SYSTEMS, METABOLIC AND NEUROPSYCHIC PARAMETERS IN CHRONIC BRAIN ISCHEMIA OF STAGE I AND II ON THE BACKGROUND OF HYPERTENSION DISEASE

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Abstract. The study aimed to develop a personalized pharmacological correction of immune, metabolic and neuropsychiatric disorders in chronic cerebral ischemia (CCI) stages I and II. The study included 104 patients, of which 76 were female and 28 were male, with CCI on the background of grade II hypertension, of which 52 patients were with stage I and 52 with stage II at the age of 50 ± 5 years. Clinical and laboratory parameters were studied in 22 healthy donors of the same age who formed a control group. Patients with CCI were randomized according to gender, age, treatment method, concomitant pathology, and duration of the disease. Evaluation of clinical and laboratory data was carried out at the beginning of treatment and 2 weeks after its end. The sorption capacity of erythrocytes and the sorption capacity of the glycocalyx (SEG), the activity of lipid peroxidation processes, the state of the antioxidant system were determined in blood plasma and erythrocytes, the level of stable metabolites of nitric oxide (SMNO), neopterin, C-reactive protein, cytokines (TNF α , IL-1 β , IL-8, IFN γ , IL-18, G-CSF, IL-4, IL-10), immunoglobulins (IgM, IgG, IgA), complement system components (C3, C4, C5, C5a), phagocytic and oxygen-dependent activity of polymorphonuclear blood leukocytes. It has been established that for patients with CCI I with high concentrations of IL-8, IL-10, SMNO and a low SEG index, the intake of Cereton and Actovegin or Ceraxon and Mexicor will be insufficient for effective correction of immunometabolic disorders, which requires additional administration of an immunomodulator. Patients with CCI II, who have a higher plasma level of TNF α , IL-10 and low SEG values, need to prescribe Ceraxon, Mexicor and Glutoxim or Ceraxon, Mexicor and Polyoxidonium in order to obtain the maximum clinical and laboratory positive effect.

Keywords: chronic cerebral ischemia, personalized pharmacological correction of immunometabolic disorders

Introduction

Today, personalized or precision medicine is a new doctrine of modern health care, which is based on the use of new methods of molecular analysis (genomics, transcriptomics, proteomics, metabolomics, microbiomics) to improve the assessment of predisposition (prediction) to diseases and their “management” (prevention and treatment).

Personalized medicine takes into account the individual's genetic program, clinical, demographic, laboratory, and instrumental predictors of response to therapy and the risks of possible complications, includes analysis of drug metabolism, immune response, which significantly affect the specifics of the body's response to a particular drug, as well as monitoring treatment using biomarkers. This allows to make medical treatment as effective and safe as possible for

a specific patient. Thus, personalized medicine can be considered as an innovative and intensive tool for modernizing the healthcare system and improving the quality of life of patients [1, 2, 5, 8].

Materials and methods

The investigation included 104 patients, including 76 female and 28 male patients, who made up the main group of hospitalized in the neurological Department of Kursk regional clinical hospital, with correction of blood pressure to the normal level, with CHEB against the background of grade II hypertension, of which 52 patients were stage I (1st main group) and 52 with stage II (2nd main group) at the age of 50 ± 5 years.

In addition, clinical and laboratory parameters were studied in 22 healthy donors (52 ± 2 years) who formed the control group 1; the results were accepted as normal.

Randomization of patients with CHEB was performed by gender, age, method of treatment, accompanied pathology, and duration of the disease.

The criteria for inclusion in the main group were: age from 40 to 60 years; the absence of accompanied diseases in the acute stage, the presence of CHEB against the background of grade II, stage II hypertension, risk 2, diagnosed 5 or more years ago in accordance with the recommendations of the world health organization and the International society on arterial hypertension (MOG, 1999).

The exclusion criteria were: symptomatic arterial hypertension; severe or moderate atherosclerotic changes in the fundus vessels; heart rhythm and conduction disorders; chronic heart failure of more than II FC in accordance with the classification of the new York heart Association (NYHA); hemodynamically significant stenoses of brachiocephalic and cerebral vessels, heart defects; myocardial infarction, postinfarction cardiosclerosis and progressive angina or indications thereof a history of diabetes or impaired glucose tolerance.

For all the patients underwent a comprehensive clinical and instrumental examination according to generally accepted standards, and in all cases, the diagnosis of stage I and II CHEB was verified. The treatment followed the principles of evidence-based medicine.

Evaluation of clinical and laboratory data in the main groups was performed at the beginning of treatment and 2 weeks after its end. From 10 ml of heparinized blood, plasma and red blood cell mass were obtained by centrifugation, with which the sorption capacity of red blood cells (SSE) and the sorption capacity of glycocalyx (SEG) were immediately determined.

The intensity of lipid peroxidation processes was assessed by the content of acyl hydroperoxides and malondialdehyde (MDA and AGP) in blood plasma and erythrocytes.

The conduction of the antioxidant system was determined by direct/competitive solid-phase enzyme immunoassay using ready-made commercial kits: the activity of superoxide dismutase (SOD) "Bender MedSystems" (Austria) and catalase "Cayman Chemical" (USA). Total antioxidant activity (AOA) was detected by a method based on the degree of inhibition of ascorbate – and ferroinduced oxidation of twin-80 to MDA. The level of stable metabolites of nitric oxide (SMNO) was detected using a set for solid-phase enzyme immunoassay by R&D (England).

In addition, plasma levels of neopterin "IBL" (Germany), endothelin-1 (EN-1) "Biomedica" (Slovakia) and erythropoietin "Biomerica" (USA) were determined by enzyme immunoassay. Ceruloplasmin (CP)

was determined by immunoturbidimetry using the sentinel kit (Spain), and C-reactive protein (CRP) using the Vector-Best kit (Russia) using the BTS-350 semi-automatic analyzer (BioSystems, Spain).

Cytokines (TNF α , IL-1 β , IL-6, IL-8, IFN γ , IL-2, IL-17, IL-18, G-CSF, IL-4, IL-10, IL-1RA) were detected by solid-phase enzyme immunoassay using sets of Vector – Best CJSC (Russia), components of the complement system (C3, C3a, C4, C5, C5a) and factor H-diagnostic set of Cytokine Ltd (Russia). The activity of the C1-inhibitor was determined by chromogenic method based on the ability to inhibit C1-esterase. Registration of all the results of enzyme immunoassay was performed using a microplate photometer "Sunrise", Tecan (Austria).

The phagocytic activity of polymorphonuclear blood leukocytes after their isolation from the blood on the phycoll-urographin density gradient ($d = 1,077$) was evaluated by determining the phagocytic index, phagocytic number, and phagocytosis activity index. The activity of oxygen-dependent neutrophil systems was evaluated using a PD 303 SApel spectrophotometer (Japan) based on the nitroblue tetrazolium reduction reaction (NBT-test), spontaneous and stimulated by zymosan, the stimulation index and the functional reserve of neutrophils.

In statistical processing results, the χ^2 (Chi-square) was used to compare qualitative parameters. The Shapiro–Wilk test was used to assess whether quantitative features belong to a distribution type. The student's t-test was used to compare normally distributed values. The statistical significance of differences in quantitative values with an abnormal distribution was assessed using the Mann–Whitney U-test and the Wilcoxon test (when comparing dependent groups). The values of normally distributed quantitative parameters are represented by the arithmetic mean (M) with the error of the arithmetic mean (m), and abnormally distributed ones are represented by the median (Me) with an interquartile interval ($Q_{0.25}$ – $Q_{0.75}$). Relationships were established based on factor analysis, cluster analysis, and Spearman's rank correlation coefficient (Gubler E.V., Genkin A.A., 1973; Lakin G.F., 1980). Differences were considered statistically significant at $p < 0.05$

Results and discussion

All drugs were administered according to the recommendations set out in the Federal guidelines for the use of medicines (formulary system) edited by A.G. Chuchalin, V.V. Yasnetsov (Moscow, 2014–2016) and in the national guidelines "Neurology" edited by E.I. Gusev, A.N. Konovalov, and A.B. Geht (Moscow, 2016).

TABLE 1. DISTRIBUTION OF PATIENTS BY THE EFFECTIVENESS OF THE PHARMACOTHERAPY

No. Group	Pharmacotherapy	Effectiveness	
		Maximum	Minimum
Chronic brain ischemia stage I			
1	Ceretone and Actovegin	6	6
2	Cerakson and Mexicor	6	6
3	Cerakson, Mexicor and Glutoxim	7	7
4	Cerakson, Mexicor and Polyoxidonium	7	7
Chronic brain ischemia stage II			
1	Ceretone and Actovegin	6	6
2	Cerakson and Mexicor	6	6
3	Cerakson, Mexicor and Glutoxim	7	7
4	Cerakson, Mexicor and Polyoxidonium	7	7

TABLE 2. NUMBER OF IMMUNE AND METABOLIC INDICATORS STATUS, DIFFERENT IN PATIENTS WITH CHEB DEPENDING ON EFFECTIVENESS OF PHARMACOTHERAPY

Group of patients	Immune status		Metabolic status of the plasma		Metabolic parameters of red blood cells	
	Σ	$p < 0.05$	Σ	$p < 0.05$	Σ	$p < 0.05$
Chronic brain ischemia stage I						
Ceretone and Actovegin or Cerakson and Mexicor	27	IL-8	11	G-CSF	8	SEG
Cerakson, Mexicor and Glutoxim or Cerakson, Mexicor and Polyoxidonium		IL-10		SMNO AGP		
Chronic brain ischemia stage II						
Ceretone and Actovegin or Cerakson and Mexicor	27		11		8	SEG AGP
Cerakson, Mexicor and Glutoxim or Cerakson, Mexicor and Polyoxidonium		TNF α IL-10		AGP		

To personalize the presented pharmacological treatment regimens for patients with CHEB, each of the patient groups was further divided into 2 subgroups depending on laboratory and clinical corrective effectiveness: maximum and minimum (Table 1).

In order to obtain reliable differences, we combined groups of patients who received Ceretone and Actovegin with patients who received Cerakson and Mexicor, and patients with prescription Cerakson,

Mexicor and Glutoxim with patients who received Cerakson, Mexicor and Polyoxidonium [3, 4, 6, 7].

We compared the indicators of immune and metabolic status before treatment in patients with CHEB with maximum and minimum clinical and laboratory effectiveness (Table 1).

Found that patients CHEB I, receiving Ceretone or Actovegin and Cerakson and Mexicor with maximum clinical effect of the pharmacotherapy of the 27 in-

indicators of immune status before treatment, only one indicator differed from a group of patients with minimal clinical and laboratory effect (IL-8); of the 11 indicators of the metabolic status of a great figure was the same one (G-CSF) (Table 1).

Patients CHEB I, treated with Ceraxon, Mexicor and Glutoxim or Ceraxon, Mexicor and Polyoxidonium, with maximum clinical-laboratory effect from the conducted pharmacotherapy of the 27 indicators of the immune status before treatment 1 was different from groups of patients with minimal clinical and laboratory effect (IL-10); the 11 indicators of the metabolic status of quality indicators were 2 (SMNO and AGP); the 8 indicators of the metabolic status of erythrocytes is an excellent indicator there was only one (SEG) (Table 2).

Patients CHEB II, receiving Cereton or Actovegin and Ceraxon and Mexicor with maximum clinical effect of the pharmacotherapy, indicators, to treatment different from patients with minimal clinical and laboratory effect was not (Table 2).

Patients CHEB II treated with Ceraxon, Mexicor and Glutoxim or Ceraxon, Mexicor and Polyoxidonium, with maximum clinical-laboratory effect of the pharmacotherapy: the 27 indicators of the immune status before treatment 2 was different from the group of patients with minimal clinical and laboratory effect (TNF α and IL-10); the 11 indicators of the metabolic status great was 1 indicator (AGP); the indicators of metabolic status of erythrocytes were excellent 2 indicator (SEG, and AGP) (Table 2).

So, the following fact that the results of the correlation, cluster and factorial analyses are the most informative in the correctional effectiveness of clinical and laboratory parameters in chronic cerebral ischemia stage I has a definition in the plasma concentration of the cytokines TNF α , IL-8,

IL-10, stable metabolites of nitric oxide and sorption capacity of the glycocalyx of erythrocytes, and with stage II disease – plasma levels of TNF α , IL-8, IL-17, IL-10, neutrophil phagocytic number and glycocalyx sorption capacity [5, 6, 7, 8], so we selected these parameters for a more detailed analysis.

In patients with CHEB I who received Cereton and Actovegin or Cerakson and Mexicor, a significantly higher concentration of IL-8 in blood plasma is determined from the minimal effectiveness of the treatment than in patients with the maximum clinical and laboratory effectiveness after the treatment.

In patients with CHEB II who received Cerakson, Mexicor and Glutoxim or Cerakson, Mexicor and polyoxidonium, with minimal effectiveness of the treatment, lower plasma concentrations of TNF α , IL-10 and higher SEG values were reliably determined than in patients with maximum clinical and laboratory effectiveness of the treatment.

Thus, it can be stated that in patients with CHEB I with high concentrations of IL-8, IL-10, SMNO and low SEG, the reception of Ceretone and Actovegin or Cerakson and Mexicor will be insufficient for effective correction of immunometabolic disorders, which requires additional administration of immunomodulator to the pharmacotherapy of scheme.

Patients with CHEB II who have higher plasma levels of TNF α , IL-10 and low SEG values should be prescribed Cerakson, Mexicor and Glutoxim or Cerakson, Mexicor and Polyoxidonium in order to obtain the maximum clinical and laboratory positive effect.

The obtained results indicate the possibility and prospects of using the principle of personalized pharmacotherapy in the treatment of patients with CHEB.

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ВЛИЯНИЕ ИММУНОТЕРАПИИ НА ОСНОВЕ АНТИГЕН-ПРАЙМИРОВАННЫХ ДЕНДРИТНЫХ КЛЕТОК НА ПРОТИВООПУХОЛЕВЫЙ КЛЕТОЧНЫЙ ИММУННЫЙ ОТВЕТ У БОЛЬНЫХ КОЛОРЕКТАЛЬНЫМ РАКОМ

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Резюме. Проблема лечения онкологических заболеваний является одной из актуальных для современной медицины. Существующие подходы лечению основаны на хирургическом, лучевом, химиотерапевтическом подходе и также использовании метода иммунотерапии, направленного на маркеры и/или специфические антигены опухолей.

Подходы, основанные на механизмах клеточной и молекулярной регуляции специфического противоопухолевого иммунного ответа, показали свою высокую эффективность (например, антитела против HER2 при раке молочной железы), но эти подходы имеют ряд побочных и нежелательных эффектов, которые ограничивают их применение. Учитывая центральную роль механизмов распознавания опухолевых антигенов и их презентации цитотоксическим клеткам в эффективной элиминации опухоли, является важным поиск и разработка подходов восстановления этих механизмов при онкологической патологии. В связи с тем, что при онкологических заболеваниях нарушается созревание, дифференцировка дендритных клеток и страдает их основная функция, ведутся научные исследования по получению зрелых дендритных клеток и восстановление естественного пути презентации антигена эффекторным клеткам.

В работе были проведены ограниченные клинические исследования (13 больных колоректальным раком), ранее разработанного протокола получения антиген-праймированных дендритных клеток больных колоректальным раком и их совместной культуры с аутологичными моноклеарными клетками в условиях *in vitro*. Из периферической крови онкобольных были получены дендритные клетки, праймированные аутологичными опухолевыми антигенами (лизат опухолевых клеток), которые сокультивировали с собственными моноклеарными клетками в присутствии иммунорегуляторных цитокинов (IL-12 и IL-18). Полученные клеточные суспензии очищались от культуральной среды и цитокинов и использовались для проведения курса иммунотерапии (еженедельно по 20-30 млн клеток

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внутривенно, капельно), состоящего из 3-5 введений. На разных сроках проведения иммунотерапии (до начала курса иммунотерапии, через 3 месяца и через 6 месяцев после окончания иммунотерапии) оценивали в периферической крови больных иммунологические показатели (иммунограмма (CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, CD19, CD16⁺CD56⁺-клетки), относительное содержание Т-регуляторных клеток (CD4⁺CD25⁺FoxP3⁺-клетки), миелоидных супрессорных клеток (CD14⁺HLA-DR⁻-клетки)) и цитотоксическую активность мононуклеарных клеток периферической крови больных против клеток опухолевой линии колоректального рака человека (Colo-320).

Полученные данные показали, что у онкологических больных на фоне проводимой иммунотерапии достоверно возрастает показатель прямого цитотоксического теста, что позволяет судить об эффективной стимуляции противоопухолевого клеточного иммунного ответа. На это также указывает возрастание относительного количества CD16⁺CD56⁺-клеток (NK-клетки) через 3 месяца после иммунотерапии. Изучение иммуносупрессорных клеток в крови онкобольных показало отсутствие значимых изменений CD14⁺HLA-DR⁻-клеток и Т-регуляторных клеток.

Таким образом, проведенные ограниченные клинические исследования иммунотерапии больных колоректальным раком на основе аутологических дендритных клеток, праймированных лизатом аутологических опухолевых клеток, продемонстрировали повышение противоопухолевого цитотоксического иммунного ответа.

Ключевые слова: иммунотерапия, дендритные клетки, опухолевые антигены, клеточная цитотоксичность, колоректальный рак

EFFECT OF ANTIGEN-PRIMED DENDRITIC CELL-BASED IMMUNOTHERAPY ON ANTITUMOR CELLULAR IMMUNE RESPONSE IN PATIENTS WITH COLORECTAL CANCER

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Abstract. The problem of treatment of oncological diseases is one of the most urgent for modern medicine. Existing treatment approaches are based on a surgical, radiation, chemotherapeutic approach, and the use of immunotherapy methods aimed at markers and / or specific antigens of tumors.

Approaches based on the mechanisms of cellular and molecular regulation of a specific antitumor immune response have shown their high efficiency (for example, antibodies against HER2 in breast cancer), but these approaches have a number of side and undesirable effects that limit their application. Considering the central role of the mechanisms of recognition of tumor antigens and their presentation to cytotoxic cells in effective tumor elimination, it is important to search for and develop approaches to restore these mechanisms in cancer pathology. Because maturation, differentiation of dendritic cells and their main function are impaired in oncological diseases, scientific research is underway to obtain mature dendritic cells and restore the natural way of antigen presentation to effector cells.

The work carried out limited clinical studies (13 patients with colorectal cancer), a previously developed protocol for obtaining antigen-primed dendritic cells of patients with colorectal cancer and their joint culture with autologous mononuclear cells *in vitro*. From the peripheral blood of cancer patients, dendritic cells primed with autologous tumor antigens (tumor cell lysate), which were co-cultured with their own mononuclear cells in the presence of immunoregulatory cytokines (IL-12 and IL-18). The resulting cell suspensions were purified from the culture medium and cytokines and used for a course of immunotherapy (weekly, 20-30 million cells intravenously, dropwise), consisting of 3-5 injections. At different periods of immunotherapy (before the start of the course of immunotherapy, 3 months and 6 months after the end of immunotherapy), immunological parameters were assessed in the peripheral blood of patients (immunogram (CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺,

CD19, CD16⁺CD56⁺-cells), the relative content of T-regulatory cells (CD4⁺CD25⁺FoxP3⁺-cells), myeloid suppressor cells (CD14⁺HLA-DR⁻-cells)) and assessed the cytotoxic activity of peripheral blood mononuclear cells of patients against cells of the tumor line of human colorectal cancer (Colo-320).

The data obtained showed that in cancer patients, against the background of ongoing immunotherapy, the indicator of the direct cytotoxic test significantly increases, which makes it possible to judge the effective stimulation of the antitumor cellular immune response. This is also indicated by an increase in the relative number of CD16⁺CD56⁺-cells (NK-cells) 3 months after immunotherapy. The study of immunosuppressive cells in the blood of cancer patients showed the absence of significant changes in CD14⁺HLA-DR⁻-cells and T-regulatory cells.

Thus, limited clinical studies of immunotherapy of patients with colorectal cancer based on autologous dendritic cells primed with lysate of autologous tumor cells demonstrated an increase in the antitumor cytotoxic immune response.

Keywords: immunotherapy, dendritic cells, tumor antigens, cellular cytotoxicity, colorectal cancer

Introduction

The incidence of cancer in the population in many countries worldwide shows a steady trend to increase. In Russia, colorectal cancer ranks the second in the pattern of malignant neoplasm incidence (11,3%) and related mortality (13,1%). Every year in Russia this nosology is diagnosed in more than 50 thousand patients. According to the histological classification of the World Health Organization, there are 4 main forms of colorectal cancer: adenocarcinoma, mucous adenocarcinoma, cricoid and squamous cell carcinoma. Adenocarcinoma accounts for 75-80% of colorectal cancer cases [5].

Surgical intervention, chemotherapy and radiation therapy are considered classical methods of treating patients with colorectal cancer, but the results of these methods, in general, remain ineffective [9]. Currently, along with other methods of conservative therapy for malignant neoplasms, specific immunotherapy is a modern and promising approach, the main purpose of which is to induce and maintain a long-term immune response aimed at recognizing and eliminating tumor cells.

The effectiveness of modern immunotherapy for oncological diseases relies on correcting impaired antigen presentation and generation of antigen-specific cytotoxic T-lymphocytes. Dendritic cells (DCs), professional antigen-presenting cells, play a key role in these processes [7, 10]. Dendritic cells are considered the most powerful stimulators of the body immune responses, able to recognize and present antigens to T- and B-lymphocytes in the context with MHCI and class II molecules, which are expressed in large quantities on the cell surface along with co-stimulatory molecules (CD80, CD86) [6]. Therefore, mature DCs demonstrate a high ability to present tumor-associated antigens *in vitro* and *in vivo* [2, 9]. Primed CD4⁺ and CD8⁺T-lymphocytes secrete cytokines such as interferon gamma (IFN γ)

and tumor necrosis factor alpha (TNF α), which promote CTL proliferation, destruction of tumor tissue and potential control and even elimination of tumor cells [8]. However, the functional activity of DCs in cancer patients is significantly reduced mainly due to impaired DC maturation to functionally active counterparts [1, 6]. In this regard, the production of mature functionally active DCs *in vitro* and their activation with tumor-associated antigens to stimulate a cytotoxic response is promising for developing dendritic cell-based antitumor vaccines and allows mobilizing the patient own defense systems using natural ways of recognizing tumor antigens and theirs subsequent elimination.

There are many methods for the production of DCs *in vitro* that stimulate the antitumor response, including loading of cells with proteins of tumor-associated antigens, as well as the introduction of DNA or mRNA encoding such antigens [3]. The loading of dendritic cells with tumor lysate allows for the presentation of the entire spectrum of patient-specific tumor antigens. The possibility of antigen-specific activation of dendritic cells with the formation of antitumor cytotoxic immune response is currently considered as one of the promising methods to fight cancer.

Thus, **the aim of the study** was to study the effect of cellular immunotherapy of patients with colorectal cancer based on autologous antigen-primed dendritic cells and mononuclear cells "trained" by them to stimulate antitumor cytotoxic immune response.

Materials and methods

The studies were carried out within the limited clinical protocol NCT0321493 (clinicaltrials.gov), dedicated to investigate clinical and laboratory effectiveness of immunotherapy based on autologous antigen-activated dendritic cells in the treatment of patients with colorectal cancer. The study was

approved by the local ethics committee at the RIFCI. The selection of patients (13 patients with colorectal cancer) was carried out according to the inclusion and exclusion criteria.

The study consisted of the following main stages:

1. Stage of clinical examination (selection of patients with colorectal cancer who meet the research criteria, and their clinical examination, review and providing signed up informed consent).

2. The stage of obtaining biological material (collection of peripheral venous blood of a cancer patient, a histological sample of a malignant tumor (colorectal cancer) during planned surgical treatment).

3. Laboratory stage for preparing cell suspension for immunotherapy.

The immunotherapy is based on the use of *in vitro*-induced autologous dendritic cells, primed with their own tumor antigens, and their co-culture with autologous mononuclear cells under *in vitro* conditions to "train" effector cells and induce an antitumor cytotoxic immune response [12]. The maturation of the induced dendritic cells and their viability were monitored by flow cytometry (BD FACS Verse). Co-culture of antigen-primed dendritic cells and MNCs was performed in the presence of recombinant IL-12 and IL-18 to obtain specifically activated lymphocytes. The final cell suspensions were cryopreserved.

4. Stage of immunotherapy.

Immunotherapy was carried out as planned (3-4 weeks after surgery; in the period between chemotherapy courses). Injections of the cell suspension were carried out weekly by using 20-30 million cells dissolved in 100 ml of 0.9% NaCl (physical solution) administered via IV drip in the hospital of the RIFCI immunopathology clinic. The number of injections (a course of cellular immunotherapy) depended on the final number of cells obtained during the laboratory stage upon preparing cell suspension for immunotherapy and ranged from 3 to 5 injections.

3 and 6 months after the end of the full course of cellular immunotherapy), repeated blood sampling was performed to assess the immunological parameters.

5. Stage of evaluating the effectiveness of the conducted immunotherapy.

For patients with IIA, IIB, IIIA, IIIB stages of colorectal cancer, it is planned to assess the quality of life, as well as overall and disease-free survival within 3 years after completing all stages of treatment.

Assessment of peripheral blood immunological parameters during immunotherapy included parameters of cellular immunity (CD3⁺, CD4⁺, CD8⁺, CD16⁺, CD19⁺, HLA-DR expression on mono-

cytes), assessment percentage of T-regulatory cells (CD4⁺CD25⁺FoxP3⁺), analyzed by flow cytometry. Monitoring of immunological parameters was carried out before vaccine administration, 3, and 6 months after the last administration. In addition, as a direct indicator of antitumor cytotoxic activity, we assessed the ability of MNCs at different periods of immunotherapy (before vaccine administration and 3 and 6 months after the last administration) to cause the death of tumor cells (human colorectal cancer cell line Colo-320) by using "CytoTox 96 Non-Radioactive" kit (Promega). The change in the level of intracellular enzyme (lactate dehydrogenase) in the conditioned environment of the co-culture between tumor cells and the cell "preparation" was used to estimate the percentage of tumor cell death in the cytotoxic test and, thereby, an effect of immunotherapy on inducing antitumor immune response.

Results and discussion

Evaluating cytotoxic test of peripheral blood mononuclear cells 6 months after immunotherapy showed significant increase in the tumor cell death (human colorectal cancer cell line Colo-320) compared to that observed before immunotherapy and 3 months after immunotherapy (Figure 1).

When assessing the parameters of the cellular immune response, a significant increase in percentage of CD16⁺CD56⁺-cells was revealed 3 months after the end of immunotherapy (Figure 2). The relative quantity of CD3⁺, CD4⁺, CD8⁺ and CD19⁺-cells in the peripheral blood of cancer patients at different timepoints before and after immunotherapy did not significantly differ.

Percentage of T-regulatory cells (CD4⁺CD25⁺FoxP3⁺) in the blood of patients with colorectal cancer decreased during immunotherapy, but showed no significant differences between the groups (data not shown).

When assessing the level of HLA-DR expression on monocytes, in particular, the density of HLA-DR-negative CD14⁺ monocytes (myeloid suppressor cells), no significant changes in were revealed (data not shown).

Thus, while applying ongoing cellular immunotherapy after co-culture of autologous antigen-primed dendritic cells and mononuclear cells, it resulted in increased anti-tumor cellular activity as shown in *in vitro* cytotoxic test and increased amount of CD16⁺CD56⁺-cells serving as an indicator of general stimulation in effector cells.

The percentage of suppressor cells (T-regulatory cells and myeloid suppressor cells) in the peripheral blood of patients did not change significantly.

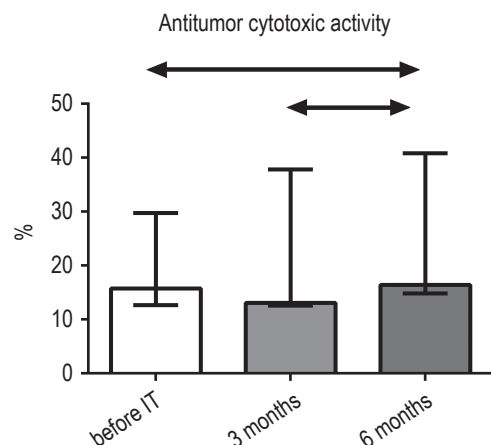


Figure 1. Antitumor cytotoxic activity of mononuclear cells in patients with colorectal cancer at different periods of immunotherapy (before immunotherapy (IT), 3 and 6 months after the end of immunotherapy)

Note. The graph shows the percentage of dead tumor cells of the Colo-320 cell line when co-cultured with mononuclear cells of patients with colorectal cancer *in vitro*. Data are presented as median and upper and lower quartiles, arrows indicate statistically significant differences between groups (n = 13).

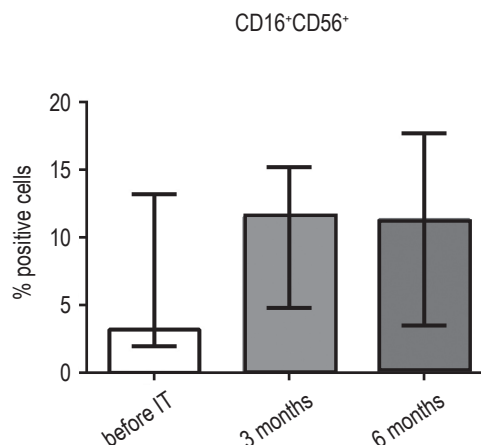


Figure 2. Relative content of CD16+CD56+ cells in the peripheral blood of patients with colorectal cancer at different periods of immunotherapy (before immunotherapy (IT), 3 and 6 months after the end of immunotherapy)

Note. Data are presented as median and upper and lower quartiles; the arrow indicates statistically significant differences between groups (n = 13).

Conclusion

The data obtained demonstrate that in patients with colorectal cancer receiving ongoing immunotherapy, the indicator of the direct cytotoxic test was significantly increased allowing to conclude about effective stimulation of the antitumor cellular immune response. This is also indicated by increased percentage of CD16+CD56+ cells (NK-cells) 3 months after immunotherapy. Assessing frequency

of immunosuppressive cells in the blood of cancer patients showed a tendency towards a decrease in the number of T-regulatory cells at 3 and 6 months after the end of immunotherapy and no significant changes in the number of CD14+HLA-DR+ cells.

Thus, limited clinical studies of immunotherapy of patients with colorectal cancer based on autologous dendritic cells primed with lysate of autologous tumor cells demonstrated enhanced antitumor cytotoxic immune response.

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ХАРАКТЕРИСТИКА CD4⁺ ЦЕНТРАЛЬНЫХ И ЭФФЕКТОРНЫХ КЛЕТОК ПАМЯТИ ПРИ ПСОРИАЗЕ

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Резюме. Псориаз — хроническое аутоиммунное заболевание, при котором в патологический процесс вовлечены кожа и суставы. Установлено, что рецидив высыпаний при данном заболевании происходит за счет резидентных клеток памяти кожи. При этом количество CD4⁺CCR3⁺ эффекторных клеток памяти периферической крови коррелирует с тяжестью заболевания. Поэтому целью нашей работы является изучение фенотипа клеток памяти периферической крови пациентов с псориазом.

В исследование включили 6 здоровых доноров: средний возраст — 45,4 (min — 29, max — 55), женщины — 3, мужчины — 3; 10 пациентов с псориазом: женщины — 4, мужчины — 6, средний возраст — 37,3 (min — 23, max — 57), из них 5 пациентов с PASI более 10 и 5 пациентов с PASI менее 10. Критериями исключения были наличие аутоиммунных, онкологических и гематологических заболеваний, системная терапия иммуносупрессивными препаратами в течение 1 месяца. Пациенты подписали добровольное информированное согласие на участие в исследовании. Выделение моноклеарных клеток периферической крови проводили в градиенте плотности фиколл-урографина ($\rho = 1,082$ г/л) (BioClot GmbH, Германия). Полученные клетки окрашивали конъюгированными с флуорохромами моноклональными антителами к поверхностным маркерам CD4⁺ центральных (Tcm) и эффекторных (Tem) клеток памяти (CD4, CD45RO, CD197), α -цепи рецептора IL-7 (CD127) и γ -цепи рецептора IL-7 (CD132). Фенотипирование клеточных популяций проводили на клеточном анализаторе FACS CantoII. Статистический анализ полученных данных проводили с использованием пакета прикладных программ Statistica 6.0 (Statsoft, США).

Доля Tcm в периферической крови доноров составляла 33,4% (min — 18,2; max — 43,7), Tem — 28,7% (min — 13,6, max — 38,9), у пациентов с псориазом: Tcm — 28,65% (min — 13,3; max — 59,6), Tem — 21,5% (min — 9,3, max — 38,6). Среди центральных CD4⁺-клеток памяти доля CD127⁺CD132⁺-клеток составляет 26,00%, CD127⁺CD132⁺ — 1,69%, CD127⁺CD132⁺ — 69,00%, CD127⁺CD132⁺ — 1,94%. Среди эффекторных CD4⁺-клеток памяти доля CD127⁺CD132⁺-клеток составляет 23,58%, CD127⁺CD132⁺ — 1,18%, CD127⁺CD132⁺ — 69,84%, CD127⁺CD132⁺ — 0,70%. Обнаружена прямая корреляционная связь между количеством CD127⁺CD132⁺ центральных клеток памяти и значением PASI ($r = 0,639$, $p < 0,05$).

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У пациентов с псориазом доля центральных клеток памяти выше, чем у здоровых доноров, при этом количество эффекторных клеток памяти — ниже. Обнаружена прямая корреляционная связь между количеством центральных клеток памяти, экспрессирующих γ -цепь рецептора IL-7, и степенью тяжести заболевания. Высокой экспрессией CD132 характеризуются активированные клетки памяти. Можно предположить, что данная популяция клеток памяти играет роль в поддержании аутоиммунного воспаления у пациентов с данным заболеванием, а также участвует в репопуляции резидентных клеток памяти кожи.

Ключевые слова: CD4⁺-клетки памяти, псориаз, аутоиммунные заболевания кожи, дерматология, резидентные клетки памяти, индекс PASI

CHARACTERIZATION OF CENTRAL AND EFFECTOR CD4⁺ MEMORY CELLS IN PSORIASIS

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Abstract. Psoriasis is a chronic autoimmune disease in which the skin and joints are involved in the pathological process. It was found that the recurrence of rashes in this disease occurs due to the resident memory cells of the skin. The number of CD4⁺CCR3⁺ effector memory cells in peripheral blood correlates with the severity of the disease. Therefore, the aim of our work is to study the phenotype of peripheral blood memory cells in patients with psoriasis.

The study included 6 healthy donors: average age — 45.4 (min — 29, max — 55), women — 3, men — 3; 10 patients with psoriasis: women — 4, men — 6, average age — 37.3 (min — 23, max — 57), of which 5 patients with PASI > 10 and 5 patients with PASI < 10. The exclusion criteria for the study were the presence of autoimmune, oncological and hematological diseases, systemic therapy with immunosuppressive drugs for 1 month. Patients signed informed consent to participate in the study. Isolation of peripheral blood mononuclear cells was performed in a density gradient of ficoll-urographin ($\rho = 1.082$ g/L). Then cells were stained with fluorochrome-conjugated monoclonal antibodies to surface markers of central (T_{cm}) and effector (T_{em}) CD4⁺ memory cells (CD4, CD45RO, CD197), the α -chain of the IL-7 receptor (CD127), and the γ -chain of the IL-7 receptor (CD132). Statistical analysis of the data obtained was performed using the Statistica 6.0 software package.

The percent of T_{cm} in the peripheral blood of donors was 33.4% (in — 18.2, max — 43.7), T_{em} — 28.7% (min — 13.6, max — 38.9), in patients with psoriasis: T_{cm} — 28.65% (min — 13.3, max — 59.6), T_{em} — 21.5% (min — 9.3, max — 38.6). In the peripheral blood of patients with psoriasis, among the central CD4⁺ memory cells, the proportion of CD127⁺CD132⁻-cells is 26.00%, CD127⁺CD132⁺ — 1.69%, CD127⁻CD132⁻ — 69.00%, CD127⁻CD132⁺ — 1.94%. Among effector CD4⁺ memory cells, the proportion of CD127⁺CD132⁻-cells is 23.58%, CD127⁺CD132⁺ — 1.18%, CD127⁻CD132⁻ — 69.84%, CD127⁻CD132⁺ — 0.70%. A direct correlation was found between the number of CD127⁺CD132⁺ central memory cells and the PASI value ($r = 0.639$, $p < 0.05$).

In patients with psoriasis, the proportion of central memory cells is higher than in healthy donors, while the number of effector memory cells is lower. A direct correlation was found between the number of central cells expressing the γ -chain of the IL-7 receptor and the severity of the disease. Activated memory cells are characterized by high expression of CD132. It can be assumed that this population of memory cells plays a role in maintaining autoimmune inflammation in patients with this disease, and also participates in the repopulation of skin resident memory cells.

Keywords: CD4⁺ memory cells, psoriasis, autoimmune skin diseases, dermatology, resident memory cells, PASI index

Introduction

The skin is normally populated by a large number of T-lymphocytes. Contact with the antigen promotes the differentiation of naive T-cells into effector cells capable of performing protective functions. Most of these cells are characterized by a short lifespan, while a small subset of them transition to the pool of long-lived memory cells [11]. Psoriatic lesions usually recur in the same locations as those at the disease onset, which indicates the possibility of the existence of immune memory. It was found that the transplantation of clinically healthy skin from psoriasis mice led to the formation of psoriatic plaques in mice with immunodeficiency [1].

There is a population of resident memory T-cells ensuring the first line of adaptive cellular defense in the skin. These cells can express both CD8 and CD4 markers being also characterized by high expression of CD103, CD69, CD49, and CD44 [5]. CD8⁺CD69⁺ resident T-lymphocytes persist in the skin of patients with psoriasis for several months after effective methotrexate therapy [13]. Secukinumab therapy led to decreased number of resident memory effector T-cells (CD8⁺CD103⁺CD49a⁺CCR7⁻) and IL-17A-producing resident memory cells. At the same time, the number of resident memory cells significantly increased in clinically healthy skin after treatment, which was accounted for by high resistance of this population to biological therapy [7]. It was also found that the number of IL-22-producing memory CD4⁺T-cells does not correlate with the duration of treatment, which indicates that the effector functions are preserved during therapy for a long time [4].

In addition to resident memory cells, there are central (Tcm) and effector (Tem) memory cells. Central CD4⁺ memory cells (Tcm) are characterized by the ability to migrate from secondary lymphoid organs to the bloodstream, and featured with high expression of homing receptors CD62L and CCR7 as well as high proliferative potential. Memory effector cells (Tem) are characterized by the ability to migrate between secondary lymphoid organs and non-lymphoid tissues, as well as the absence or low expression of CD62L and CCR7, while adhesion molecules are highly expressed [2]. The use of alefacept led to apoptosis of effector CD4⁺ memory cells, while the level of cell reduction correlated with the magnitude of the PASI index indicating the role of CD4⁺ memory cells in the development of the disease [6]. Gaide et al. showed the common clonal origin of central and resident memory cells, which suggested that Tcm are capable of repopulating the pool of resident memory cells [8]. CD4⁺ and CD8⁺ circulating memory cells of patients with psoriasis are characterized by high expression of CCR4 (a chemokine receptor responsible for the migration of lymphocytes into the skin), and their count correlates with the severity of the disease [12].

It was found that central CD8⁺ memory vs effector cells are characterized by a higher CCR4 expression. At the same time, the central CD8⁺CCR4⁺ and CD4⁺CCR4⁺-lymphocytes had the peak expression of the cutaneous lymphocyte antigen (CLA) necessary for skin tropism [3].

IL-7 plays a fundamental role in the development and maintenance of memory cells. It promotes cell survival due to its antiapoptotic action [10]. Therefore, **the aim of our work** was to study the phenotype of memory cells (expression of α - and γ -chains of the IL-7 receptor) in the peripheral blood of patients with psoriasis.

Materials and methods

There were enrolled 6 healthy volunteers (males and females), aged 28 to 58 years (mean age 48 ± 2.7 years), and 10 patients with psoriasis aged 27 to 58 years, of which 5 patients had PASI more than 10 and 5 patients with PASI less than 10. The exclusion criteria were concomitant autoimmune, oncological and hematological diseases, systemic therapy with immunosuppressive drugs. Both groups were assigned after signing informed consent.

PBMCs were isolated by centrifugation in a Ficoll-Urographin ($\rho = 1.082$ g/L) density gradient. Next, cells were stained with fluorochrome-conjugated monoclonal antibodies to surface markers of central and effector CD4⁺ memory cells, the IL-7 receptor α -chain, and the common γ -chain. The following antibody panel was used: CD4-PE (Sorbent LLC), CD45RO-PE/Cy7, CD197 (CCR7)-APC/Cy7, CD127-PerCP/Cy5.5, CD132-APC (BioLegend, USA). Staining was performed with a combination of antibodies according to the manufacturer's recommended protocol. Phenotyping of cell populations and the study of proliferative activity were performed on a FACS CantoII cellular analyzer (BD, USA) by using the FACS Diva 6.1 software.

Statistical data analysis was carried out by using the Statistica 6.0 software package. The Mann–Whitney test was used to evaluate unrelated variables. The revealed differences were considered significant in case of $p < 0.05$.

Results and discussion

Patients with psoriasis were found to have significantly lower count of CD123⁺CD132⁺-lymphocytes, as well as double positive CD127⁺CD132⁺-cells both in central and effector memory cells (Table 1). It can be assumed that, in this disease, cells expressing IL7R replenish the population of resident skin memory cells involved in the maintenance of local inflammation, which accounts for low number of CD123⁺CD132⁺ Tcm and Tem

TABLE 1. PHENOTYPE OF CD4⁺ CENTRAL AND EFFECTOR MEMORY CELLS OF HEALTHY DONORS AND PATIENTS WITH PSORIASIS

	Control	Psoriasis	
Tcm, %	33.4±10.14	40.80±11.07	p > 0.05
CD127 ⁺ CD132 ⁻ , %	29.40±14.96	26.90±17.08	p > 0.05
CD127 ⁺ CD132 ⁺ , %	10.55±1.49	1.68±1.94	p < 0.05
CD127 ⁻ CD132 ⁻ , %	50.31±9.63	69.46±18.01	p > 0.05
CD127 ⁻ CD132 ⁺ , %	9.73±6.78	1.94±2.26	p < 0.05
Tem, %	28.70±9.51	23.83±10.10	p > 0.05
CD127 ⁺ CD132 ⁻ , %	27.45±12.05	23.57±16.67	p > 0.05
CD127 ⁺ CD132 ⁺ , %	12.26±5.83	1.17±2.05	p < 0.05
CD127 ⁻ CD132 ⁻ , %	48.87±10.07	69.84±26.61	p < 0.05
CD127 ⁻ CD132 ⁺ , %	11.14±4.86	0.70±1.00	p < 0.05

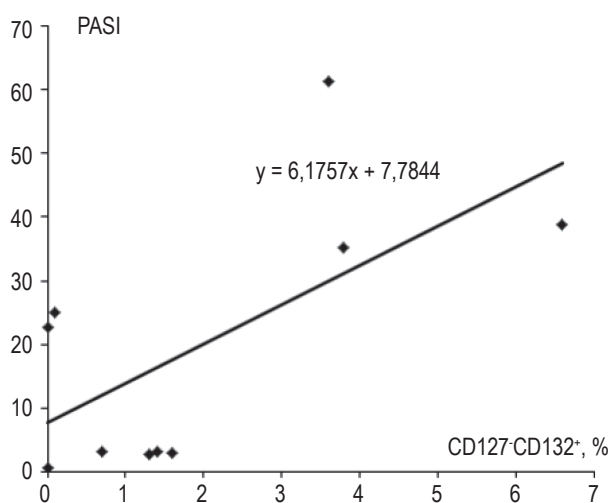


Figure 1. Direct correlation between the percentage of CD127⁻CD132⁺ central memory cells and the PASI value ($r = 0.639$, $p < 0.05$).

compared to apparently healthy donors. Expression of the IL-7 receptor γ -chain is characteristic of activated memory cells, which decreased number in the peripheral blood may also be due to the migration to skin foci of the disease. In view of the aforementioned, cell migration, percentage of CD123⁺CD132⁺ effector CD4⁺ memory cells naturally increases. In the Tcm population, the number of double negative cells tends to decrease compared to the control group.

An inverse correlation was found between the proportion of CD4⁺ central memory cells expressing the IL-7 receptor γ -chain (CD123⁺CD132⁺) and the PASI value ($r = 0.639$, $p < 0.05$) (Figure 1). We assume that this population of memory cells plays a role in maintaining autoimmune inflammation in patients with this disease, and also participates in the repopulation of skin resident memory cells.

Based on the study results, we conclude that CD4⁺ central memory cells comprise one of the factors in developing severe psoriasis, which is indicated by a direct correlation between the percentage of activated Tcm expressing the IL-7 receptor γ -chain and magnitude of the PASI index. Also, this cell population is characterized by the peak expression of homing skin receptors [11].

According to the linear model of the immunological memory development [1], the central memory cells represent early stage. It is assumed that at this stage, the cells are transferred to the pool of skin resident memory cells [10]. Our data provide another argument in favor of this hypothesis. The dependence of the disease severity on the number of peripheral blood central CD4⁺CD123⁺CD132⁺ memory cells, despite the fact that the proportion of this cell population is significantly lower in patients with psoriasis compared vs apparently healthy donors, indicates that CD132⁺Tcm are involved in maintaining inflammation during psoriasis, and migrate from the bloodstream to peripheral tissues (presumably the skin).

Memory effector cells are characterized by minimal expression of CCR4 and CLA, but at the same time they express a higher level of CXCR3 and CCR5 molecules as compared to central memory cells [11, 13]. It is assumed that the expression of the above markers emphasizes the difference

between the migration potential between central and effector memory cells: to the skin and to the areas of inflammation, respectively [13]. This accounts for significantly decreased number of activated Tcm and Tem in the peripheral blood of patients with psoriasis vs control subjects.

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РАННЯЯ ДИАГНОСТИКА АУТОВОСПАЛИТЕЛЬНЫХ НАРУШЕНИЙ, АССОЦИИРОВАННЫХ С ПОСТВИРУСНЫМ СИНДРОМОМ ХРОНИЧЕСКОЙ УСТАЛОСТИ И КОГНИТИВНЫМИ РАССТРОЙСТВАМИ ПРИ ХРОНИЧЕСКИХ МИКСТ ГЕРПЕС-ВИРУСНЫХ ИНФЕКЦИЯХ

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Резюме. Неуклонный рост числа аутоиммунных заболеваний и иммуноопосредованных аутовоспалительных процессов вызывает повышенный интерес врачей всех специальностей к этой теме и делает крайне актуальным вопрос раннего выявления аутоиммунных расстройств/аутоиммунного синдрома (АС). Зачастую эти нарушения развиваются на фоне атипичного течения хронических активных вирусных инфекций, вызываемых персистирующими вирусами, в частности вирусами семейства Herpesviridae (АХА ГВИ) и остаются не диагностированными вследствие полисимптомности опосредуемых ими нарушений и различными «клиническим масками» вызываемых расстройств.

Разработанный нами полуколичественный метод скрининговой оценки содержания аутоантител в сыворотке крови пациентов, страдающих АХА ГВИ, вызываемой герпес-вирусами, с использованием метода ИФА (Immunodot), является высокоспецифичным, скрининговым методом, который может позволить проводить объективную оценку динамики течения аутоиммунного процесса, а также осуществлять контроль за эффективностью проводимой комплексной противовирусной и иммуномодулирующей терапии.

Детекция аутоантител различной специфичности в сыворотке крови пациентов, страдающих атипичной хронической активной инфекцией, вызываемой герпес-вирусами (АХАИ), является ранним диагностическим маркером, необходимым, в первую очередь, для выявления аутоиммунной патологии нервной системы, которая ассоциирована с длительным течением активного микст-герпес-вирусного процесса.

Ключевые слова: герпесвирусы, аутоантитела, ганглиозиды, аутоиммунитет, иммунная дисфункция, пост-вирусный синдром хронической усталости

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EARLY DIAGNOSTICS OF AUTOINFLAMMATORY DISORDERS ASSOCIATED WITH POST-VIRAL CHRONIC FATIGUE SYNDROME AND COGNITIVE IMPAIRMENTS IN CHRONIC MIXED HERPES VIRAL INFECTIONS

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Abstract. The steady increase in the number of autoimmune diseases and immune-mediated auto-inflammatory processes causes an increased interest of doctors of all specialties in this topic and makes the issue of early detection of autoimmune disorders / autoimmune syndrome (AS) extremely urgent. These disorders often develop against the backdrop of an atypical stream of chronic active viral infections caused by persistent viruses, in particular those of the Herpesviridae family, and remain undiagnosed due to polysymptomatic disease, and various “clinical masks” of the disorders caused by them. The semi-quantitative method developed by us for screening assessment of the content of autoantibodies in the blood serum of patients suffering from ACAI caused by herpes viruses using the ELISA method (Immunodot) is a highly specific screening method that can allow for an objective assessment of the dynamics of the autoimmune process, as well as control the effectiveness of the ongoing complex antiviral and immunomodulatory therapy. The detection of autoantibodies of various specificity in the blood serum of patients suffering from an atypical chronic active infection caused by herpes viruses (ACAI) is an early diagnostic marker, necessary, first of all, to identify autoimmune pathology of the nervous system, which is associated with a long course of the active mixed herpes-viral process.

Keywords: herpesviruses, autoantibodies, gangliosides, autoimmunity, immune dysfunction, post-viral chronic fatigue syndrome

Introduction

The steady increase in the number of autoimmune diseases and immune-mediated auto-inflammatory processes provokes growing interest of doctors of all specialties in this topic and makes the issue of early detected autoimmune disorders/autoimmune syndrome (AS) extremely urgent [2, 9]. These disorders often develop in parallel with aberrant input of chronic active viral infections caused by persistent viruses, particularly those of the Herpesviridae family, and remain undiagnosed due to polysymptomatic picture, as well as diverse related “clinical masks” [3, 4, 7]. It is shown, that viruses particularly the Epstein-Barr virus (EBV) is able to bind to B-lymphocyte receptors, participate in epigenetic regulation, DNA methylation in B lymphocytes that may be the basis for inducing autoantibody formation of different specificities, thereby promoting autoimmune process [1, 5]. Besides, the phenomenon of cross-reactivity between viral epitopes and human own protein epitopes has been described, which is both a trigger for various autoimmune disorders and a cornerstone that for formation of immunopathological disorders particularly mono- or polyvalent autoimmune processes with varying loci. Herpes-induced interferon system (IFN) suppressor develops altered response to viral invasion and contributes to a defect

in system-induced IFN alpha and IFN gamma products in vitro, promoting long-term active replication of herpesviruses and pathological changes such as autoimmune disorders and various types of immunopathology [8].

Currently, there have been described 8 human herpesviruses (HHV) divided into 3 subfamilies: Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae. All Herpesviridae viruses are characterized by pronounced tissue tropism, high mutability and a rapid transition from acute latent-to chronic infection with lifelong persistence in various human cells, including those of the monocytic-macrophage lineage and effector immune cells, thereby impairing immunobiological surveillance. Cases of co- and mixed infections caused by viruses of this family have been described at increasingly higher rate.

The effect on the immune system of any damaging factor, both exogenous and endogenous, including host damaged, infected or modified cells by viruses triggers activation of the immune system [10]. Moreover, it may be both local and systemic at the early stages with nonspecific character and manifested by produced pro-inflammatory factors, changes in microvasculature permeability and migration to the focus of damaged immune cells, primarily neutrophils – the main effectors of inflammation.

Neutrophil degranulation in the focus of inflammation is accompanied by released proteolytic enzymes and reversed focus of damage. However, the ongoing activation of inflammatory factors leads to cell and tissue damage, release of autoantigens accompanied by produced of relevant autoantibodies and development of autoimmune reactions. The detection of autoantibodies of various specificities in patients with ACA HVI can be considered as an early novel preclinical marker of impaired autoimmune homeostasis and development of pathological processes as well as immune-mediated diseases and conditions [11].

In connection with the data noted above, an important and relevant aspect is the search for biological markers that objectively reflect features of the clinical state (clinical and biological correlations). The potential of using such indicators as predictors is also of great importance, allowing to reliably assess probability of positive or negative dynamics in developing disease even during the initial clinical examination.

The aim: our study is dedicated to determine markers of autoimmune disorders/autoimmune syndrome (AS) in patients suffering from mixed herpesvirus infections (HSVI) using a semi-quantitative method to screen for serum autoantibodies of various specificities in patients with ACA HVI.

Materials and methods

There were followed up 548 subjects, of both sexes, aged 25 to 68 years, suffering from atypical chronic active mono- and mixed HVI. Among them were selected 20 patients with mixed HVI suffering from severe disorders of the mnestic sphere, post-viral syndrome of chronic fatigue and immune dysfunction, clinical signs of developing autoimmune syndrome – fibromyalgia, cephalgia, neuropathic pain and paresthesia, etc. comprising a study group.

The inclusion criteria were: clinical signs of the immunocompromised state characteristic of ACA HVI. Criteria for immunocompromised state are presented by repeated acute respiratory viral infections of bacterial-viral etiology with incidence rate up to 10-12 times a year, chronic recurrent herpes-viral infections (HSV1, HSV2), chronic CMV, EBV and HHV6 infections, chronic bacterial and fungal co-infections. The comparison group included 20 sex- and age-matched apparently healthy adults. The study was approved by the Ethics Commission, and informed consent was obtained from all patients for participation in the study, in accordance with the World Medical Association's Declaration of Helsinki (WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects, 2013) and for the processing personal data. There was organized a single-centre, prospective, non-

randomized study conducted at the “Clinical and Diagnostic center MEDSI Belorusskaya”, Moscow.

Along with routine methods (collection of anamnesis, methods of physical examination, CBC, etc.), there were used serologic diagnostic methods to detect herpes virus infections (IgM VCA EBV, IgG VCA EBV, IgM CMV, IgG CMV IgM HSV1/2, IgG HSV1/2) by ELISA test systems NPO “Diagnostic Systems” (Russia).

The PCR method of the AmpliSens test system (Russia) was used to detect virus genome in biomaterial samples (blood, saliva, urine, scraping from the tonsils and the posterior pharyngeal wall). Methods of flow cytometry, ELISA were used to assess parameters of antiviral immunity (immunogram, INF-status, etc.). To detect autoantibodies in the blood serum, the ELISA (Immunodot-point solid-phase ELISA), Medipan GMBH, Germany, was used: Anti-Phospholipid 10 Dot detecting IgM/G antibodies against phospholipids and β 2-glycoprotein; ANA 12 Line Dot for detecting IgG antibodies to nuclear and cytoplasmic antigens; Anti-Gangliosid Dot specific to antiganglioside antibodies against a wide range of gangliosides (GM1-4; GD1a, b; GD2,3; GT1a, b; GQ1b; sulfatides). Based on the use of Immunodot technology, we have developed and implemented a semi-quantitative method for screening the level of serum autoantibodies of various specificities in patients suffering from herpes virus-caused ACAI.

Statistical analysis was performed by using the Microsoft Excel 2010 software package.

Results and discussion

Among the 548 examined patients, 36.6% of patients suffer from monoherpesvirus infections, of which 55.5% are patients with EBV infection; 35.3% – with HSV type 1 and 11.1% – with either HSV-2 and CMV, respectively.

63.7% cases were presented with mixed herpesvirus infections.

The pattern of such mixed infections is dominated by combination of HSV1 + HSV2; EBV + HHV 6, EBV + CMV + HHV6, as well as EBV + CMV + HHV6 + HSV-1 – per 11.2% in each case. Further, the distribution of mixed infections by the occurrence of combinations is as follows: EBV + CMV (9.7%); EBV + CMV + HSV1 (4.8%); EBV + CMV + HSV2 (3.2%).

According to our data, the patients in the study group were characterized by some symptom complex, including complaints of a prolonged sensation of severe weakness and chronic fatigue.

Patients were concerned with increased sweating, intermittent pain in the throat, muscles (transient myalgias) and joints (arthralgia), headache, long-term subfebrile fever, regional or extended lymphadenopathy, sleep disorders (insomnia), loss of

memory, attention, impaired intellect, with less often psychogenic depression. Moreover, in 100% of cases, this pathology was associated with chronic latent/persistent HVI (HSV-1/HSV-2, EBV, CMV, HHV6). Most common dominant viruses in the mixed HVI were EBV and HHV6. Multiple symptoms and syndromes detected in 100% of patients with mixed ACA HVI pose great diagnostic difficulties for doctors of all specialties, which complicates timely diagnostics and administration of proper therapy.

We have shown that it is necessary to carry out PCR diagnostics aimed at detecting herpesviruses in various biomaterials: in saliva, in scraping from the tonsils, in scraping from the oropharynx to determine activity of the viral process. It is mandatory to quantify this viral load necessary to assess the dynamics of antiviral and immunotropic therapy.

The frequency of detected EBV genome in diverse samples from patients with ACA EBV infection (PCR) was as follows: 76.3% – saliva; 12.4% – blood; 63.8% – scrapings from the back of the throat; 52.7% – tonsil scrapings; and 18.3% – urine.

However, up to the present time, the detection of the EBV genome in various biological environments of the body in such patients poses substantial obstacles for interpretation by doctors of other specialties, and often such patients remain neglected receiving no proper and timely therapy.

While studying the parameters of autoimmune homeostasis in patients with ACA HVI by using the innovative Immunodot technology, we developed criteria for the semi-quantitative assessment of the autoantibody level to a wide range of ganglioside antigens (GM1-4; GD1a, b; GD2,3; GT1a, b; GQ1b; sulfatides), phospholipids (cardiolipin, phosphatidic acids, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, annexin V, prothrombin, β 2-glycoprotein I), nuclear and cytoplasmic antigens (dsDNA, nucleosomes, Sm, ribosomes, RNP histones, SS-A 60 kD, SS-A 52 kD, SS-B, Scl-70, CENP-B, Jo-1). According to our data, the results of determining the level of autoantibodies were interpreted in accordance with the proposed reference range shown in arbitrary units (U) and interpreted as: negative result; low level; average level; high level; very high level. The magnitude of detectable antibody level shown in U are presented for each Immunodot test (Table 1).

Patients of the study group were found to have serum autoantibodies of various specificity in 100% of cases. Among them, they were dominated by antiganglioside antibodies IgM and IgG (Anti-Gangliosid Dot) found in the blood serum of 60% patients. Gangliosides are glycopospholipids containing a sialic acid residue found in the membrane of many cells, but their amount is specifically increased in neurons. They are necessary for cell growth and

differentiation, transmission of nerve impulses, and mounting immune responses. Among them, autoantibodies to GM1 ganglioside are found in 80% of blood samples. Autoantibodies against the GM1 ganglioside initiate damage to the axolemma and also block voltage-gated calcium channels. It should be noted that often it is possible to identify a combination of various autoantibodies to gangliosides in the blood of patients with various clinical forms of the syndrome. The second place in the frequency of detected autoantibodies is held by antibodies to phospholipids and β 2-glycoprotein (Anti-Phospholipid 10 Dot), among which IgG to phosphatidylcholine predominates (45%). β 2-glycoprotein I is a serum protein with natural anticoagulant activity. Circulating AFLA recognizes antigenic structures formed by the interaction of β 2-glycoprotein I and cardiolipin. Antibodies to cardiolipin and β 2-glycoprotein I are more often found in patients with the primary APS. IgG antibodies against nuclear and cytoplasmic antigens (ANA 12 Line Dot) were found in 16.7% of patients predominantly against dsDNA. Antinuclear antibodies (ANA) against extractable nuclear antigens consist of antibodies to soluble components of the cell nucleus (ribonucleoproteins). Normally, ANAs are undetected in vivo. However, during autoimmune pathology, the immune system starts to produce specific immunoglobulins to its own cells and related components. It is believed that immune complexes consisting of double-stranded DNA and specific antibodies (IgG and IgM) are involved in developing microvasculitis (Table 2).

It was shown that the largest amount of serum autoantibodies in examined patients was specific to a wide range of gangliosides. Among them, antibodies to GM1 ganglioside were determined – in 70%; GM3 – 43%; GD2,3-21.4%; sulfatides – 32%. Autoantibody levels varied in a wide range from low (30 U) to high (100 U) and correlated with the activity of the viral process and clinical symptoms.

Besides, our study allowed simultaneously identify autoantibodies to several types of autoantigens (antiganglioside and antiphospholipid) in several patients, which allows us to interpret emerging auto-aggression as multivalent reaction. Hence, it opens up wide avenues for understanding etiopathogenetic mechanisms underlying comorbid conditions and syndromes in patients with ACA HVI.

Thus, the prevailing spectrum and a certain level of anti-ganglioside autoantibodies identified by us in patients suffering from ACAI caused by herpes viruses allow us to assess not only the activity of virus-induced autoaggression but also to dynamically monitor effectiveness of the ongoing complex antiviral and immunomodulatory therapy.

TABLE 1. VALUES OF THE LEVEL OF DETECTABLE ANTIBODIES FOR EACH IMMUNODOT TEST

Dot	Negative result	Low level	Average level	High level	Very high level
Anti-Phospholipid 10 Dot	<30 U	30-40 U	40-70 U	70-100 U	>100 U
Anti-Gangliosid Dot	<20 U	20-40 U	40-70 U	70-100 U	>100 U
ANA 12 Line Dot	<1.0 U	1-1.5 U	1.5-3.0 U	3.0-3.5 U	>5.0 U

TABLE 2. DETECTION RATE OF THE SPECIFIC ANITBODIES

Antibody specificity	Detection rate (%)
Antiganglioside antibodies IgM and IgG, among them:	60.0 %
AutoAB to GM1 ganglioside	80.0 %
Antibodies to phospholipids and β 2-glycoprotein, among which: IgG to phosphatidylcholine	45.0 %
IgG to nuclear and cytoplasmic antigens	16.7 %

Conclusion

In the present study, a predominant spectrum of autoantibodies of a certain specificity was revealed, an increase in various anti-ganglioside autoantibodies was demonstrated with adjusting their level in patients suffering from ACAI caused by herpes viruses and associated with a post-viral syndrome consisting of chronic fatigue, cognitive disorders, polyneuropathy, fibromyalgia. The data obtained indicate that the long-term course of active mono- and mixed HVI is associated with developing mono- or poly-autoimmune syndrome, which can lead later to the manifested autoimmune diseases.

The semi-quantitative method developed by us for assessment of serum autoantibodies in patients with ACAI caused by herpes viruses by using ELISA (Immunodot) is a highly specific screening method that can allow to objectively assess dynamics of autoimmune process, as well as control effectiveness of the ongoing complex antiviral and immunomodulatory therapy.

The detection of serum autoantibodies of various specificity in patients suffering from atypical chronic active infection caused by herpes viruses (ACAI) is an early diagnostic marker, necessary, primarily to verify autoimmune pathology of the nervous system, which is associated with a long course of the active mixed herpes-viral process.

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ВЛИЯНИЕ СУБКЛИНИЧЕСКОГО КЕТОЗА КОРОВ НА ФОРМИРОВАНИЕ КОЛОСТРАЛЬНОГО ИММУНИТЕТА ТЕЛЯТ

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Резюме. Здоровье новорожденного всецело зависит от состояния организма матери на протяжении всей беременности. Обеспечение оптимальных условий содержания беременных животных базируется, в первую очередь, на полноценном кормлении и обеспечении санитарно-гигиенических условий окружающей среды. Большую нагрузку организм коровы претерпевает в транзитный период, который начинается за 3 недели до отела и продолжается в течение шести недель. При нарушении технологии кормления и содержания у коров в данный период часто возникают метаболические нарушения, которые проявляются повышенной выработкой кетонов.

Известно, что становление иммунитета в ранний постнатальный период у телят во многом зависит от своевременной выпойки молозива. Материнские иммуноглобулины из молозива попадают непосредственно в системный кровоток новорожденного в тонком отделе кишечника по тубулярной системе эпителиальных клеток путем пиноцитоза. Задача исследования — изучить влияние субклинического кетоза у коров-матерей на формирование колострального иммунитета у родившихся от них телят. Для исследования были отобраны стельные коровы 3-6 лет за 3-7 дней до родов. У коров были взяты пробы мочи и крови. С целью выявления субклинического кетоза у коров было проведено исследование мочи на содержание кетонов. По результатам исследования были сформированы две группы по 10 голов — в первой группе (подопытной) уровень кетоновых тел в моче составил от 1,8 до 3,7 ммоль/л, во второй группе (контрольной) кетоны в моче не обнаружены. Сразу после отела у коров отбирали порции молозива, а у новорожденных телят брали кровь через сутки после первой выпойки молозива. В обезжиренном молозиве и в сыворотке крови новорожденных телят исследовали содержание иммуноглобулинов. В сыворотке крови суточных телят также определяли содержание общего белка биуретовым методом, альбуминов — фотометрическим методом с бромкрезоловым зеленым.

По результатам проведенного исследования было обнаружено снижение классов иммуноглобулинов G, M и A в сыворотке крови коров перед отелом на 19,1-23,5%, в молозиве — на 23,7-34,4% и в сыворотке крови суточных телят — на 21,7-27,6%. В наибольшей степени определялось снижение концентрации IgM. На содержание в крови телят альбуминов субклинический кетоз коров-матерей практически не оказывал влияние.

Ключевые слова: коровы, телята, иммуноглобулины, молозиво, колостральный иммунитет, кетоз

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INFLUENCE OF SUBCLINICAL KETOSIS IN COWS ON FORMATION OF COLOSTRAL IMMUNITY IN CALVES

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Abstract. The health of the newborn depends entirely on the state of the mother's body throughout the pregnancy. Ensuring optimal conditions for keeping pregnant animals is based, first of all, on adequate feeding and ensuring the sanitary and hygienic conditions of the environment. The cow's body undergoes a great load during the transition period, which begins 3 weeks before calving and lasts for six weeks. When the technology of feeding and housing is violated, during this period, metabolic disorders often occur in cows, which are manifested by increased production of ketones. It is known that the development of immunity in the early postnatal period in a calf largely depends on the timely feeding of colostrum. Maternal immunoglobulins from colostrum enter the systemic circulation of the newborn in the small intestine through the tubular system of epithelial cells by pinocytosis. The aim of the study is to study the effect of subclinical ketosis in mothers cows on the formation of colostrum immunity in calves born from them.

For the study, pregnant cows 3-6 years old were selected 3-7 days before delivery. Urine and blood samples were taken from the cows. In order to identify subclinical ketosis in cows, urine was tested for ketones. According to the results of the study, two groups of 10 animals were formed – in the first group (experimental) the level of ketone bodies in the urine ranged from 1.8 to 3.7 mmol/l, in the second group (control) ketones were not found in the urine. Immediately after calving, portions of colostrum were taken from the cows, and blood was taken from newborn calves a day after the first colostrum was fed. The content of immunoglobulins was studied in skim colostrum and in the blood serum of newborn calves. In the blood serum of day-old calves, the content of total protein was also determined by the biuret method, albumin – by the photometric method with bromocresol green.

According to the results of the study, a decrease in the classes of immunoglobulins G, M and A was found in the blood serum of cows before calving by 19.1-23.5%, in colostrum – by 23.7-34.4%, and in the blood serum of day old calves – by 21.7-27.6%. The decrease in IgM concentration was determined to the greatest extent. Subclinical ketosis of mothers had practically no effect on the content of albumin in the blood of calves.

Keywords: cows, calves, immunoglobulins, colostrum, colostrum immunity, ketosis

Introduction

The neonatal health depends entirely on the state of the mother's body throughout the pregnancy. Ensuring optimal conditions for keeping pregnant animals is based, first of all, on adequate feeding and ensuring the sanitary and hygienic environmental conditions. These factors are very broad: optimizing feeding means not only introducing all the necessary nutrients into the diet – proteins, lipids, carbohydrates, as well as vitamins and minerals in certain proportions, but also accurately tracking changes in the animal's need for nutrients and the energy value of the diet in connection with changing the phases of lactation [4, 14]. What matters is the frequency of feeding, the degree of feed crushing, moisture content, and taste. It should be also noted that it is important to check the quality of all feed, especially that of our own production, such as silage and haylage. An important component of an animal's optimal health is its habitat. The organization of the manure removal system,

the choice of litter, milking technology, hierarchical relationships in the herd – all this is important for creating an optimal herd ecosystem. There is evidence that limitation of mobility in cows associated with lameness can lead to metabolic disorders [2, 4, 5]. The use of highly productive dairy cows in livestock farms in our country is unthinkable without adherence to livestock management technology and competent herd management. Errors made by livestock breeders will affect the health of cows and calves anyway. The consequences of violating conditions of feeding and keeping cows during the so-called transit, or transitional period, are especially pronounced [7, 9, 13].

The transit period is extremely important in the cow's production cycle, starting three weeks before calving and continuing for six weeks. This period is associated with a serious restructuring of metabolic processes, with a sharp change in the cow's need for nutrients after calving. Despite the intensive increase

in fetal weight during deep pregnancy, energy and plastic costs before calving are not as high as during lactation. Breeding work in dairy farming has led to a significant increase in the milk production of modern cows. So, in the first month of lactation, they produce 40–45 liters of milk per day. With such milk yields, up to 50 kg of pure protein and fat, up to 60 kg of lactose, up to 2 kg of calcium per pure element are removed from the body in a month. Therefore, during the transit period, the cow's body is most prone to metabolic diseases [7, 13, 14]. A big problem is the temporary lack of feed energy immediately after calving. The digestive system of the cow plays a prominent role in the absorption of nutrients from the diet, the microflora of the proventriculus is especially important. In the proventriculus, chemical processing of feed nutrients – carbohydrates, fats, proteins – occurs under the influence of enzymes from bacteria, fungi, protozoa. The ecosystem of the ruminant proventriculus includes a community of a large number of microorganisms – bacteria (50–90%), protozoa (10–50%), fungi (5–10%), the density of which exceeds that of known natural ecosystems. The number of bacterial species in the rumen reaches 150 and up to 60 species of protozoa. In the contents of the cow rumen, the bacterial mass reaches up to 1.5–2.0 kg. The system of symbiosis macroorganism-microflora ensures the assimilation of both feed nutrients and microorganisms as necessary metabolites for the host animal [14]. The active participation of microorganisms as metabolites of nutrients in the digestion of cellulose and starch in the rumen of ruminants makes this process more effective in this species of animals, in comparison with one-hoofed herbivores.

The introduction of ruminant nutrients into the diet is normalized depending on the phase of productivity. The maximum introduction of nutrients in terms of metabolizable energy and dry matter is carried out at the peak of lactation. Milking of cows is forcibly stopped two months before calving, and during this period, which is called dry-period, the feeding of energy-intensive feed is minimized. Thus, during the dry period, the nutritional value of the rations is minimal. With a decrease in the nutritional value of diets at the end of pregnancy, the concentration of cicatricial microflora naturally declines. Therefore, the conversion of feed in the early postpartum period is physiologically reduced. Today it is believed that a temporary negative balance after calving is a completely normal physiological phenomenon that every cow goes through to one degree or another [7]. However, if the cow is not properly provided with easily digestible high-energy feed, then a cascade of events quickly develops, leading to the overproduction of ketone bodies – acetoacetic, beta-hydroxybutyric acids and acetone. Ketosis develops in response to carbohydrate

starvation, its biological meaning is to compensate for glucose deficiency in brain cells. The onset of ketosis in late pregnancy is exacerbated by a significant increase in fetal glucose intake [11]. Mobilization of own fat reserves partially covers the energy deficit, but fatty acids are not able to pass through the blood-brain barrier, therefore they cannot be used for oxidative metabolism of brain cells. A significant disadvantage is the rate of fatty acid beta-oxidation, which is not high enough due to the hydrophobicity of substrates, and under conditions of energy starvation cannot quickly replenish ATP regeneration at the proper level. On the other hand, activation of fatty acid oxidation results in an excess of acetyl-CoA molecules, which are not only substrates of the tricarboxylic acid cycle, but also sources for ketogenesis. Cows with high fatness, which received an excessively high-calorie diet during the dry period, are especially prone to ketosis [13, 15]. In the period of preparation for calving, the mobilization of fats from the depot increases and the concentration of non-etherified fatty acids in the blood sharply increases, which has an inhibitory effect on appetite. As a result, a vicious circle occurs – cows with high fatness and large reserves of subcutaneous fat become more prone to metabolic disorders during the transition period. Thus, a complex of factors arises that lead to the development of subclinical or clinical ketosis, the first signs of which may appear during pre-calving period. It is known that the development of immunity in the early postnatal period in a calf largely depends on the timely feeding of colostrum [1, 10, 12]. Maternal immunoglobulins from colostrum enter the systemic bloodstream of the newborn in the small intestine through the tubular system of epithelial cells by pinocytosis, i.e., in an unsplit form [3, 10, 16]. The ability for effective pinocytosis of immunoglobulins is very limited in time and last only for few hours. It is recommended to drink the first colostrum within the first two hours after delivery. The rate and degree of absorption of immunoglobulins in the small intestine continuously decreases during the first days of the calf life. The possibility of absorption of uncleaved antibody molecules is explained by the absence of hydrochloric acid in the abomasum, the presence of a trypsin inhibitor in colostrum, and the immaturity of the small intestinal epithelial cells [10].

Colostrum secret is extremely enriched in immunoglobulins: according to diverse studies, from 50 to 100 g/l is found in the first portion of colostrum after calving, which is several times higher than their concentration in the blood serum.

It is known that transplacental transmission of maternal antibodies to the fetus in ruminants is practically impossible due to the syndesmochorial structure of the placenta in ruminants, which rules out the possibility of their transport across the placenta [11]. In contrast, in primates and rodents

with a hemochorial type of placenta, it is quite possible to transfer of maternal immunoglobulins to the fetus.

During the period of deep pregnancy, the cow's body intensively prepares the udder for lactation [3]. Under the influence of hormones, mainly prolactin and cortisol, during this period, synthetic processes are activated in the alveolar epithelial cells. In addition to organizing the biosynthesis of nutritional components of milk secretion, intensive immunobiological processes take place in the mammary gland. So, there is a migration of various leukocyte and macrophage cells into the alveolar cavity. The transport of immunoglobulins from the bloodstream to the secretion of the mammary gland is also activated [16]. Since the concentration of immunoglobulins peaks in the first portion of colostrum after calving, the most intensive processes of globulin protein enrichment of milk secretion occurs several days before calving. Contribution to the formation of the colostrum immunoglobulin pool is provided not only by proteins exported from the bloodstream, but also by its own immunoglobulins, synthesized by immunocompetent cells directly in the mammary gland tissues.

The importance of timely feeding of colostrum lies not only in the ability of the newborn epithelium to absorb native immunoglobulins, but also in reducing their secretion by prolactin-influenced alveolar epithelial cells, an inhibitor of colostrogenesis. As a result, the secretory epithelium of the alveoli intensively synthesizes milk components and completely stops the immunoglobulin transfer [1, 12].

Harbingers of metabolic disorders in cows appear even before calving, while the intensive development of metabolic pathology, accompanied by ketonemia, decreased gluconeogenesis, and liver lipidosis, manifests itself already in the postpartum period. Considering the fact that the formation of the colostrum immunoglobulin pool occurs before calving, the question arises about the effect of ketonemia of pregnant cows on the protein composition of colostrum secretion and on formation of colostral immunity in calves.

Materials and methods

The studies were carried out in the livestock farm of the Leningrad region (Slantsevsky district). For the study, pregnant cows 3-6 years old were selected 3-7 days before delivery. Urine and blood samples were collected from the cows. In order to identify subclinical ketosis in cows, urine was tested for ketones. According to the study data, two groups of 10 animals were formed – in the first group (experimental) the level of ketone bodies in the urine ranged from 1.8 to 3.7 mmol/l, in the second group (control) ketones were not found in the urine. Immediately after calving, portions of colostrum were obtained from the cows, and blood was collected from newborn calves a day

after the first colostrum was fed. Colostrum secretions were centrifuged, then frozen followed by separation of lipid fraction. The content of immunoglobulins was studied in skim colostrum and in the blood serum of newborn calves. Before laboratory examination, colostrum secretion was diluted with saline at 1: 5. The study of ketones in urine was carried out using a CL-50 semi-automatic optoelectronic urine analyzer. In the blood serum of cows, the content of immunoglobulin classes A, M and G was investigated by the method of discrete sedimentation.

The essence of the method implies salting out certain classes of immunoglobulins with solutions of different ionic strengths at certain pH values and subsequent nephelometric measurement of the turbidity degree. It is known that at a certain degree of heterogeneity, each class of immunoglobulins consists of a complex of proteins with similar physicochemical properties. In particular, each class shows common features in folding of the molecular tertiary and quaternary structure based on a certain set of light and heavy chains, they have a certain sedimentation constant, molecular weight. A certain class of immunoglobulins has a specific set of heavy chains, which unites them into several classes.

The method of discrete deposition of immunoglobulins according to Zh.Badin and F.Rousellet (1964) modified by E.G. Larsky and N.P. Kravchenko (1968) with additions by M.A. Kostyna (1983) [8] found its application in veterinary laboratory diagnostics as well as in research work.

For the analysis, working solutions were prepared under the conditions of a clinical and biochemical laboratory using analytical grade reagents and analytical balances VL-220S, in strict accordance with the procedure. Measuring the concentration of immunoglobulins was carried out by using a KFC-3 photoelectric colorimeter at a wavelength of 450 nm. In the blood serum of one-day-old calves, the content of total protein was also determined by the Biuret method, albumin – by the photometric method with bromocresol green. Statistical data processing was carried out by using the Microsoft Excel package. The significance of differences was assessed by the method of paired comparisons using the Student's t-test, and a significance was set at $p < 0.05$.

Results and discussion

Considering the data obtained, a significant difference can be noted in the content of all studied serum immunoglobulin classes in the cows with subclinical ketosis. Analyzing the data presented in table 1, it can be noted that the concentration of IgG, IgM and IgA in cows with ketonuria was lowered by 19.1, 23.5 and 22.3% ($p < 0.05$), respectively, in comparison with cows of the control group. Metabolic disturbances in the body of pregnant cows,

TABLE 1. CONCENTRATION OF IMMUNOGLOBULINS IN THE BLOOD SERUM OF COWS, CALVES AND COLOSTRUM SECRETIONS

Study groups	Investigated indicators	Immunoglobulin content:		
		in the blood serum of cows (before calving)	in colostrum secretion	in the blood serum of calves (one day after drinking colostrum)
Group 1	IgG, g/l	14.71±0.51	36.88±1.83	13.86±0.47
	IgM, g/l	2.51±0.19	7.16±0.34	2.02±0.16
	IgA, g/l	1.67±0.14	3.12±0.29	1.92±0.09
Group 2	IgG, g/l	18.19±0.61	48.32±2.44	17.70±0.47
	IgM, g/l	3.28±0.13	10.91±0.55	2.79±0.18
	IgA, g/l	2.15±0.12	4.15±0.29	2.60±0.14

TABLE 2. CONCENTRATION OF TOTAL PROTEIN, ALBUMIN AND GLOBULINS IN CALVES

Investigated indicators	Group 1	Group 2
Total protein, g/l	49.77±1.00	56.18±1.22
Albumins, g/l	25.48±0.79	26.71±1.35
Globulins, g/l	24.29±0.32	29.47±0.95

associated with the ketone hyperproduction had a negative effect on antibody production. As a result, the colostrum secretion of cows with subclinical ketosis showed a decrease in all studied immunoglobulin classes by 23.7–34.4% ($p < 0.05$). To the greatest extent, the difference was observed for serum and colostrum IgM of cows, because proteins of this class are characterized by a short half-life period. It can be assumed that under conditions of pathologically altered metabolism, the production of antibodies in response to natural antigenic stimuli from the external environment is impaired. A decrease in the concentration of immunoglobulins in colostrum secretions influenced their blood levels in calves. As our studies have shown, the blood serum of calves born to cows with subclinical ketosis had level of IgG, IgM and IgA lowered by 21.7, 27.6 and 26.2%, respectively, compared to calves of the control group ($p < 0.05$). It was found that ketosis has the least effect on the decrease in the serum and colostrum content of class G immunoglobulins of cows and in the blood serum of calves born to them. The unevenness of the degree of reduction for different immunoglobulin classes in the studied biological fluids may result from different half-life periods for these proteins [10].

While studying the concentration of total protein, albumin and globulins in the blood serum of newborn calves one day after drinking colostrum (Table 2),

there may be noted quantitative and qualitative differences in the protein spectrum. Thus, the concentration of total protein in calves of the first group was significantly lowered by 11.4% compared to the control group. It might be due to the proteins of the globulin fraction (by 17.6%, $p < 0.05$), while the difference in the albumin concentration in calves was not significant ($p > 0.05$). Most of the serum albumin is synthesized directly in the liver of the calf, so that only a small amount is supplied with colostrum. Therefore, differences in the colostrum immunoglobulin profile do not affect the serum albumin content in newborn calves. The subclinical ketosis that develops at the end of pregnancy does not significantly affect the synthesis of albumin in the fetal liver.

Thus, subclinical ketosis that develops in cows before calving affects colostrogenesis in the mammary gland, suppressing the formation of immunoglobulins and their inclusion in colostrum. A decrease in the content of immunoglobulins in colostrum secretion leads to decreased colostrum immunity in calves. Subclinical ketosis in mother cows has a significant effect on the serum content of globulins in calves, but virtually does not affect the albumin concentration, which level depends on their synthesis in the liver, but almost does not depend on the protein spectrum of colostrum secretion.

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ИММУНОБИОЛОГИЧЕСКИЕ СВОЙСТВА ВАГИНАЛЬНОГО СЕКРЕТА У ЗДОРОВЫХ И БОЛЬНЫХ МИКОПЛАЗМОЗОМ КОРОВ

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Резюме. Генитальный микоплазмоз у коров — заболевание, сопровождающееся латентным течением, что затрудняет его своевременную диагностику и назначение адекватной терапии. Неспецифическая симптоматика микоплазмоза в совокупности с периодами бессимптомного течения приводит к развитию функциональных и морфологических изменений в органах половой системы коров, заканчивающихся в итоге бесплодием. Мониторинговые исследования животноводческих хозяйств Северо-Западного региона РФ показали, что инфицированность гениталий коров микоплазмами может составлять от 20% до 40% поголовья. Причем прослеживается четкая зависимость между высокой инфицированностью поголовья и низкими показателями воспроизводства. В связи с этим животноводческие предприятия несут существенный экономический ущерб. Одним из важных механизмов, предотвращающих внедрение различных патогенов в репродуктивный тракт, является резистентность слизистой оболочки влагалища. Среди этих факторов наиболее важная роль отводится вагинальной аутофлоре, десквамации эпителия, фагоцитозу, кислотности вагинального секрета, содержанию в нем иммуноглобулинов, лизоцима и ряду других неспецифических факторов защиты. Нами было проведено изучение изменения иммунобиологических характеристик вагинального секрета при субклиническом течении генитального микоплазмоза. Для исследования были отобраны здоровые коровы с отрицательным PCR-тестом на *Mycoplasma* spp. и инфицированные *Mycoplasma* spp., без клинических признаков вагинита. У обеих групп животных проводили определение в вагинальном секрете концентрации водородных ионов, активности лизоцима и иммуноглобулинов классов IgG, IgM, IgA и sIgA. Несмотря на то, что персистенция микоплазм во влагалище не сопровождается выраженными клиническими признаками вагинита, но состояние факторов защиты слизистой оболочки претерпевает значительные изменения. Проведенные исследования позволили установить, что длительное нахождение микоплазм во влагалище коров проявляется достоверным увеличением концентрации водородных ионов на 41% и снижением лизоцимной активности вагинального секрета в 2 раза, то есть наблюдается угнетение основных факторов предотвращающих колонизацию слизистой патогенной микрофлорой. Кроме того, происходит перераспределение классов иммуноглобулинов в вагинальном секрете. Это проявляется достоверным увеличением содержания IgM и sIgA на фоне тенденции к снижению IgA. Отмеченные изменения в совокупности создают благоприятные условия для внедрения секундарной микрофлоры и развитию бактериально-микоплазменных вагинитов, усугубляющих морфофункциональные изменения в репродуктивном тракте и повышающих риск возникновения бесплодия.

Ключевые слова: коровы, иммуноглобулины, вагинальный секрет, микоплазмоз, лизоцим

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IMMUNO-BIOLOGICAL PROPERTIES OF VAGINAL DISCHARGE IN HEALTHY AND MYCOPLASMOSIS-INFECTED COWS

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Abstract. Genital mycoplasmosis in cows is a disease accompanied by a latent course, which complicates its timely diagnosis and the appointment of adequate therapy. The nonspecific symptomatology of mycoplasmosis, combined with periods of asymptomatic course, leads to the development of functional and morphological changes in the organs of the reproductive system of cows, resulting in infertility. Monitoring studies of livestock farms in the North-West region of the Russian Federation have shown that infection of the genitals of cows with mycoplasmas can be from 20 to 40% of the livestock. Moreover, there is a clear relationship between the high infection rate of the livestock and low reproduction rates. In this regard, livestock enterprises incur significant economic damage. One of the important mechanisms preventing the introduction of various pathogens into the reproductive tract is the resistance of the vaginal mucosa. Among these factors, the most important role is assigned to vaginal autoflora, epithelial desquamation, phagocytosis, acidity of vaginal secretions, the content of immunoglobulins, lysozyme and a number of other nonspecific protective factors in it. We studied the changes in the immuno-biological characteristics of vaginal secretions in the subclinical course of genital mycoplasmosis. Healthy cows with a negative PCR test for *Mycoplasma* spp. were selected for the study. and infected with *Mycoplasma* spp., without clinical signs of vaginitis. In both groups of animals, the concentration of hydrogen ions, the activity of lysozyme and immunoglobulins of the classes IgG, IgM, IgA and sIgA were determined in the vaginal secretions. Despite the fact that the persistence of mycoplasmas in the vagina is not accompanied by pronounced clinical signs of vaginitis, but the state of protective factors the mucous membrane undergoes significant changes. The conducted studies made it possible to establish that the long-term presence of mycoplasmas in the vagina of cows is manifested by a significant increase in the concentration of hydrogen ions by 41% and a decrease in the lysozyme activity of vaginal secretions by 2 times, that is, inhibition of the main factors preventing the colonization of mucous pathogenic microflora is observed. In addition, there is a redistribution of classes of immunoglobulins in the vaginal secretion. This is manifested by a significant increase in the content of IgM and sIgA against the background of a tendency towards a decrease in IgA. The noted changes in aggregate create favorable conditions for the introduction of secondary microflora and the development of bacterial-mycoplasma vaginitis, aggravating morpho-functional changes in the reproductive tract and increasing the risk of infertility.

Keywords: cows, immunoglobulins, vaginal secretions, mycoplasmosis, lysozyme

Introduction

In recent years, the attention of veterinary specialists has increasingly been drawn to diseases that are not accompanied by specific clinical manifestations and characterized by a long latent course. These features complicate early diagnostics and, as a result, the timely administration of adequate therapy. As a result, irreversible functional and morphological changes develop in the body, leading to the premature exclusion of animals from economic use, causing significant economic damage to livestock enterprises. One of such diseases is the genital form of mycoplasmosis [3, 8]. It is known that long-term mycoplasma persistence in the genitourinary system has a negative effect, including the reproductive function of animals [6, 7]. Genital mycoplasmosis can manifest not only by chronic urethritis and vaginitis, but also by infertility, fetal mortality, and early abortion. Quite often, farm veterinarians find no connection between these manifestations and mycoplasmosis. A study

on the major genital infections provided a negative result, and a biochemical blood test indicates a good metabolic status in animals. As a result, mycoplasmas continue to persist in the herd, increasing the number of infected animals. Monitoring of livestock farms in the North-West region of the Russian Federation showed that urogenital mycoplasmosis is widespread and shows a clear correlation with low reproduction rates [10].

The vaginal mucous membranes display a wide range of protective factors to resist invasion and reproduction of pathogenic microorganisms. Among them, a significant role is played by the vaginal microflora, desquamation of the epithelium, phagocytosis, acidity of the vaginal secretion and level of immunoglobulins, lysozyme and other nonspecific protective factors therein. Thus, the natural vaginal microbiota in combination with a certain range of hydrogen ions concentration prevents the penetration and reproduction of most pathogenic microorganisms. Immunoglobulins contained in vaginal secretions,

especially secretory immunoglobulin A (sIgA), prevent the adhesion of infectious agents to epithelial cells of the mucous membrane via formation of immune complexes, and also activate the complement system neutralizing the pathogen biological activity. Lysozyme hydrolyzes murein contained in the cell wall of many microorganisms.

Taking into account the features of the mycoplasma biology, which allows to escape from some arms of the immune response, it would be worth studying what changes are observed in the cow local immunity of the vaginal mucosa during their long-term persistence.

Materials and methods

The studies were carried out in JSC "Osminskoye" of the Slantsevsky district of the Leningrad region on non-pregnant cows aged 3–4 years old. There were formed 2 groups per 8 animals in each. The group 1 contained healthy cows with negative PCR test for *Mycoplasma* spp.; group 2 consisted of cows with a positive PCR test for *Mycoplasma* spp., without overt clinical signs of vaginitis. For the diagnosis of mycoplasmosis, the test system "MIC-COM", LLC "InterLabService", Russia was used. *M. bovis genitalium* was identified by the serological method in such animals. In both groups, vaginal discharge was collected from the walls of the vagina using a special spoon in the area of the cervix.

The concentration of hydrogen ions (pH), the activity of lysozyme, level of total immunoglobulins and their classes were determined in the samples. Concentration of hydrogen ions was measured by using the test system "Colpo-test pH" LLC "Biosensor AN", Russia. For this, the test strip was immersed in the vaginal sample for 2 seconds, removed, excess material was removed, and the result was assessed 15 seconds later. The level of immunoglobulins classes IgG, IgM, IgA and sIgA was determined by the method of radial gel immunodiffusion according to Mancini, using antisera against cattle immunoglobulins manufactured by Hangzhou Sunlong Biotech Co., Ltd. China. Lysozyme activity was determined by the nephelometric method according to V.G. Dorofeychuk method using a test culture of *Micrococcus lysodeikticus* strain No. 2665. The results obtained were statistically processed by using the SPSS 22.0 computer software.

Results and discussion

The studies carried out have established significant changes in the immuno-biological characteristics of vaginal discharge in cows with mycoplasmosis.

Thus, the concentration of hydrogen ions in the vaginal discharge of healthy cows ranged from 4.2 to 5.5 and averaged 4.65 ± 0.02 . In cows with genital mycoplasmosis, it ranged from 6.0 to 7.0 and averaged 6.56 ± 0.02 .

The study of the immunoglobulin class level in the vaginal discharge showed the multidirectional pattern of the changes. The concentration of IgG in healthy cows and cows with mycoplasmosis practically did not differ and reached to 0.19 ± 0.008 g/l and 0.183 ± 0.008 g/l, respectively. The IgM level in healthy cows was 0.039 ± 0.002 g/l, while in those infected with mycoplasma it was increased – 0.054 ± 0.003 g/l. The level of IgA in the vaginal secretion of healthy animals was 0.024 ± 0.003 g/l, while in animals with mycoplasmosis it was slightly decreased – 0.018 ± 0.001 g/l. The data on the level of secretory immunoglobulin A were as follows: 0.067 ± 0.005 g/l – in healthy cows and 0.096 ± 0.007 g/l – in infected cows. It should be noted that the total level of immunoglobulins in the vaginal discharge of healthy and mycoplasma-infected cows did not differ significantly – 0.32 g/l and 0.351 g/l, respectively.

Assessing the lysozyme activity in vaginal discharge showed that in healthy animals it was $11.71 \pm 0.41\%$, whereas in animals with mycoplasmosis it was significantly lowered – $5.86 \pm 0.33\%$.

Although the persistence of mycoplasmas in the vagina was not accompanied by pronounced clinical signs of vaginitis, the state of the mucous protection underwent significant changes [2, 5].

The concentration of hydrogen ions in vaginal secretions in cows infected with mycoplasmas increased by 41% ($p < 0.001$) and became close to neutral pH value. Such a shift in pH value exerted a suppressive effect on the vaginal microflora and, at the same time, was more favorable for pathogenic microorganisms. As a result, an environment is created in the vagina promoting to development of secondary bacterial vaginitis.

The study of the immunoglobulin level showed that its total amount in the vaginal discharge in healthy and mycoplasma-infected cows was virtually similar suggesting that they do not actively migrate through the histohematogenous barrier. However, the immunoglobulin class composition was markedly altered. The level of IgG in the discharge of sick animals was by 0.007 g/l lower than in healthy animals, which was insignificant ($p > 0.05$). Such changes indicate that the persistence of mycoplasmas in the vaginal mucosa does not trigger the opsonization mechanism nor activate the complement system. The decrease in IgA level by 25% observed in cows with mycoplasmosis was also insignificant. Along with this, the sIgA level in infected animals was higher by 43% than in healthy cows, and such changes had a high degree of significance ($p < 0.001$). Since secretory immunoglobulin A prevents the adhesion of pathogens on the mucous membranes and stimulates the phagocytic activity of macrophages and neutrophils, an increase in its concentration indicates potentiated protection of the mucous membranes

from their further colonization by mycoplasmas [9]. A slight decrease in the amount of IgA is probably associated with its transport into epithelial cells to form sIgA. Changes in the concentration of class A immunoglobulins of similar nature on some mucous membranes of cows with mycoplasmosis were also noted elsewhere [1, 4]. While studying the content of IgM, its significant ($p < 0.01$) increase by 38.5% in the vaginal discharge of mycoplasma-infected cows was established. Since IgM is produced first in response to contacting any antigen, long-term maintenance of its high concentration suggest about systematic mycoplasma antigenic stimulation of the vaginal mucosa. The antigenic polymorphism of the pathogen can contribute to long-term stimulation, and the high homology to the structural proteins of the animal host allows it to evade the pre-synthesized antibodies.

While studying the lysozyme activity of vaginal discharge, it was found that in cows infected with mycoplasmas, it was lowered by 2-fold compared to healthy animals. It might be due to the fact that the

structure of the mycoplasma cytoplasmic membrane differs significantly from the structure of the bacterial cell wall, destruction of which is directed by lysozyme, as well as the possible production of metabolites that suppress the activity of lysozyme-producing cells.

Thus, the persistence of mycoplasmas in the vaginal mucous membrane of cows causes multidirectional changes in protective properties. This is manifested by a significant increase in the concentration of hydrogen ions and decreased lysozyme activity of vaginal discharge, i.e., inhibition of the main factors preventing the colonization of the mucous layers by pathogenic microflora is observed. In addition, immunoglobulin classes were redistributed in the vaginal discharge. Such changes along with favorable conditions were generated for invasion of secondary microflora and the development of bacterial-mycoplasma vaginitis. The established changes will allow to outline a rational protocol for treatment of cows with genital mycoplasmosis.

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РОЛЬ МИТОХОНДРИАЛЬНОЙ ПОРЫ В НЕТОЗЕ НЕЙТРОФИЛОВ ЧЕЛОВЕКА

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Нейтрофилы образуют нейтрофильные внеклеточные ловушки (НЕТ) как средство борьбы с патогенами в очаге воспаления. Однако избыточное образование НЕТ является причиной развития многих аутоиммунных и воспалительных заболеваний. Индукция НЕТ специфическими рецепторами ведет к повышению концентрации $[Ca^{2+}]_i$. В связи с этим применение кальциевого ионофора A23187 можно рассматривать как модель образования НЕТ. Известно, что индукция НЕТ различными стимулами зависит от образования активных форм кислорода (АФК) NADPH-оксидазой. Однако образование НЕТ, индуцированное кальциевыми ионофорами, происходит с участием митохондриальных АФК (мтАФК) или без АФК вообще.

В работе был применен широкий ингибиторный анализ. Оценка окислительного взрыва проводилась с помощью регистрации люминолзависимой хемилюминесценции, а образование НЕТ оценивалось методом флуоресцентной микроскопии.

С использованием митохондриально-направленного антиоксиданта SkQ1 было показано, что мтАФК участвуют в образовании НЕТ и окислительном взрыве, индуцированных A23187, но не активатором протеинкиназы C ФМА. Используя специфические ингибиторы, мы показали, что АФК, образованные NADPH-оксидазой, также участвуют в A23187-индуцированном НЕТозе. Таким образом, нами было впервые показано, что повышение Ca^{2+} индуцирует взаимодействие двух источников образования АФК, NADPH-оксидазы и митохондрий. Один из механизмов образования мтАФК, опосредованного повышением $[Ca^{2+}]_i$, может быть связан с открытием митохондриальной поры, mPTP.

Применив специфические ингибиторы поры, мы впервые показали, что mPTP участвует в передаче сигнала от митохондрий к NADPH-оксидазе, в образовании мтАФК, НЕТозе и окислительном взрыве, индуцированных A23187.

ОПРЕДЕЛЕНИЕ УРОВНЯ ИНТЕРФЕРОНА-ГАММА КАК КРИТЕРИЯ ТЯЖЕСТИ ГЕРПЕСВИРУСНОЙ ИНФЕКЦИИ 6-ГО ТИПА У ДЕТЕЙ ПЕРВЫХ ТРЕХ МЕСЯЦЕВ ЖИЗНИ

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Внутриутробная герпесвирусная инфекция является одной из важнейших медико-социальных проблем неонатологии. Один из важнейших механизмов, посредством которого иммунокомпетентные клетки участвуют в защите от инфекции, вызванной вирусом герпеса, связан с продукцией интерферона- γ (IFN γ).

Цель. Изучение содержания IFN γ при тяжелой форме инфекции, вызванной вирусом герпеса 6-го типа (HHV-6), у новорожденных и в динамике заболевания как критерия тяжести заболевания.

Материалы и методы. Обследовано 58 новорожденных с инфекцией, вызванной HHV-6. Контрольную группу составили 31 здоровый новорожденный. Уровень IFN γ в сыворотке крови определялся с помощью набора реагентов для твердофазного иммуноферментного анализа ProCon If gamma (ООО «Протеиновый контур», Санкт-Петербург, Россия), на 1-м месяце жизни и в возрасте 3 месяцев.

Результаты. У детей с тяжелой формой HHV-6 на 1-м месяце жизни отмечалось статистически значимое снижение уровня IFN γ в сыворотке крови (0,8 (0,6-0,9) пг/мл) по сравнению с группой контроля (1,04 (0,95-1,5) пг/мл) ($p < 0,00002$). В динамике заболевания в возрасте 3 месяцев отмечалось статистически значимое ($p < 0,05$) повышение исследуемого параметра по сравнению с 1-м месяцем жизни, однако по-прежнему отсутствовали статистически значимые различия с контрольной группой (0,9 (0,6-1,4) и 2,2 (1,6-2,8) пг/мл соответственно).

Заключение. Снижение продукции IFN γ при тяжелой форме HHV-6 у новорожденных, сохраняющееся до 3 месяцев, свидетельствует о дефиците IFN γ и может рассматриваться как показание для длительной интерферон-заместительной терапии.

К 70-ЛЕТИЮ СО ДНЯ РОЖДЕНИЯ ЛЮДМИЛЫ ВИКТОРОВНЫ ГАНКОВСКОЙ



Людмила Викторовна Ганковская поступила на медико-биологический факультет 2-го МОЛГМИ им. Н.И. Пирогова в 1968 году. В 1974 году она пришла на первую в стране кафедру иммунологии, созданную академиком Р.В. Петровым, где выполнила и защитила дипломную работу. Иммунология в эти годы начала активно развиваться и стала одной из ключевых дисциплин медицины. Именно с этой уникальной увлекательной наукой связана вся последующая научная и педагогическая деятельность Людмилы Викторовны. В 1977 году она закончила аспирантуру и защитила кандидатскую диссертацию по теме: «Генетический контроль иммунного ответа на туберкулин у мышей». Людмила Викторовна прошла путь от ассистента до доцента и профессора кафедры. В 1983 году Л.В. Ганковская защитила докторскую диссертацию по теме: «Цитокиновая регуляция функциональной активности макрофагов; новые подходы к цитокинотерапии».

Ее учителем и соратником все эти годы был заведующий кафедрой профессор Л.В. Ковальчук, воспитавший не одно поколение отечественных иммунологов. В 2012 году Л.В. Ганковская возглавила кафедру иммунологии. Все эти годы она сохраняет традиции, заложенные основателями кафедры.

В настоящее время на кафедре создан профессиональный коллектив, состоящий из высококвалифицированных преподавателей фундаментальной и клинической иммунологии, включающий 10 профессоров — докторов медицинских наук, 12 доцентов — кандидатов медицинских наук, 7 ассистентов, прошедших обучение в ординатуре и в аспирантуре. За эти годы расширилось преподавание дисциплины иммунологии в РНИМУ им. Н.И. Пирогова. Помимо МБФ, ЛФ и ПФ, иммунология стала преподаваться на стоматологическом и международном факультетах. При непосредственном участии Людмилы Викторовны созданы новые элективные курсы по иммуногенетике, персонализированной медицине, разрабатывается учебный план по молекулярной иммунологии. Кафедра является ведущей в стране, разработаны 24 программы по иммунологии для всех факультетов, включая программы ординатуры и аспирантуры. Л.В. Ганковская является одним из авторов учебника «Клиническая иммунология и аллергология с основами общей иммунологии», ряда учебно-методических пособий, 3 монографий. Она читает полный курс лекций для студентов МБФ. Педагогическую деятельность Л.В. Ганковская успешно сочетает с научной работой: в течение 7 лет она была одним из руководителей приоритетного направления развития Университета «Персонализированная медицина». В рамках этого направления создан НКЦ по персонализированной медицине. Под руководством Л.В. Ганковской проводятся исследования по изучению роли механизмов врожденного иммунитета в патогенезе социально-значимых заболеваний человека (аллергии, аутоиммунные заболевания, вирусные и бактериальные инфекции и др.). Сфера научных интересов — молекулярная иммунология, иммунология репродукции, персонализированная иммунотерапия. Под руководством Л.В. Ганковской защищено более 30 кандидатских диссертаций, 2 докторские диссертации. Она является автором более 400 научных работ и более 25 патентов.

Л.В. Ганковская активно участвует в работе российских и международных конференций, является организатором круглых столов по преподаванию иммунологии, членом 3 защитных советов, экспертом РНФ, ВАК.

Л.В. Ганковская награждена Почетной грамотой Министерства здравоохранения Российской Федерации за заслуги в области здравоохранения и многолетний добросовестный труд, нагрудным знаком «Отличник здравоохранения», а также грамотой Министерства образования.

Редколлегия журнала «Медицинская иммунология» поздравляет Людмилу Викторовну с юбилеем и желает ей крепкого здоровья, счастья, долгих лет жизни, оптимизма, дальнейших творческих и трудовых успехов!

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В журнал принимаются следующие виды публикаций:

Оригинальная статья

Статья должна описывать результаты законченного исследования. Допускается объем статьи до 20 машинописных страниц, включая рисунки, таблицы. Статья должна содержать: 1) введение; 2) материалы и методы; 3) результаты исследований; 4) обсуждение результатов; 5) благодарности.

- **Введение** содержит обоснование цели и задач проведенного исследования.
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Ссылки на литературные источники в тексте статьи, в рисунках и таблицах обозначаются арабскими цифрами в квадратных скобках [1, 2, 3,...]. не допускаются ссылки на диссертации, авторефераты диссертаций, публикации в сборниках, методические документы местного уровня. Количество источников не ограничено. В каждой ссылке приводятся все авторы работы. Неопубликованные статьи в список не включаются.

Обозначения, сокращения и единицы измерения

Для сложных терминов или названий, наиболее часто используемых в тексте статьи, можно ввести (в круглых скобках после первого упоминания полного названия термина) не более 3–5 нетрадиционных сокращений. Узаконенные международными номенклатурами сокращения используются в соответствующей транскрипции. Например, для термина «интерлейкин» используется сокращение «IL», а не русскоязычный вариант «ИЛ»; аналогично этому используются сокращения: «TNF», а не «ТНФ» или «ФНО»; «CD», а не «СД». Названия микроорганизмов приводятся в оригинальной транскрипции с использованием курсива (*E. coli*, *Streptococcus pyogenes*). Единицы измерения приводятся без точки после их сокращенного обозначения (с, ч, см, мл, мг, kDa и т.д.), регламентированного международными правилами.

Оформление иллюстративного материала

Иллюстративный материал должен быть оригинальным, то есть ранее нигде не опубликованным. Общее количество иллюстраций (таблиц и рисунков) не должно превышать восьми. При большом количестве иллюстраций их публикация оплачивается автором. Публикация цветных иллюстраций (независимо от их количества) также оплачивается автором. Весь иллюстративный материал присылается в двух экземплярах и на диске в виде отдельных файлов.

Размеры иллюстраций:

- максимальная высота — 210 мм
- максимальная ширина для 1 столбца — 82 мм, для 2 столбцов — 170 мм

Таблицы. Каждая таблица печатается на отдельном листе (в отдельном файле на диске) через 2 интервала. Нумерация таблиц дается арабскими цифрами отдельно от нумерации рисунков (графиков и фотографий). Название печатается над таблицей. Весь текст на русском языке, содержащийся в таблице, включая единицы измерения, должен быть переведен на английский язык; при этом перевод следует помещать в ячейку с соответствующим русским текстом отдельной строкой. Название таблицы и текст примечания к ней также должны быть

переведены на английский язык и приведены под русским текстом с новой строки. Для пометок в таблицах следует использовать одну или несколько (*). Пояснения печатаются после соответствующего количества (*) под таблицей. Единицы измерения, при необходимости, включаются в заголовки строк или столбцов.

Рисунки (графики и фотографии). В тексте статьи названия рисунков (графиков, фотографий) и таблиц размещаются сразу после абзаца, где на них дается первая ссылка. Все рисунки нумеруются последовательно арабскими цифрами по мере их использования в тексте статьи. Названия рисунков и подписи к ним выносятся в виде списка на отдельную страницу. В списке указываются: номер рисунка, название (с большой буквы), текст примечаний (для микрофотографий должно быть указано увеличение). Подписи к рисункам даются краткие, но достаточно информативные. Названия рисунков и примечаний к ним, нарисовочные подписи, текст легенды должны быть переведены на английский язык и размещены под соответствующим текстом с новой строки. На обороте каждой иллюстрации подписывается фамилия первого автора, название статьи и порядковый номер. Для публикации в журнале принимаются только оригиналы фотографий (не ксерокопии) хорошего качества, максимально приближенные к вышеуказанным размерам. Фотографии не должны иметь больших полей, т. е. фотографический материал должен занимать всю площадь фотографии. Рисунки могут быть представлены в графических форматах с расширением .tiff (разрешение не менее 300 dpi при 100% масштабе), .eps или .ai. Изображения, встроенные в документы Word, не принимаются. Графики и диаграммы предоставляются вместе с таблицами, на основе которых они были созданы, или с численными обозначениями показателей, отображаемых соответствующими графическими элементами (столбиками, секторами и т.п.) в виде файлов с расширениями .doc или, предпочтительнее, .xls.

Плата за публикацию статей

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Подготовка статей

Для представления статьи авторы должны подтвердить нижеследующие пункты. Статья может быть отклонена, если она им не соответствует.

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- В. Файл отправляемой статьи представлен в формате .doc, .docx, .rtf.
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 - Название учреждения, где работает ответственный автор (в русском и официально принятом английском вариантах).
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 - Полное название статьи, направляемой в редакцию.
 - Количество страниц текста, количество рисунков, количество таблиц.
 - Указать, для какого раздела журнала предназначена работа: оригинальные статьи, лекции, обзоры, «точка зрения», краткие сообщения, новые иммунологические методы, случаи из практики, дневник иммунолога, книжное обозрение.
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 - сокращенное название статьи для верхнего колонтитула (не более 35 символов, включая пробелы и знаки препинания) (на русском и английском языках);
 - не менее 6 ключевых слов на русском и английском языках;
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 - 5) Рисунки, если они есть - каждый отдельным файлом (при загрузке в систему каждому рисунку присваивается имя «Рисунок. Название рисунка (где название рисунка соответствует содержащемуся в файле рисунку. Порядковый номер рисунка»)
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Порядковый номер ссылки	Авторы, название публикации и источника, где она опубликована, выходные данные	ФИО, название публикации и источника на английском	Полный интернет-адрес (URL) цитируемой статьи
Размещаются в таблице в алфавитном порядке, вначале русскоязычные, затем на языках с латинской графикой	Указывать по библиографическому стандарту, представленному выше	Официальное англоязычное название публикации и источника, где она опубликована - для русскоязычных статей. В редких случаях, когда не существует официальных англоязычных названий (это возможно для таких типов публикаций, как тезисы, книги и др.) - редакция просит предоставить их перевод, используя красный цвет шрифта. Для англоязычных публикаций и источников в этом столбце ставится прочерк	В том случае, если информация о статье не размещена на официальном сайте издания, допустимо использовать URL статьи со сторонних сайтов, в том числе системы www.e-library.ru

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