ORIGINAL RESEARCH

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Obtaining and Characterization of the Monoclonal Antibodies Against G-Protein of the Respiratory Syncytial Virus

Natalia A. Demidova[1], Regina R. Klimova[1], Alla A. Kushch[1]✉, Ekaterina I. Lesnova[1], Olga V. Masalova[1], Anastasia D. Dorofeeva[2], Alexandra A. Nikonova[2], Natalia E. Fedorova[1], Vitaly V. Zverev[2,3]

[1]National Research Centre for Epidemiology and Microbiology named after the honorary academician N.F. Gamaleya, Moscow 123098, Russia;

[2]Mechnikov Research Institute for Vaccines and Sera, Moscow 105064 , Russia;

[3]I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow 127994, Russia

The **aim** of this study was to obtain hybridomas producing monoclonal antibodies (Mabs) to the G-protein of the respiratory syncytial virus (RSV), and to evaluate their immunological characteristics and virus-neutralizing activity.

Material and methods. Mouse Mabs were obtained using hybridoma technology. The properties of Mabs were studied by enzyme-linked immunosorbent assay (ELISA), immunofluorescence staining (IF) of infected cells, as well as by biological neutralization test *in vitro* (NT). To identify epitopes recognized by the Mabs on G protein ELISA additivity test was used.

Results. Hybridization of splenocytes with Sp2/0 myeloma cells and primary screening showed that 75 hybridomas produce antibodies interacting with purified virus, 17 of them also react with the recombinant G-protein in ELISA. In NT 4, hybridomas suppressed *in vitro* RSV infection by more than 50%. Cloning of these hybridomas revealed 4 monoclones producing the most active Mabs. Mab 1C11 was IgG2a, 3 others (5D4, 5G11 and 6H4) were IgM. Three IgM Mabs actively reacted with both RSV A2 and Long, and with G-protein; Mab 1C11 was less reactive with all antigens tested. All Mabs suppressed RSV infection, while Mab 5D4 supressed it almost completely (98%). IF analysis showed that all Mabs detected RSV G-protein in the cell cytoplasm, the largest number of infected cells was detected using Mab 5D4 (80%). It was shown that the isolated Mabs were directed to two non-overlapping epitopes on the RSV G-protein.

Conclusion. The isolated Mabs can be used to detect RSV in clinical samples by ELISA and IF. The isolated Mabs can be used for humanized recombinant antibodies construction and for the treatment of RSV infection in future.

Keywords: *respiratory syncytial virus; G-protein; monoclonal antibodies; ELISA; immunofluorescence staining of infected cells; non-overlapping epitopes.*

Acknowledgments. This work was supported by a grant from the Russian Science Foundation 17-15-01525.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Demidova N.A., Klimova R.R., Kushch A.A., Lesnova E.I., Masalova O.V., Dorofeeva A.D., Nikonova A.A., Fedorova N.E., Zverev V.V. Obtaining and Characterization of the Monoclonal Antibodies Against G-Protein of the Respiratory Syncytial Virus. *Zhurnal mikrobiologii, epidemiologii i immunobiologii = Journal of microbiology, epidemiology and immunobiology, Russian journal.* 2020; 97(1): 7–14.

DOI: https://doi.org/10.36233/0372-9311-2020-97-1-7-14

Received 3 December 2019
Accepted 18 December 2019

Получение и характеристика моноклональных антител к G-белку респираторно-синцитиального вируса

Демидова Н.А.[1], Климова Р.Р.[1], Кущ А.А.[1]✉, Леснова Е.И.[1], Масалова О.В.[1], Дорофеева А.Д.[2], Никонова А.А.[2], Федорова Н.Е.[1], Зверев В.В.[2,3]

[1]ФГБУ «Национальный исследовательский центр эпидемиологии и микробиологии имени почетного академика Н.Ф. Гамалеи» Минздрава России, 123098, Москва, Россия;

[2]ФГБНУ «Научно-исследовательский институт вакцин и сывороток имени И.И. Мечникова», 105064, Москва, Россия;

[3]ФГАОУ ВО «Первый Московский государственный медицинский университет им. И.М. Сеченова» Минздрава России (Сеченовский Университет), 127994, Москва, Россия

Цель работы состояла в получении гибридом, продуцирующих моноклональные антитела (МКА) к G-белку респираторно-синцитиального вируса (РСВ), и в изучении их иммунологических характеристик и вируснейтрализующей активности.

Материал и методы. Мышиные МКА получали с помощью гибридомной технологии. Свойства МКА изучали методами иммуноферментного анализа (ИФА), иммунофлюоресцентного окрашивания (ИФл) зараженных клеток культуры, а также в реакции биологической нейтрализации *in vitro* (РБН). Взаимное расположение эпитопов, выявляемых МКА на G-белке, определяли с помощью теста аддитивности в ИФА.

Результаты. Гибридизация спленоцитов с клетками миеломы Sp2/0 и первичный скрининг показали, что 75 гибридом продуцируют МКА, взаимодействующие с очищенным вирусом, 17 из которых реагируют также и с рекомбинантным G-белком в ИФА. В РБН 4 гибридомы подавляли РСВ-инфекцию *in vitro* более чем на 50%. Клонирование этих гибридом позволило выявить 4 моноклона, продуцирующих наиболее активные МКА. МКА 1С11 принадлежали к IgG2a, 3 других (5D4, 5G11 и 6H4) — к IgM. 3 МКА IgM активно реагировали как с РСВ А2 и Long, так и с G-белком, МКА 1С11 были менее активны со всеми антигенами. Все МКА подавляли РСВ-инфекцию, причем МКА 5D4 — практически полностью (98%). ИФл-анализ показал, что все МКА выявляли G-белок РСВ в цитоплазме клеток культуры, наибольшее количество зараженных клеток детектировали с помощью МКА 5D4 (80%). Полученные МКА направлены к двум неперекрывающимся эпитопам на G-белке РСВ.

Заключение. Полученные МКА могут использоваться для обнаружения РСВ в клиническом материале методами ИФА и ИФл. Активность в РБН создает предпосылки для получения на основе МКА гуманизированных рекомбинантных антител и возможность их использования для терапии РСВ-инфекции.

Ключевые слова: *респираторно-синцитиальный вирус; G-белок; моноклональные антитела; иммуноферментный анализ; иммунофлюоресцентное окрашивание зараженных клеток; неперекрывающиеся эпитопы.*

Источник финансирования. Работа выполнена при поддержке гранта РНФ 17-15-01525.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Для цитирования: Демидова Н.А., Климова Р.Р., Кущ А.А., Леснова Е.И., Масалова О.В., Дорофеева А.Д., Никонова А.А., Федорова Н.Е., Зверев В.В. Получение и характеристика моноклональных антител к G-белку респираторно-синцитиального вируса. *Журнал микробиологии, эпидемиологии и иммунобиологии*. 2020; 97(1): 7–14.
DOI: https://doi.org/10.36233/0372-9311-2020-97-1-7-14

Поступила 03.12.2019
Принята в печать 18.12.2019

Introduction

Acute respiratory infections are among the leading causes of morbidity in Russia. Childhood morbidity rates are on average 3.5-4 times as high as the rates among adults, and the number of respiratory cases among children keeps increasing. From 2004 to 2016, the number of acute respiratory infection cases increased by 33.4% [1].

Respiratory syncytial virus (RSV) is the most common pathogen identified in young children with acute lower respiratory infection (ALRI) [2]. Those at greatest risk of severe RSV cases include premature infants with a very low birth weight and extremely low birth weight, senior people (older than 65), and people with cardiovascular diseases and impaired immune system. Based on the data from the World Health Organization, globally, RSV affects an estimated 64 million people and causes 160 thousand deaths each year [3].

Palivizumab is currently the only approved agent for prevention of RSV infection; it is based on humanized monoclonal antibodies (MAbs) specific for the RSV F-protein. Palivizumab administration reduces the rate and severity of RSV infections. The efficacy of palivizumab administered to premature infants with bronchopulmonary dysplasia was confirmed by Russian researchers who conducted multicenter studies: Among the infants who were at high risk of severe RSV infection because of their premature birth, the hospitalization rate decreased to 0.3% after 3 injections of palivizumab [4]. However, Palivizumab does not prevent the risk of recurrent disease; the efficacy of Palivizumab is much lower when administered to mature newborn infants and older children; the antibodies having a higher binding affinity to the F-protein (motavizumab) caused allergies [5]. In addition, the preparation is too expensive for many people to afford. The emergence of resistant strains is another disadvantage of the F-protein specific antibody-based drugs [6].

Several approaches have been studied to overcome these drawbacks both to improve the F-protein-specific MAbs and to develop MAbs specific to other RSV proteins such as nucleoprotein (N), polymerase (L), surface G protein [7]. The G-protein is considered the most promising option; it is one of the two RSV proteins (along with the F-protein) that induce neutralizing antibodies. Animal studies showed that antibodies specific for the G-protein have a therapeutic effect, even in conditions where F-protein-specific antibodies are not efficient [8].

The purpose of this study was to develop RSV G protein specific MAbs and to study their immunological properties and virus-neutralizing activity.

Materials and methods

Two reference strains of RSV were included in this study (A2 and Long, subgroup A) from the collection of the Mechnikov Research Institute for Vaccines and Sera. The RSV A2 strains were cultured in a continuous HEp-2 cell line (a human, laryngeal cancer), in the RPMI medium containing 5% of fetal calf serum, glutamine and gentamicin (PanEco LLC, Russia), at 37°C and 5% CO_2. The RSV Long strains were cultured with

DOI: https://doi.org/10.36233/0372-9311-2020-97-1-7-14

a continuous MA-104 cell line (the embryonic rhesus monkey kidney) in the Eagle's MEM medium.

The viruses from the culture media (CM) of infected cells were concentrated and purified [9]. The virus titration was performed by using MA-104 cells, which are ideal for demonstration of the RSV classical cytopathogenic effect (formation of syncytia) that can be visualized under microscope.

The virus titer was calculated by using the Spearman-Kärber method. After the concentration, the RSV A2 titer was 9 lg of the tissue 50% cytopathic dose per 1 ml (TCD_{50}/ml); the PSV Long titer was 7 lg of TCD_{50}/ml. The protein concentration in the virus preparation was measured with the NanoDrop spectrophotometer (Thermo Scientific, USA) at the 280 nm wavelength against 20% sucrose or with the Bradford reagent (Sigma, USA) in accordance with the manufacturer's instructions.

The BALB/c mice (females of 18–20 grams in weight) received from the Pushchino breeding farm (Moscow Region) were immunized intraperitoneally 3 times at a 2-week interval: the 1st immunization — RSV with complete Freund's adjuvant; the other immunizations were performed with incomplete Freund's adjuvant (Sigma, USA). In each injection, the dose of RSV A2 was 100 µg/mouse. The studies were performed in compliance with the Guidelines for Care and Use of Experimental Animals[1].

Myeloma and hybridoma cells were cultured in the RPMI-1640 medium containing 4.5 g/l of glucose, 3 mM of glutamine, 60 µg/ml of gentamicin (PanEco LLC, Russia) with supplement of 20% fetal calf serum (Gibco, Invitrogene, USA) and 0.2 U/ml of insulin (Lilly, France). The cells were cultured in the CO_2-incubator at 37°C and 5% CO_2. The Sp2/0 mouse myeloma cells were used for fusion, as they do not produce their own immunoglobulins.

The suspension of mouse splenocytes was mixed with the Sp2/0 cells at 2:1 and diluted with PEG/DMSOSolution (Hybri-Max™, Sigma, USA). After the fusion, the cells were resuspended in the selective hypoxanthine-aminopterin-thymidine medium (Hybri-Max™, Sigma, USA), placed onto 96-well plates and incubated at 37°C and 5% CO_2. The cells were cultured in the selective hypoxanthine-aminopterin-thymidine medium for 14 days after the fusion. CL from the wells containing hybrid cells was tested for antibodies specific for RSV and the G-protein by using the enzyme-linked immunoassay (ELISA).

The ELISA test of RSV-specific antibodies in mouse blood sera, in CL from hybrid cells as well as MAbs was conducted in its indirect version: RSV in the amount of 50 µl and in concentration of 10 µg/ml in 0.1 M of the phosphate-buffered saline, pH 7.2 were added to each well of the 96-well MAXISORP NUNC-IMMUNO plates (Thermo Scientific, USA) for 24 hours at 4°C. After the washing with the phosphate-buffered saline combined with 0.1% Tween-20 solution, serially diluted mouse sera or CL from the wells with hybrids were added to the wells and incubated for 1 hour at 37°C. After the washing, peroxidase-labeled goat antibodies were added to the mouse immunoglobulins (Sorbent, Russia) in a dilution of 1:500. Tetramethylbenzidine hydrochloride was used as a substrate (US Biomedical, USA). The 1N sulfuric acid solution was used to inhibit the reaction. The optical density (OD) was measured with a Sunrise ELISA plate reader (Tecan, Switzerland) at the 450 nm wavelength, where the reference wave is 620 nm. The antibody titer was expressed as the inverse value of the highest dilution, where OD was 2 times as high as the ODs of negative controls — pre-immune mouse sera or CL from the wells not containing hybrid cells.

To analyze the RSV G-protein, recombinant G-protein (Sino Biological, Republic of Korea) RSV A2 was added to the wells of 96-well MAXISORP NUNC-IMMUNO plates (Thermo Scientific, USA) at a concentration of 0.5 µg/ml in the phosphate-buffered saline to be further incubated for 18 hours at 4°C. Then, CL from the wells with hybrid cells or MAbs was added for the ELISA test.

The Mab additivity test was conducted as previously described [10] and was used to determine whether MAbs recognize different or similar epitopes. The plates were sensitized with the concentrated purified RSV A2, as described above. Each pair of MAbs, in saturating concentrations selected during the previous tests, was added to separate wells or to the same well of the plate. The indirect ELISA was used to measure the quantity of virus-associated MAbs. The results were expressed as the additivity index (AI), which compares ODs obtained in both options (for a single MAb and for combined MAbs) under the standard reaction conditions. AI was calculated for each pair of MAbs by the following formula:

$$AI = \{[2 \times A1{+}2/(A1 + A2)] - 1\} \times 100\%,$$

where A1 and A2 are the OD of MAbs applied separately; A1+2 — the OD of the MAb combination. AI will tend toward zero, if both MAbs recognize the same epitope, and will tend toward 100%, if 2 epitopes are not linked topographically.

To conduct a reaction of biological neutralization *in vitro*, CL from the monoclones was incubated with RSV A2 (the multiplicity of infection 10^4 TCD_{50}/ml) for 2 hours at 37°C in the presence of 5% CO_2 and then was placed onto an MA-104 cell monolayer. One hour after, the treated cells were washed with a serum-free medium; then, the cell culture medium was added in combination with 2% fetal calf serum containing glutamine in double concentration. The cytopathogenic ef-

[1] Decree No. 266 of the Ministry of Health of Russia, 19/6/2003.

fect produced by the virus on the cells was assessed in 72–96 hours, when it developed in the infected, rather than in treated culture.

The infected cells were further used for indirect immunofluorescence assay (IFA). The MA-104 cells at a concentration of 4 × 10^4 cells/ml were placed into wells of 24-well plates with cover slips; on the next day, they were infected with RSV at a multiplicity of infection equal to 10^4 TCD_{50}/ml. Four days after the infection, the cells were fixed with methanol for 20 minutes at –20°C. The MAbs were applied onto the fixed preparations and incubated for 1 hour at 37°C. Then, they were washed with the phosphate-buffered saline before application of the FITC-labeled anti-mouse serum (Dako, Denmark). The cells were counterstained with Evans blue (Biochem, France). The staining was monitored with the Axio Scope A1 fluorescence microscope (Carl Zeiss, Germany) at the wavelength of 520–560 nm. The results were assessed by the number of the stained cells and were expressed with symbols: +++ corresponded to the number of stained cells >50%; ++ — 10–50%; + — single stained cells.

The MAb subtypes and types of light chains were identified by using the ELISA test and the Pierce Rapid ELISA Mouse mAb isotiping Kit (Thermo Scientific, USA) in accordance with the manufacturer's instructions: CL from MAbs was added to wells of the plate pre-coated with antibodies specific for mouse immunoglobulins of class G (IgG1, IgG2a, IgG2b, IgG3), IgA and IgM, as well as for subtypes of the kappa and lambda light chains. After the incubation, the polyvalent conjugate was added to peroxidase-labeled IgG+IgA+IgM to be followed by the tetramethylbenzidine hydrochloride substrate. The MAb specificity, together with the immunoglobulin subtype and light chain type, was identified by the peak signal.

Results

The analysis of the immune response in five mice to injection of the purified RSV A2 showed that the minimum activity of RSV-specific antibodies corresponded to the $4×10^{-5}$ titer, while the maximum activity corresponded to the $7.8×10^{-5}$ titer in ELISA. MAbs in blood sera of all mice neutralized the RSV infective activity in titers ranging from 1:40 to 1:160. Tests in cell hybridization were performed on 2 mice selected for their high activity in both reactions. The tests in fusion of splenocytes of the selected mice with myeloma cells produced 236 hybrid cultures stable to the selective hypoxanthine-aminopterin-thymidine medium. 7–14 days after, the CL-present MAbs were analyzed by using the ELISA test, which showed the presence of MAbs interacting with RSV A2 in 75 CL (32%) from hybrid cells. The analysis of CL from hybrid cells in ELISA with the G-protein showed that 17 (23%) out of 75 clones were reactive with the G-protein (**Fig. 1, *a***). CL from these hybridomes was studied in the reaction of biological neutralization *in vitro* (Fig. 1, b).

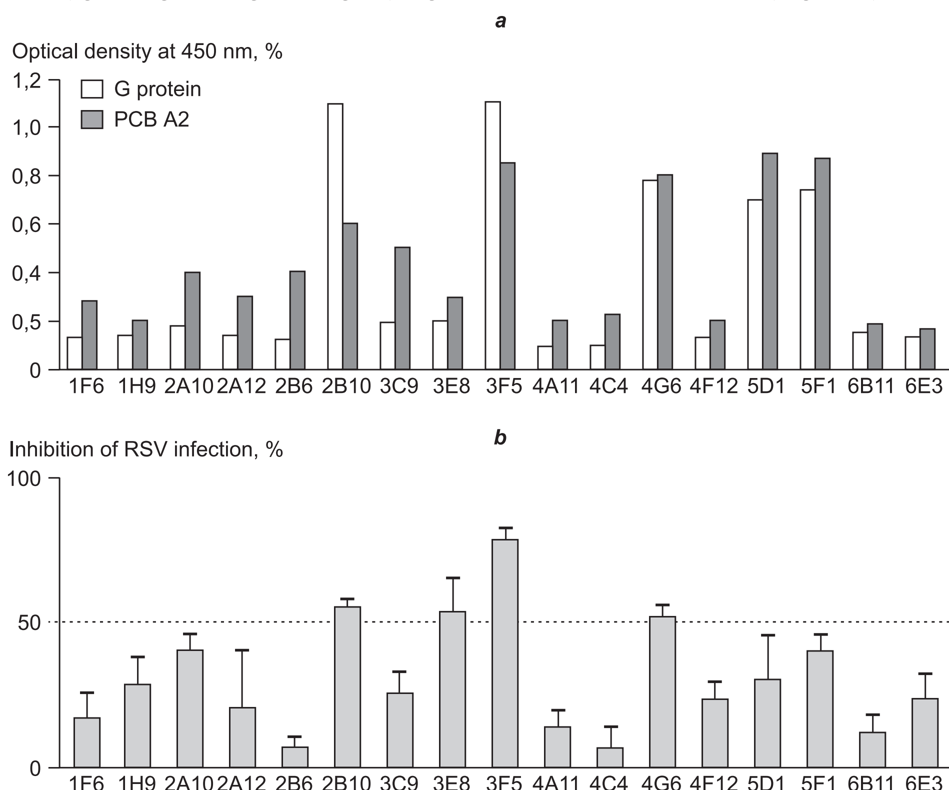

Fig. 1. The activity of the interaction of antibodies produced by hybrid cultures, with whole RSV and with RSV G protein.
a — interaction of antibodies in supernatants with whole RSV A2 virus and G protein in ELISA;
b — virus-neutralizing activity of supernatants from hybridomas producing antibodies to the G protein of RSV.

DOI: https://doi.org/10.36233/0372-9311-2020-97-1-7-14

Table 1. The properties of the Mabs to the RSV G protein

Mabs	Ig isotype, light chain type	ELISA: optical density with supernatants			Reaction of neutralization *in vitro*, % inhibition	Immunofluorescence assay**
		PCB A2	PCB Long	G protein		
1C11	Ig2a, κ	1,48	0,34	0,31	60	+
5D4	IgM, κ	2,50	2,39	2,02	98	+++
5G11	IgM, κ	2,30	2,58	2,36	60	++
6H4	IgM, κ	2,38	2,46	2,31	50	+

Note. *Optical density indicators in the analysis of Mabs in supernatants from hybridomas; Optical density of negative controls = 0.03; **Immunofluorescence assay — a comparative evaluation of the detection efficiency of infected cells infected with RSV A2 in the immunofluorescence reaction.

For the further cloning, we selected 4 hybrid cultures (2B10, 3E8, 3F5 and 4G6) interacting with the G-protein and neutralizing the RSV-infection by more than 50%. The cloning tests helped select one monoclone from each culture — 4 hybridomes producing MAbs specific for the G-protein. The immunological, immunocytochemical and biological characteristics of MAbs are given in **Table 1** and **Fig. 2**.

The subtypes of monoclonal immunoglobulins were identified. The MAb isotyping showed that MAbs 1C11 belonged to the IgG2a isotype, while the 3 other belonged to the IgM class. All the monoclonal Igs had a kappa-type light chain.

The ELISA results showed that MAbs 5D4, 5G11 and 6H4 demonstrated equal intensity at OD ≥ 2.0 in their reaction with their specific RSV A2 strain, the RSV Long strain and the RSV G-protein. The MAbs 1C11 interacted more actively with the RSV A2 than with the RSV Long and the G-protein. It should be pointed out that 3 out of 4 MAbs displayed significant virus-neutralizing activity, having suppressed the development of the RSV-infection by 60–98%.

All the received clones recognized RSV in infected cells, though with different activity (Table 1, **Fig. 2**). The IFA test showed that MAbs detected RSV G-proteins in the cell cytoplasm; most of the infected cells were detected with MAbs 5D4 and 5G11.

The topographic links between epitopes were identified by the additivity test (**Table 2**). The method requires antibodies used in specially selected concentrations to completely saturate antigens. Low AIs were obtained for all MAbs in their self-combinations (from 1.6 to 4.2%), which proves the adequate conditions of the reaction performance. For the MAbs 5D4-6H4, 5D4-5G11 and 6H4-5G11 pairs, the AIs were 8.3–12.1%, which is indicative of overlapping epitopes or epitopes closely located on the G-protein. The AIs for MAbs 1C11 paired with other MAbs were >40%, therefore, the epitope binding to MAbs 1C11 is located at a distance from epitopes for MAbs 5D4, 6H4 and 5G11.

Discussion

During the present study, we received 4 hybridomes producing MAbs specific for the RVS G-protein. The G-protein is more variable in comparison with the F-protein, which is frequently used in preparing products for prevention of RSV-infection; at the same time, when administered, it produces a protective effect. In the last years, the G-protein has been attracting researchers' attention due to its properties that make it a promising candidate for development of therapeutic products and vaccines. The protein contains a small, though highly conserved central domain (CCD) [7] and is immunogenic when administered to healthy people

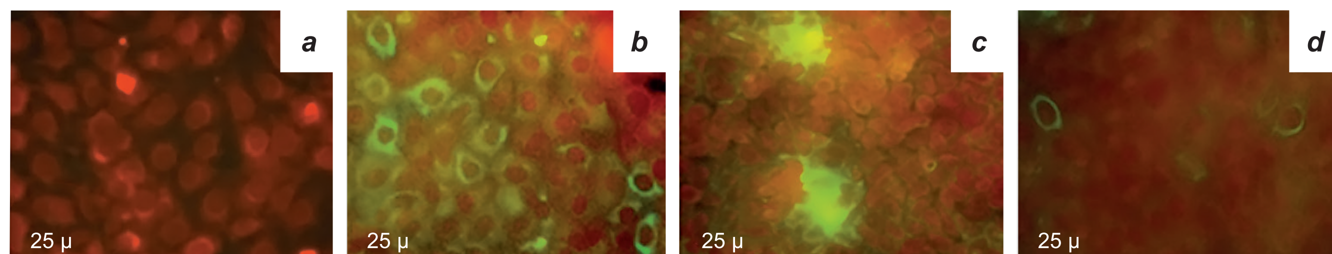

Fig. 2. IF analysis of the interaction of Mabs with MA-104 cells infected with RSV A2.

a — Mabs for hepatitis virus, negative control; *b* — Mabs 5D4, the number of stained cells 80% (+++); *c* — Mabs 5G11, the number of stained cells 20% (++); *d* — Mabs 6H4, single stained cells (+); green color — FITC, red — Evans blue. The picture is duplicated on the cover of the magazine.

Table 2. Additivity index for Mabs to G-protein of RSV (*M* ± *m*, %)

МКА	5D4	6H4	5G11	1C11
5D4	1,6 ± 0,3	8,3 ± 2,8	11,3 ± 0,5	41,6 ± 3,1
6H4	–	2,1 ± 0,6	12,1 ± 0,8	40,7 ± 4,2
5G11	–	–	3,5 ± 0,7	42,4 ± 2,6
1C11	–	–	–	4,2 ± 3,1

[11]. The CCD of the G-protein has a CX3C chemokine motif, which interacts with the CX3CR1 chemokine receptor on host cells, thus interfering with the antiviral response [12]. It has been found that MAbs targeted at CCD produce an antiviral effect on infected mouse models in RVS-infection by blocking the development of the disease and improving the immune response [13]. The G-protein interacts with epithelial cells of the patients' respiratory tract and induces an infection process. It has been found that G-protein specific antibodies efficiently suppress inflammation [14]. The further study of the antigenic structure and functions of the G-protein is required for defining a new strategy in the battle against RSV-infection [15].

The MAbs received during this study bind to two epitopes of the G-protein, displaying an additive effect during the interaction; they detect the virus antigen in infected cells of the culture and can be used for detection of RSVs in clinical material, in combination with immunofluorescence assays, immune-peroxidase staining and ELISA. The virus-neutralizing activity of MAbs offers opportunities for receiving humanized recombinant antibodies. Further studies will show possibilities and feasibility of using G-protein specific MAbs in combination with MAbs specific for RSV F-protein for prevention and treatment of RSV-infection.

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Information about the authors:

Natalia A. Demidova — junior researcher, N.F. Gamaleya National Research Center of Epidemiology and Microbiology, 123098, Moscow, Russia. ORCID ID: http://orcid.org/0000-0003-1961-9789.
E-mail: ailande@yandex.ru

Regina R. Klimova — Cand. Sci. (Biol.), senior researcher,, Laboratory of cell engineering, D.I. Ivanovsky Institute of Virology, N.F. Gamaleya National Research Center of Epidemiology and Microbiology, 123098, Moscow, Russia.
ORCID ID: http://orcid.org/0000-0002-4147-8444.
E-mail: regi.k@mail.ru

Alla A. Kushch✉ — Doct. Sci. (Biol.), Prof., Head, Laboratory of cell engineering, Department of molecular virology, D.I. Ivanovsky Institute of Virology, N.F. Gamaleya National Research Center of Epidemiology and Microbiology, 123098, Moscow, Russia.
ORCID ID: http://orcid.org/0000-0002-3396-5533.
E-mail: vitallku@mail.ru

Ekaterina I. Lesnova — researcher, Laboratory of cell engineering, D.I. Ivanovsky Institute of Virology, N.F. Gamaleya National Research Center of Epidemiology and Microbiology, 123098, Moscow, Russia.
ORCID ID: https://orcid.org/0000-0002-2801-6843.
E-mail: wolf252006@yandex.ru

Olga V. Masalova — Doct. Sci. (Biol.), leading researcher,, Laboratory of cell engineering, D.I. Ivanovsky Institute of Virology, N.F. Gamaleya National Research Center of Epidemiology and Microbiology, 123098, Moscow, Russia.
ORCID ID: https://orcid.org/0000-0001-5571-5669.
E-mail: ol.mas@mail.ru

Информация об авторах:

Демидова Наталья Андреевна — м.н.с. ФГБУ «Национальный исследовательский центр эпидемиологии и микробиологии имени почетного академика Н.Ф. Гамалеи», 123098, Москва, Россия.
ORCID ID: http://orcid.org/0000-0003-1961-9789.
E-mail: ailande@yandex.ru

Климова Регина Рафаиловна — к.б.н., с.н.с. лаборатории клеточной инженерии Института вирусологии им. Д.И. Ивановского ФГБУ «Национальный исследовательский центр эпидемиологии и микробиологии имени почетного академика Н.Ф. Гамалеи», 123098, Москва, Россия.
ORCID ID: http://orcid.org/0000-0002-4147-8444.
E-mail: regi.k@mail.ru

Кущ Алла Александровна✉ — д.б.н., проф., рук. отдела молекулярной вирусологии, зав. лабораторией клеточной инженерии Института вирусологии им. Д.И. Ивановского ФГБУ «Национальный исследовательский центр эпидемиологии и микробиологии имени почетного академика Н.Ф. Гамалеи», 123098, Москва, Россия.
ORCID ID: http://orcid.org/0000-0002-3396-5533.
E-mail: vitallku@mail.ru

Леснова Екатерина Ивановна — н.с. лаборатории клеточной инженерии Института вирусологии им. Д.И. Ивановского ФГБУ «Национальный исследовательский центр эпидемиологии и микробиологии имени почетного академика Н.Ф. Гамалеи», 123098, Москва, Россия.
ORCID ID: https://orcid.org/0000-0002-2801-6843.
E-mail: wolf252006@yandex.ru

DOI: https://doi.org/10.36233/0372-9311-2020-97-1-7-14

Anastasia D. Dorofeeva — junior researcher, Mechnikov Research Institute for Vaccines and Sera, 105064, Moscow, Russia.
ORCID ID: https://orcid.org/0000-0002-2501-2847.
E-mail: d0r0feeva@yandex.ru

Alexandra A. Nikonova — Cand. Sci. (Biol.), Head, Laboratory of molecular biotechnology, Department of virology, Mechnikov Research Institute for Vaccines and Sera, 105064, Moscow, Russia.
ORCID ID: https://orcid.org/0000-0001-9610-0935.
E-mail: sana80@list.ru

Natalia E. Fedorova — Cand. Sci. (Biol.), leading reasecher, Laboratory of cell engineering, D.I. Ivanovsky Institute of Virology, N.F. Gamaleya National Research Center of Epidemiology and Microbiology, 123098, Moscow, Russia.
ORCID ID: http://orcid.org/0000-0001-8466-7993.
E-mail: ninani@mail.ru

Vitaly V. Zverev — Doct. Sci. (Biol.), prof., Academician of RAS, Scientific Director, Mechnikov Research Institute for Vaccines and Sera, 105064, Moscow, Russia; Head, Microbiology, virology, immunology department, I.M. Sechenov First Moscow State Medical University oh Ministry of Health of Russian Federation (Sechenov University), 127994, Moscow, Russia.
ORCID ID: http://orcid.org/0000-0002-0017-1892.
E-mail: vitalyzverev@outlook.com

Contribution: the authors contributed equally to this article.

Масалова Ольга Владимировна — д.б.н., в.н.с. лаборатории клеточной инженерии Института вирусологии им. Д.И. Ивановского ФГБУ «Национальный исследовательский центр эпидемиологии и микробиологии имени почетного академика Н.Ф. Гамалеи», 123098, Москва, Россия.
ORCID ID: https://orcid.org/0000-0001-5571-5669.
E-mail: ol.mas@mail.ru

Дорофеева Анастасия Дмитриевна — м.н.с. ФГБНУ «НИИ вакцин и сывороток им. И.И. Мечникова», 105064, Москва, Россия.
ORCID ID: https://orcid.org/0000-0002-2501-2847.
E-mail: d0r0feeva@yandex.ru

Никонова Александра Александровна — к.б.н., зав. лабораторией молекулярной биотехнологии отдела вирусологии ФГБНУ «НИИ вакцин и сывороток им. И.И. Мечникова», 105064, Москва, Россия.
ORCID ID: https://orcid.org/0000-0001-9610-0935.
E-mail: sana80@list.ru

Федорова Наталья Евгеньевна — к.б.н., в.н.с. лаборатории клеточной инженерии Института вирусологии им. Д.И. Ивановского ФГБУ «Национальный исследовательский центр эпидемиологии и микробиологии имени почетного академика Н.Ф. Гамалеи», 123098, Москва, Россия.
ORCID ID: http://orcid.org/0000-0001-8466-7993.
E-mail: ninani@mail.ru

Зверев Виталий Васильевич — д.б.н., проф., акад. РАН, науч. рук. ФГБНУ «НИИ вакцин и сывороток им. И.И. Мечникова», 105064, Москва, Россия; зав. кафедрой микробиологии, вирусологии, иммунологии, ФГАОУ ВО «Первый Московский государственный медицинский университет им. И.М. Сеченова» Минздрава России (Сеченовский Университет), 127994, Москва, Россия
ORCID ID: http://orcid.org/0000-0002-0017-1892.
E-mail: vitalyzverev@outlook.com

Участие авторов: все авторы сделали эквивалентный вклад в подготовку публикации.