ИССЛЕДОВАНИЕ АДЪЮВАНТНЫХ СВОЙСТВ ПРЕПАРАТОВ, СОДЕРЖАЩИХ РЕКОМБИНАНТНЫЙ ГРАНУЛОЦИТАРНО-МАКРОФАГАЛЬНЫЙ КОЛОНИЕСТИМУЛИРУЮЩИЙ ФАКТОР ЧЕЛОВЕКА

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Резюме. Актуальность поиска новых вакцинных адъювантов растет вместе с ростом количества новых вакцинных препаратов, особенно созданных на основе белков либо нуклеиновых кислот. Известно, что некоторые цитокины обладают адъювантными свойствами. Представленная работа посвящена изучению адъювантной активности рекомбинантного гранулоцитарно-макрофагального колониестимулирующего фактора человека (rhGM-CSF) и конструкций на его основе. Ранее нами была разработана технология выделения и очистки rhGM-CSF, а также технология получения конъюгатов полиглюкин-спермидин с рекомбинантным rhGM-CSF. Для получения молекулярных конструкций на основе конъюгата rhGM-CSF использовали двуспиральную РНК. Для сборки конструкций соотношение компонентов рассчитывали таким образом, чтобы в одной дозе препарата содержалось 5-40 мкг белка с rhGM-CSF и 100 мкг двуспиральной РНК. Эффективность сборки молекулярной конструкции оценивали в 1%-ном агарозном геле по снижению подвижности двуспиральной РНК. Эффективность полученных адъювантов определяли измерением титров специфических антител в сыворотках мышей методом ИФА с использованием в качестве антигенов овальбумина либо рекомбинантного рецептор-связывающего домена поверхностного белка коронавируса SARS-CoV-2 (вариант B.1.617.2 (Delta)). В работе использовали самцов мышей линии BALB/c массой 16-18 г в количестве 100 особей. Иммунизацию проводили двукратно с интервалом 14 суток, внутримышечной инъекцией по 200 мкл на животное. Рекомбинантный рецептор-связывающий домен поверхностного белка коронавируса SARS-CoV-2 вводился в дозе 50 мкг на животное, овальбумин в двух дозах – 1 и 5 мкг на животное. В качестве положительного контроля использовали соответствующий антиген. В качестве отрицательного контроля – физиологический раствор. Показано, что максимальный эффект был достигнут при иммунизации конструкцией на основе конъюгата полиглюкин-спермидин с rhGM-CSF с двуспиральной РНК, использование в качестве адъюванта конъюгата без двуспиральной РНК так

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же приводило к усилению гуморального ответа. Использование нативного с рекомбинантным гранулоцитарно-макрофагальным колониестимулирующим фактором человека не привело к повышению показателей титров специфических антител. Таким образом, установлено, что rhGM-CSF в составе конъюгата с полисахаридом либо молекулярной конструкции обладал способностью усиливать гуморальный иммунный ответ на белковые антигены.

Ключевые слова: адъювант, ГМКСФ, иммунный ответ, S-белок, овальбумин, иммунизация

STUDY OF THE ADJUVANT PROPERTIES OF PREPARATIONS CONTAINING RECOMBINANT HUMAN GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR

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Abstract. The relevance of the search for new vaccine adjuvants is growing along with the increase in the number of current vaccine preparations, especially those developed on the basis of proteins. Some cytokines are known to exert adjuvant properties. The present work is devoted to the study of adjuvant activity of recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) and constructs based on it. Earlier, we developed a technology for isolation and purification of GM-CSF from the E. coli SG20050/p280_2GM producer strain, as well as a technology for conjugating polyglucin:spermidine complexes with rhGM-CSF. Double-stranded RNA was used to obtain molecular constructs on the basis of rhGM-CSF conjugate. To assemble constructs, the ratio of the components was calculated for one dose of the preparation to contain 5-40 μg of rhGM-CSF and 100 μg of double-stranded RNA. The effectiveness of the formation of molecular constructs was evaluated by dsRNA electrophoretic mobility shift in a 1% agarose gel. The effectiveness of the resulting adjuvants was determined in ELISA assays by measuring the titers of specific antibodies in mouse sera against ovalbumin or recombinant receptor-binding domain of the surface S protein of the severe acute respiratory syndrome coronavirus 2 (Delta variant (B.1.617.2). The experiments were carried out in 100 male BALB/c mice weighing 16-18 g. Mice were immunized twice, with a 14-day interval, by intramuscular injection of 200 μL per animal. Recombinant receptor-binding domain of the surface protein of SARS-CoV-2 was administered at a dose of 50 μg/animal, ovalbumin – at two doses – 1 μg or 5 μg/animal. Corresponding antigen was used as a positive control, a saline solution – as a negative control. It was shown that the maximum effect was achieved by immunization with a construct based on double-stranded RNA and rhGM-CSF conjugated to polyglucin-spermidine. The use of a conjugate without double-stranded RNA as an adjuvant also improved humoral response. The use of native rhGM-CSF did not increase the titers of specific antibodies. Thus, it was found that rhGM-CSF being a part of a polysaccharide conjugate or a molecular construct exerted an ability to enhance the humoral immune response to protein antigens.

Keywords: adjuvant, GM-CSF, immune response, S protein, ovalbumin, immunization

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Introduction

The relevance of the search for new vaccine adjuvants is growing along with the increase in the number of new vaccine preparations, especially those developed on the basis of proteins (subunit or peptide vaccines) or nucleic acids (RNA-, DNA-vaccines). Some cytokines are known to exert adjuvant properties [1, 14], which allows them to be considered as promising adjuvants of current vaccines.

Preventive vaccines based on GM-CSF elicited strong antitumor and antiviral immune responses in preclinical experiments [1, 10, 14]. However, in clinical studies, these effects were not always reproduced, moreover, at times they contradicted the

results of animal studies [15]. One of the reasons for such ambiguous results may probably be the rapid degradation of cytokine in the bloodstream and, as a consequence, the use of its high doses, which stimulate hematopoiesis, but at the same time inhibit the immune response [2, 9, 10]. In this regard, it is relevant to search for the ways to stabilize GM-CSF, which is possible, in particular, by its conjugation with various carriers or inclusion in the composition of corpuscular structures.

Earlier, an original system for depositing and transporting proteins was designed at the State Research Center of Virology and Biotechnology "Vector", which is a molecular construct containing yeast double-stranded RNA (dsRNA) in the central part, protected by an envelope of polyglucin-spermidine conjugate [8]. The introduction of dsRNA into the core of the construct, on the one hand, solves the problem of particles self-assembly, and on the other – potentiates the activity of the protein component due to immunomodulating activity of polynucleotide complex. Double-stranded RNAs used as the "core" of constructs were shown to be able to enhance the immunogenicity of vaccines [3, 7].

The aim of the present work was to obtain preparations containing recombinant human GM-CSF as part of conjugates or molecular constructs, and to study their adjuvant activity.

Materials and methods

Earlier, we developed a technology for the isolation and purification of GM-CSF from the *E. coli SG20050/p280_2GM* producer strain [5], as well as a technology for conjugating polyglucin: spermidine (PGS) complexes with rhGM-CSF [6], used herein. The resulting rhGM-CSF-PGS conjugates were analyzed using electrophoresis in 15% PAAG, the concentration of the protein within the conjugate was determined by the Lowry method.

To obtain molecular constructs based on rhGM-CSF-PGS conjugates, the substance of the Ridostin drug (Sodium salt of double-stranded ribonucleic acid, FSP R No. 002021/01 – 07 04 20090769-08), containing 21% of dsRNA (produced by the Institute of medical biotechnology of the State Research Center of Virology and Biotechnology "Vector" of Rospotrebnadzor) was used. To assemble constructs, the ratio of the components was calculated for one dose of the preparation to contain 5-40 μg of rhGM-CSF and 100 μg of dsRNA. The effectiveness of the formation of molecular constructs was evaluated by dsRNA electrophoretic mobility shift in a 1% agarose gel.

Adjuvant activity of the obtained conjugates and constructs was assayed in ELISA and evaluated by changing the titers of specific antibodies in blood sera of mice immunized with ovalbumin (OVA, cat. No. A5503-5G, Sigma) or receptor-binding domain (RBD) of the surface S-protein of the severe acute respiratory syndrome coronavirus 2 (Delta variant (B.1.617.2).

The experiments were carried out in 100 male BALB/c mice weighing 16-18 g. The animals were kept and manipulated in compliance with the principles of humane treatment of laboratory animals in accordance with Appendix A of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS № 123, Strasbourg, 1986). All the experimental procedures were approved by the Bioethics Committee of the State Research Center of Virology and Biotechnology "Vector" (SRC VB Vector/September 10, 2020, approved by the protocol of Bioethics Committee No. 5 as of October 1, 2020). BALB/c mice were immunized twice, with a 14-day interval, by intramuscular injection of 200 μL per animal. RBD was administered at a dose of 50 μg/animal, OVA – at a dose of 5 μg/animal, adjuvants – at a dose of 40 μg (GM-CSF). Corresponding antigen was used as a positive control, a saline solution – as a negative control.

On day 10 after the second immunization, blood samples were collected from the retro-orbital sinus of mice in a volume of 0.5 ml using a Pasteur pipette as described in [4]. Blood sera were obtained by standard methods and stored at (6 ± 2) °C for no more than 7 days. For longer storage, sera were frozen at -20 °C.

After blood sampling, mice were euthanized by cervical dislocation as described in [11]. Before the analysis, sera from 6 mice in the group were thawed and combined into one sample (total sera). The level of specific antibodies in the sera of immunized mice was determined by ELISA assays. In the 96-well plates, antigens (100 μL/well) were sorbed in phosphate-buffered saline (PBS) (pH 7.4) for 2 hrs at 37 °C and for 16 hrs at 4 °C. The unbound antigen solution was removed, and 200 μL of the blocking buffer (1% solution of bovine serum albumin (BSA) in PBS, pH 7.4) was added to the wells. The plates were incubated for 2 hrs at 37 °C, and then washed three times with 350 μL/well of PSBT (PBS supplemented with 0.05% Twin-20). 200 μL of PBS was added to each well of row A, and 100 μL of PSBT supplemented with 0.5% BSA – to each well of rows B – H. 12 samples of total sera of immunized animals were added to the wells of row A (1-12 strips), and the titration in the vertical rows was performed. To control the conjugate, a buffer dilution solution (single PBS (pH 7.4) supplemented with 0.05% Twin-20 and 0.5% BSA) was added to two wells, without sera samples. The plates were incubated at 310 rpm in a thermoshaker PST-60HL-4 for 1.5 hrs at 37 °C. After incubation, the wells were washed four times, as described above. Next, 100 μL of goat anti-mouse IgG-HRP antibodies conjugate

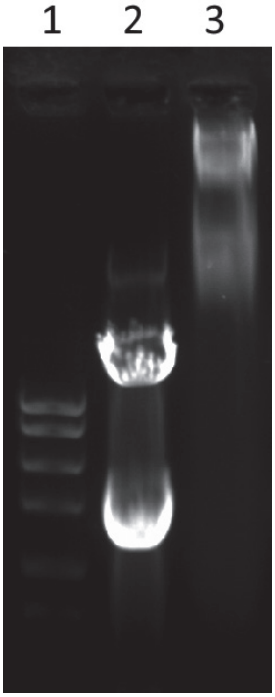

Figure 1. Electrophoresis of preparations containing dsRNA in 1% agarose gel, staining with ethidium bromide

Note. Lanes: 1, DNA ladder 250-2000 bp; 2, dsRNA (100 μg); 3, dsRNA (100 μg) incubated for 60 minutes with GM-CSF-PGS conjugate (40 μg of protein).

solution (Sigma, USA), diluted at 1:5000 in a buffer solution for dilution, was added to each well, and the plates were incubated at 310 rpm in a thermoshaker for 1 hr at 37 °C. The contents of the wells were then removed and washed five times, as described above. To produce a color reaction, the plates were coated with chromogen 3,3',5,5'-tetramethylbenzidine solution (a one-component ready-made solution), 100 μL per well, and incubated for 30 min at RT protected from direct light. The reaction was stopped by adding 50 μL of "stop reagent" (1M solution of sulfuric acid) to each well. Absorbance was measured at 450 nm using a Varioskan Lux multimode microplate reader (Thermo Fisher Scientific, USA). The results were processed using GraphPad Prism 6.0 software.

Results and discussion

The need to use adjuvants in current subunit vaccines is often associated with low immunogenicity or side effects at high doses. Therefore, ways to increase the immunogenicity of such vaccines remain urgent task in the development of new vaccine preparations.

We evaluated adjuvant properties of native GM-CSF and GM-CSF preparations. To stabilize GM-CSF, conjugates with a polysaccharide matrix (PGS complex) were synthesized according to [12],

TABLE 1. GEOMETRIC MEAN TITERS OF SPECIFIC ANTIBODIES AGAINST OVA IN THE BLOOD SERUM OF MICE AFTER A TWO-TIME IMMUNIZATION WITH OVA, OR OVA + GM-CSF PREPARATIONS

Preparation	OVA	OVA + GM-CSF	OVA + GM-CSF-PGS conjugate	OVA + (GM-CSF-PGS + dsRNA) construct
Dose, μg/mouse	5	5 μg (OVA) 40 μg (GM-CSF)	5 μg (OVA) 40 μg (GM-CSF)	5 μg (OVA) 40 μg (GM-CSF) 100 μg (dsRNA)
GMT	500	125 000	500 000	3 000 000
N		250	1 000	6 000

Note. GMT, geometric mean titer of specific antibodies against OVA; N, multiplicity of titers of antibodies against OVA in the blood serum of mice immunized with OVA combined with a preparation in comparison with OVA alone.

TABLE 2. GEOMETRIC MEAN TITERS OF SPECIFIC ANTIBODIES AGAINST RBD IN THE BLOOD SERUM OF MICE AFTER A TWO-TIME IMMUNIZATION WITH RBD, OR RBD + GM-CSF PREPARATIONS

Preparation	RBD	RBD + GM-CSF	RBD + GM-CSF- PGS conjugate	RBD + (GM-CSF-PGS + dsRNA) construct
Dose, μg/mouse	50	50 μg (RBD) 40 μg (GM-CSF)	50 μg (RBD) 40 μg (GM-CSF)	50 μg (RBD) 40 μg (GM-CSF) 100 μg (dsRNA)
GMT	3 000	625 000	1 000 000	15 625 000
N		208	333	5 208

Note. GMT, geometric mean titer of specific antibodies against RBD; N, multiplicity of titers of antibodies against RBD in the blood serum of mice immunized with RBD combined with a preparation in comparison with RBD alone.

and molecular constructs containing GM-CSF-PGS conjugate were obtained as in [6, 13].

The constructs based on dsRNA molecules and conjugate molecules assembled due to the formation of ionic bonds between positively charged spermidine and negatively charged dsRNA. Therefore, the effectiveness of the constructs formation was evaluated by dsRNA electrophoretic mobility decrease in 1% agarose gel as a result of formation of a polyglucin complex, which demonstrated in Figure 1.

The results of studying adjuvant properties of the obtained preparations (Table 1) indicate that a two-time immunization with OVA in combination with various adjuvants containing GM-CSF and dsRNA led to the appearance of specific antibodies in the blood of animals in titers from 125 000 to 3 000 000, while the values for immunization with OVA alone did not exceed 1:500.

The administration of GM-CSF in combination with OVA increased the titer of specific antibodies by 250 times, compared to the comparison group (OVA). The preparation of GM-CSF with PGS conjugate increased antibody titers by 1000 times compared to the mice immunized with OVA; the preparation of a molecular construct containing GM-CSF-PGS and dsRNA – by 6000 times.

Table 2 shows the values of geometric mean titers of specific antibodies against RBD protein. As one can see, the immunization of mice with RBD in combination with GM-CSF and dsRNA preparations led to a significant increase in titers of specific antibodies, similarly as in case with OVA. The GMT of antibodies after administration of GM-CSF increased by 208 times, GM-CSF-PGS conjugate – by 333 times. The administration of GM-CSF as part of a dsRNA construct led to even more pronounced stimulation. The titers of specific antibodies increased by 5208 times compared to RBD, by 15.6 and 25.0 times, compared to GM-CSF-PGS conjugate and GM-CSF, respectively.

Conclusion

Thus, the experimental data obtained confirm that GM-CSF preparations possess the ability to enhance humoral immune response to immunization with various antigens (OVA, RBD). The use of dsRNA as a component of the adjuvant construct additionally contributes to the effectiveness of vaccination.

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