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RESEARCH ARTICLE

Method for obtaining recombinant antibodies produced by a cell line transduced with recombinant adenoviruses

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Abstract

Objectives. *To develop a technology for obtaining recombinant antibodies in a suspension culture of human HEK293 cells using transduction with recombinant adenovirus serotype 5 (rAd5) carrying genes expressing heavy and light chains of antibodies on the example of two broad-spectrum anti-influenza antibodies 27F3 and CR9114.*

Methods. *Ad5-27F3-H, Ad5-CR9114-H, and Ad5-27F3-L recombinant adenoviruses carrying the 27F3 antibody heavy chain gene, CR9114 antibody heavy chain gene, and 27F3 light chain gene, respectively, were generated using the AdEasy™ Adenoviral vector system. To accumulate preparative amounts of recombinant r27F3 and rCR9114 antibodies, the HEK293 suspension cell line was transduced with recombinant adenoviruses carrying genes for heavy and light chains of antibodies. The cells were cultured in a wave-type bioreactor. Chromatography was used to purify recombinant antibodies from the culture medium. After analyzing the molecular weights of purified antibodies using protein electrophoresis, their ability to interact with influenza A and B viruses was analyzed using the Western blot technique, while their ability to neutralize influenza A and B viruses was evaluated using the virus neutralization assay.*

Results. *A method for the accumulation and purification of recombinant r27F3 and CR9114 antibodies from the culture medium of a suspension culture of human cells following transduction with its recombinant adenoviruses carrying the genes for heavy and light chains of these antibodies was developed. The ability of the r27F3 antibody to interact with and neutralize influenza A viruses of group 1 (except influenza A virus subtype H2) and group 2 was shown. The ability of the rCR9114 antibody to interact with influenza A viruses of group 1 and influenza B viruses, as well as to neutralize influenza A viruses of group 1, was demonstrated.*
Conclusions. *A technology for obtaining recombinant antibodies in a suspension culture of HEK293 cells using transduction with recombinant adenoviruses carrying genes expressing heavy and light chains of antibodies was developed along with a confirmation of their specificity.*

Keywords: *recombinant antibodies, recombinant human adenovirus 5 serotype, suspension cell culture*

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НАУЧНАЯ СТАТЬЯ

Способ получения рекомбинантных антител, продуцируемых клеточной линией, трансдуцированной рекомбинантными аденовирусами

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Аннотация

Цели. *Разработать технологию получения рекомбинантных антител в суспензионной культуре клеток человека HEK293 с помощью трансдукции рекомбинантными аденовирусами человека пятого серотипа, несущими гены, экспрессирующие тяжелые и легкие цепи антител, на примере двух противогриппозных антител широкого спектра действия 27F3 и CR9114.*
Методы. *Рекомбинантные аденовирусы Ad5-27F3-H, Ad5-CR9114-H и Ad5-27F3-L, несущие ген тяжелой цепи антитела 27F3, ген тяжелой цепи антитела CR9114 и ген легкой цепи 27F3, были получены с помощью набора AdEasy™ Adenoviral vector system. Для накопления препаративных количеств рекомбинантных антител r27F3 и rCR9114 суспензионную клеточную линию HEK293 трансдуцировали рекомбинантными аденовирусами, несущими гены тяжелых и легких цепей антител, и культивировали клетки в биореакторе волнового типа. Рекомбинантные антитела очищали из культуральной*

жидкости хроматографическим методом. Молекулярную массу полученных антител анализировали с помощью белкового электрофореза, их способность взаимодействовать с вирусами гриппа А и В методом вестерн-блот анализа, а способность нейтрализовать вирусы гриппа А и В с помощью реакции вирус-нейтрализации.

Результаты. *Отработана методика накопления и очистки рекомбинантных антител r27F3 и CR9114 из культуральной жидкости суспензионной культуры клеток человека после трансдукции ее рекомбинантными аденовирусами, несущими гены тяжелых и легких цепей этих антител. Показана способность антитела r27F3 взаимодействовать с вирусами гриппа А подгруппы 1 (кроме вируса грипп А субтипа H2) и подгруппы 2 и нейтрализовать их. Показана способность антитела rCR9114 взаимодействовать с вирусами гриппа А подгруппы 1 и вирусами гриппа В, а также нейтрализовать вирусы гриппа А подгруппы 1.*

Выводы. *Отработана технология получения рекомбинантных антител в суспензионной культуре клеток HEK293 с помощью трансдукции рекомбинантными аденовирусами, несущими гены, экспрессирующие тяжелые и легкие цепи антител, и показана их специфичность.*

Ключевые слова: *рекомбинантные антитела, рекомбинантный аденовирус человека пятого серотипа, суспензионная культура клеток*

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INTRODUCTION

Due to their useful properties, including high affinity and specificity for a particular antigen, monoclonal antibodies are widely used in laboratory practice for the detection, purification, and characterization of target proteins, as well as for diagnostic and therapeutic purposes in medicine [1, 2]. For this reason, antibody-based biologics are one of the best-selling classes of biomolecules on the market today.

Modern recombinant technologies are used to produce antibodies in preparative quantities. For the production of recombinant antibodies (rAB), as well as their fragments, the following expression systems are currently used: bacterial, yeast, expression using baculovirus vectors in insect cells, as well as expression in mammalian cells [3]. All of these expression systems imply obtaining a high-performance producer for constant expression of the target protein. While bacterial [4] and yeast [5] expression systems are characterized by a high yield of the target product (20–1000 mg of antibody per 1 L of culture liquid), these approaches are only suitable for obtaining non-glycosylated antibody fragments (bacteria) or highly mannosylated forms of antibodies that are rapidly excreted from the body (yeast) [6]. Glycosylation of antibodies obtained in yeast and baculovirus expression systems [7] differs from native glycosylation of mammalian antibodies [8]. Today, most therapeutic antibodies are produced in mammalian cells, which are best suited for the

production of rABs due to offering the greatest biological similarity to antibodies produced in the human body. The amount of rAB obtained in Chinese hamster ovary (CHO) cells can reach approximately 10 g/L of culture fluid [9]. An even higher antibody titer of up to 27 g/L can be achieved in a perfusion bioreactor by expression in the human embryonic retinal cell line Per.C6 (*Crucell*, Leiden, the Netherlands). However, the creation of high-yielding cell lines remains expensive, laborious, and time consuming [9].

A simpler and cheaper method is the transient expression of antibody genes in mammalian cells, since it does not require the creation of producer cell lines [10]. This method of production of antibodies is suitable for small-scale and intralaboratory production. For example, the most commonly-used method for generating rAB at the present time is the transient co-transfection of a CHO cell line with two plasmids carrying light- and heavy-chain antibody expression cassettes. When using this technology, the expression cassette does not integrate into the cell genome; accordingly, the long-term expression of antibodies cannot be maintained. In this case, genes are expressed for several days, after which the transgene is lost due to cell division or environmental factors [11].

Despite its widespread use, transient expression of rAB in CHO cells also has disadvantages. It has been shown that the glycosylation profile differs between proteins obtained in CHO cell culture and proteins obtained in human cell cultures [12]. Therefore, such human cell lines as Per.C6 and HEK293 are also widely used to produce monoclonal antibodies. The HEK293 cell line is capable of providing optimal post-translational modifications of human antibodies, including their glycan profile [13, 14]. Target antibody yields of tens and hundreds of milligrams per liter of culture medium can be achieved using expression systems based on human suspension cells in serum-free media maintaining a high suspension density [6]. However, transfection of suspension cell cultures is a labor-intensive method that requires the use of rather expensive reagents and highly purified plasmids [9], which is not justified for small-scale and intralaboratory production.

In connection with the foregoing, the development of new or improvement of existing technologies for the production and purification of rAB is currently relevant. One such technology involves the use of recombinant viral vectors that allow the genetic information to be effectively delivered to many types of cells, including suspension cultures of human cells [16]. Due to the exclusion of the transfection stage, the use of recombinant viral vectors for the delivery of genetic information encoding target antibodies to the producer cells makes it possible to reduce the cost and simplify the technology. The most commonly-used and well-studied viral vector for delivering genetic information to human cells is recombinant human adenovirus serotype five (rAd5). In the paper, we present a developed method for obtaining rABs by transducing the human embryonic kidney cell line HEK293 rAd5 carrying the heavy and light chain genes of target antibodies. This approach is used for the first time to develop a technology for obtaining preparative amounts of rABs. 27F3 [17] and CR9114 antibodies to broad-spectrum influenza virus hemagglutinin were selected as target antibodies [18].

MATERIALS AND METHODS

Viruses

In this work, the following viruses were used: Influenza A viruses A/California/04/2009 (H1N1) (GenBank numbers KY238116–KY238123), A/BlackDuck/NewJersey/1580/1978(H2N3) (GenBank numbers KX879562–KX879569), A/Aichi/ 2/1968 (H3N2) (GenBank numbers KX879570–KX879577), A/Duck/Pensylvania/10218/1984 (H5N2) (GenBank numbers KX879578–KX879585), A/Chicken/Moscow/NJ294598-12/20017 (H7N2) (GenBank numbers MN400380–MN400388), A/Swine/HongKong/9A-1/1998 (H9N2) (GenBank numbers KX879586–KX879593), and influenza B viruses B/Phuket/3073/2013 and B/Washington/20/2019 obtained from the State Collection of Viruses of the Russian Federation (subdivision of the D.I. Ivanovsky Institute of Virology of the N.F. Gamaleya National Center for Epidemiology and Microbiology of the Ministry of Health of Russia).

Cell cultures

Adhesive and suspension cell cultures of the human embryonic kidney HEK293 were obtained from the Cell Culture Collection of the Tissue Culture Laboratory, Division of the D.I. Ivanovsky Institute of Virology of the N.F. Gamaleya National Center for Epidemiology and Microbiology of the Ministry of Health of Russia. The cell culture of intestinal adenocarcinoma Caco-2 was obtained from the Russian collection of cell cultures of the Institute of Cytology of the Russian Academy of Sciences.

Antibodies

An anti-species immunoperoxidase conjugate Anti-Human IgG (Fc specific)–Peroxidase antibody (A0170, *Merck*, Germany) was used.

Generation of rAd5 expressing the heavy and light chain genes of the human antibody 27F3, as well as the heavy chain gene of the human antibody CR9114

The nucleotide sequences encoding the heavy and light chains of the target antibodies were chemically synthesized (*Evrogen*, Russia) and cloned into the pAL-TA plasmid. Next, the target genes were cloned into the pShuttle-CMV plasmid from the AdEasy™ Adenoviral vector system kit (*Agilent Stratagene*, USA). Ad5-27F3-H, Ad5-CR9114-H, and Ad5-27F3-L recombinant adenoviruses carrying the 27F3 antibody heavy chain gene, the CR9114 antibody heavy chain gene, and the 27F3 antibody light chain gene, respectively, were prepared according to the kit instructions. rAd5 titers were determined on an adhesive HEK293 cell culture using the plaque formation reaction [19].

Accumulation of preparative amounts of r27F3 and rCR9114 rAB in culture liquid

To accumulate preparative amounts of r27F3 and rCR9114 antibodies, the HEK293 suspension cell line was transduced with rAd5 Ad5-27F3-L and Ad5-27F3-H or Ad5-27F3-L and Ad5-CR9114-H. For this, the cells were cultivated in a wave-type bioreactor in Biostat® CultiBag RM 5L cell culture bags (*Sartorius AG*, Germany) in CDM4HEK293 HyClone™ culture medium (*Cytiva*, USA) supplemented with 2 g/L of sodium bicarbonate (*PanEco*, Russia), 1 g/L of Poloxamer 188 (*Corning*, USA), and 4 mM of L-glutamine (*PanEco*, Russia) at 37°C and 5% CO_2. After reaching a concentration of 2×10^6 cells/mL, 100 mL of a viral suspension containing Ad5-27F3-Ladenovirus (2×10^8 PFU/mL) (plaque-forming units in a mL) and 200 mL of a suspension containing Ad5-27F3-H adenovirus (10^8 PFU/mL) were aseptically added to the culture. Cells together with recombinant adenoviruses were incubated for 3 days until 50–60% cytopathic effect (CPE) was achieved. The percentage of dead cells was determined by visual counting an aliquot of the cell suspension stained with trypan blue in a hemocytometer. The resulting cell suspension was centrifuged at 7700g for 20 min at room temperature (20°C), and the supernatant was collected and stored −70°C in sterile test tubes.

Preparative isolation of r27F3 and rCR9114 rAB from culture fluid

The clarified culture liquid of 4 L was filtered through a Sartopore® 2 0.45/0.2 µm capsule filter (*Sartorius AG*, Germany). The pH of the culture liquid was adjusted to 7.2 by adding 1 M of Tris-HCl pH 8. The conductivity was adjusted to 17 mS with a 5-M sodium chloride solution. An XK26/20 chromatographic column (*Cytiva*, USA) packed with 20 mL of MabSelect SuRe™ sorbent (*Cytiva*, USA) was equilibrated with a buffer containing 20 mM of sodium phosphate and 150 mM of sodium chloride, pH 7.2. The culture liquid was applied at a linear speed of 500 cm/h. After loading, the column was washed with 10 column volumes of a buffer containing 20 mM of sodium phosphate and 150 mM of sodium chloride, pH 7.2. Antibodies were eluted isocratically with a buffer containing 150 mM of sodium chloride, 0.1% Tween® 20, 200 mM of glycine-HCl, pH 3.5, and neutralized by adding 1 M of 10% Tris-HCl, pH 8 to the eluate. Additional cleaning and replacement of the buffer with a buffer, containing 20 mM of sodium phosphate, 150 mM of sodium chloride, 0.05% Tween® 20, pH 7.2, was performed using gel filtration on an XK 26/100 chromatography column (*Cytiva*, USA) packed with Superdex 200 prep grade sorbent (*Cytiva*, USA).

To remove the residual amount of a possible admixture of rAd5, antiviral filtration of rAB preparations was performed using Viresolve® Pro Micro filters (*Millipore*, USA). Filtration was carried out at a constant pressure of 0.9 bar using compressed air. Used filters were sterilized by autoclaving before disposal.

Dot-enzyme-linked immunosorbent assay (dot-ELISA)

Dots were placed along the radius on the nitrocellulose filter for subsequent application of samples and visual control of adsorption, including points for positive and negative controls. Samples in a volume of 1.5 µL were applied to the dot and allowed to dry, controlling the light. Then, the nitrocellulose filter was washed 3 times to remove unbound proteins with a solution of 0.1% Tween® 20. The filter was blocked from nonspecific binding for 30 min on a shaker with working phosphate buffer pH 7.2 with 2% inert protein Blosker™ Casein in phosphate buffered saline (*Thermo Fisher Scientific*, USA). Following incubation, the filter was washed three times with distilled water, then three times for 10 min with a solution of 0.1% Tween® 20 on a shaker. After washing, the filter was incubated in a working dilution of 1/5000 of the anti-species immunoperoxidase-conjugated Anti-Human IgG (Fc specific)-Peroxidase antibody (*Merck*, Germany) for 30–40 min at 37°C on a shaker. Following this incubation, washing was again carried out. Staining was performed with a ready-made solution of

3,3'-diaminobenzidine Pierce™ DAB Substrate Kit (*Thermo Fisher Scientific*, USA). The reaction was stopped after 10 min by transferring the filter to distilled water.

Protein electrophoresis in polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE)

Protein fractionation was performed by electrophoresis in 10% SDS-PAGE, in a Laemmli buffer system, using a Mini-Protean® 3 Cell vertical mini-gel device (*Bio-Rad*, USA). In order to analyze the monomeric and multimeric forms of the protein, the samples were treated under denaturing (+) and non-denaturing (–) conditions before being added to the gel. To the sample (+), an equal volume of a twofold dissociating 2× Laemmli Sample Buffer (*Merck*, Germany) containing mercaptoethanol was added, then the samples were heated at 95°C for 7 min. An equal volume of 2× buffer containing no mercaptoethanol was added to the sample (−) without further heating. Samples were added to gel wells and electrophoresis was performed at a constant current of 20 mA/gel in an electrode buffer with the following composition: 25 mM of Tris-HCl, 0.2 M of glycine, 0.1% sodium dodecyl sulfate, pH 8.3. At the end of electrophoresis, the gel was scanned using a Gel Doc™ EZ imager (*Bio-Rad*, USA). PageRuler™ Unstained Protein Ladder (*Thermo Fisher Scientific*, USA) and PageRuler™ Plus Prestained Protein Ladder (*Thermo Fisher Scientific*, USA) were used as markers for electrophoresis.

High performance liquid chromatography and gel filtration (HPLC-HF)

To study the purified rAB preparations r27F3 and rCR9114, HPLC with an Agilent 1260 Infinity II chromatographic system (*Agilent Technologies*, USA) and a Phenomenex SEC-s3000 chromatographic column (particle size: 5 μm, pore size: 290 Å, geometry: 300 × 7.8 mm) (*Phenomenex*, USA) was used. Chromatography was carried out in isocratic mode, with a flow rate of 0.3 mL/min, 25°C, using an ultraviolet detector at a wavelength of 280 nm. The results were calculated using the OpenLAB CDS Chemstation VL Edition software (*Agilent Technologies*, USA).

Study of the functional activity of rAB using Western blot analysis

To set up a Western blot analysis, electrophoresis was carried out in SDS-PAGE, and then proteins were transferred from the gel to the membrane by semi-dry transfer on a Trans Blot® SD Semi-dry Transfer cell (*Bio-Rad*, USA) at a constant current of 250 mA per gel area 5 × 8 cm. Upon completion of the transfer, the Nitrocellulose membranes 0.2 (*Bio-Rad*, USA) was washed three times in distilled water for 10 min and the free sites of the membrane were blocked with a buffer containing the inert protein Blosker™ Casein in phosphate buffered saline (*Thermo Fisher Scientific*, USA) for 30 min at 37°C on a shaker. Then the membrane was incubated with a buffer solution containing the r27F3 antibody or the CR9114 antibody in a working dilution of 1/50 on a shaker at 22°C overnight (16 h). At the end of incubation, the membrane was washed with distilled water, then three times for 10 min with phosphate buffer pH 7.2 with 0.1% Tween® 20 on a shaker. Then, incubation was carried out with secondary antibodies to the human IgG Fc fragment conjugated with horseradish peroxidase at a dilution of 1/5000 for an hour at 37°C on a shaker. After incubation, the membrane was washed again and the chemiluminescent detection of the bound complex was performed using ECL™ Prime Western Blotting Detection Reagent (*GEHealthcare*, USA) followed by scanning of the immunoreplica on an Amersham™ Imager 680 device (*GEHealthcare*, USA).

Accumulation and concentration of influenza viruses

Influenza viruses A and B were accumulated in the allantoic fluid of 9-day-old chicken embryos at 37°C for 48 h. The virus-containing allantoic fluid was stored at –70°C.

Virus-containing allantoic liquid after clarification by centrifugation at 3000 rpm, 15 min, 4°C was layered on 4 mL of 20% sucrose in a buffer solution containing 0.15 M of NaCl and 0.01 M of Tris-HCl, pH 7.2, and precipitated by ultracentrifugation in a SW-27 rotor (*Beckman Coulter*, USA) at 22000 rpm for 90 min at 4°C. The obtained precipitate was resuspended in a Dounce homogenizer in the same buffer solution in 1/64 of the original volume and re-clarified by centrifugation at low speed (3500 rpm, 8 min, 4°C). The purified and concentrated viral preparation was titrated using the hemagglutination assay [20], and the 50% tissue cytopathic effect of TCDmL was also evaluated on the cell culture of intestinal adenocarcinoma Caco-2 and stored at −80°C.

Virus neutralization assay

The virus neutralization assay was carried out on a cell culture of intestinal adenocarcinoma Caco-2. Cells were cultured on Dulbecco's modified

Eagle medium (DMEM) with a high glucose content (*HyClone*, USA) supplemented with 10% fetal serum (*HyClone*, USA), 2 g/L of sodium bicarbate (*PanEco*, Russia), 25000 U of penicillin/streptomycin (*PanEco*, Russia) and 4 mM of L-glutamine (*PanEco*, Russia) at 37°C and 5% CO_2. The virus neutralization assay was carried out in a 96-well plate at a cell concentration of (4–6) × 10^4 cells/well. The target influenza virus was preliminarily mixed in a 96-well plate in an amount of 10^2 TCD_{50}/well and two-fold dilutions of sera made in a medium with 0.5% lactalbumin hydrolyzate with Hanks' salts (*PanEco*, Russia). The resulting mixture was incubated for 40 min at 37°C and added to the cells. After 40 min, the medium was changed to serum-free DMEM. After 4–5 days, the medium was taken from the cell culture plate and the presence of influenza virus particles in it was assessed using hemagglutination assay [20]. The analysis was carried out with a quadruple repetition. The results of the reaction were expressed using 50% inhibitory concentration (IC_{50}), which is the amount of antibody that inhibits the virus in 50% of cases.

RESULTS AND DISCUSSION

Production of rAd5 expressing human antibody genes 27F3 and CR9114

To develop a new technology for the production of rAB in suspension eukaryotic cells transduced with recombinant viral vectors expressing rAB genes, we chose the system "recombinant viral vector based on human adenovirus serotype 5 for transduction of a suspension culture of human cells HEK293." Such a system makes it possible to produce antibodies with the glycosylation profile closest to the native human one and does not require expensive reagents and laborious method of cell suspension transfection with plasmids. Previously, we published works where the possibility of expressing the recombinant mini-antibody gene using an adenoviral vector in eukaryotic cells both *in vitro* [21] and *in vivo* [22] was shown.

We conducted a literature search for monoclonal antibodies that could serve as a model for the technology being developed. As a model, we decided to choose antibodies specific to influenza virus hemagglutinin, with a known tertiary structure and a wide spectrum of activity, which makes it possible to detect influenza viruses of various subtypes. As a rule, antibodies interacting with the conservative, stem part of hemagglutinin have the greatest breadth of the spectrum of action. Of the many known monoclonal antibodies to the hemagglutinin stem domain, four antibodies (CR9114, 27F3, CR6261, F10) interact with hemagglutinin only in complementary determining regions of heavy chains [23], which makes them a convenient model for preparative production using recombinant adenoviruses, since it does not require the creation individual constructs encoding light chains for each antibody. We chose anti-influenza antibodies 27F3 and CR9114 as model ones, since they have the widest range of recognition. The literature data shows the ability of the 27F3 antibody to interact with the hemagglutinins of the H1, H5, H6, H9, H11, H12, H13, and H16 influenza viruses of the H1 subgroup, and the H3, H7, and H10 hemagglutinins of the H3 subgroup [17]. The CR9114 antibody is able to interact with the hemagglutinins of the H1, H5, H9, H12, H13, H16 influenza viruses of the H1 subgroup, with the hemagglutinins of the H3, H7, H10, and H15 influenza viruses of the H3 subgroup, as well as with hemagglutinins of various influenza B viruses [18].

Antibodies 27F3 and CR9114 interact with the stem domain of influenza virus hemagglutinin and belong to the VH1-69 class of antibodies. The interaction of this class of antibodies with their epitope occurs with the participation of only the heavy chain [17]. According to the literature data, preparative accumulation with a high yield of an antibody heavy chain in eukaryotic expression systems requires the expression of a light chain in equivalent amounts [7]. Therefore, we constructed three rAd5: rAd5 carrying the heavy chain gene of the 27F3 antibody, rAd5 carrying the heavy chain gene of the CR9114 antibody, and rAd5 carrying the light chain gene of the 27F3 antibody, which was designed to obtain transient expression of both the full-length 27F3 antibody and full length antibody CR9114.

The amino acid sequences of the Fab fragments of the heavy and light chains of the 27F3 antibody and the heavy chain of CR9114 were obtained from the protein database of tertiary structures—Protein Data Bank ID[1] (5WKO and 4FQH). Based on the obtained amino acid sequences, nucleotide sequences with codons optimized for expression in human cells were constructed. Genetic constructs were developed that included the heavy or light chain gene of the target antibody and the leader sequence of the secreted alkaline phosphatase (SEAP), which is necessary for the release of the antibody from the cell into the culture medium. Schemes of the obtained structures are shown in Fig. 1.

[1] https://www.rcsb.org/docs/general-help/identifiers-in-pdb. Accessed January 20, 2021.

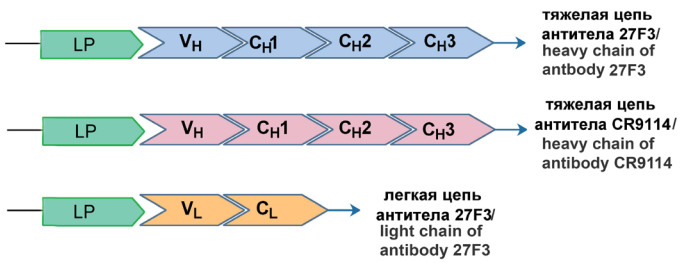


Fig. 1. Schematic representation of the genetic constructs encoding the heavy chain of the 27F3 antibody, the heavy chain of the CR914 antibody, and the light chain of the 27F3 antibody. LP – secreted alkaline phosphatase SEAP leader peptide; V_H – heavy chain variable domain; V_L – light chain variable domain; C_H – heavy chain constant domain; C_L – light chain constant domain.

Recombinant adenoviruses Ad5-27F3-H, Ad5-CR9114-H, and Ad5-27F3-L were obtained in accordance with the instructions for the AdEasy™ Adenoviral vector system kit (*Agilent Stratagen*, USA). Preparative accumulation of recombinant adenoviruses was carried out in an adhesive culture of eukaryotic cells of the HEK293 line. As a result, cell lysates were obtained containing recombinant adenoviruses Ad5-27F3-L, Ad5-CR9114-H and Ad5-27F3-H, having titers of 2×10^8, 1×10^8 and 1×10^8 PFU/mL (plaque-forming units per mL), respectively.

Selection of conditions for obtaining a preparative amount of rAB in HEK293 cell culture

At the next stage of the work, optimal conditions were selected for the accumulation of the maximum level of rAB in the culture liquid. The selection of conditions was carried out using the r27F3 rAB example during transduction of a suspension HEK293 cell line with recombinant Ad5-27F3-L and Ad5-27F3-H adenoviruses. A cell suspension with a concentration of 1×10^6 cells/mL was transduced with different amounts of cell lysates containing Ad5-27F3-L and Ad5-27F3-H adenovirus particles so that the particle ratio was 1 : 1. RAd5 doses of 2 PFU/cell of each adenovirus, 20 and 200 PFU/cell were used. The amount of the target antibody in the culture liquid was estimated at 30–40%, 50–60%, and 100% CPE. The resulting culture fluid samples were analyzed in dot-ELISA using peroxidase-conjugated antibodies to the Fc fragment of human IgG. Each point of the experiment was repeated three times. The results of dot-ELISA were divided into three groups: "–" means the absence of a colored dot on the membrane at the site of the sample, "+" — the presence of a colored dot, "++" — the presence of a bright, clearly visible colored dot. The results of the selection of conditions are presented in Table 1.

Table 1. Selection of conditions for the preparative accumulation of the r27F3 antibody in the culture fluid after transduction of the HEK293 Ad5-27F3-L and Ad5-27F3-H cell culture

Number of viral particles of each rAd, PFU/cell	Mean CPE, %	Incubation time, days	Dot-ELISA results
2	36.5	6	–
2	56.2	7	–
2	100	9	–
20	37.2	4	–
20	53.4	5	+
20	100	7	–
200	40.1	2	+
200	56.5	3	++
200	100	5	–

Table 1 shows that the optimal parameters for the accumulation of the r27F3 antibody are a dose of 200 PFU/cell of each Ad5-27F3-L and Ad5-27F3-H rAd5, and the onset of 50–60% CPE, which corresponds to 3 days of incubation.

Further accumulation of the r27F3 antibody was performed by transducing a cell suspension of 200 PFU/cell of each Ad5-27F3-L and Ad5-27F3-H rAd5 and of rCR9114 antibody by transducing a suspension of 200 PFU/cell of Ad5-27F3-L and Ad5-CR9114-H. The culture fluid was collected on the third day of incubation at the onset of 50–60% CPE.

Obtaining preparative amounts of r27F3 and rCR9114 rAB, chromatographic purification, and analysis of the polypeptide spectrum of purified antibodies

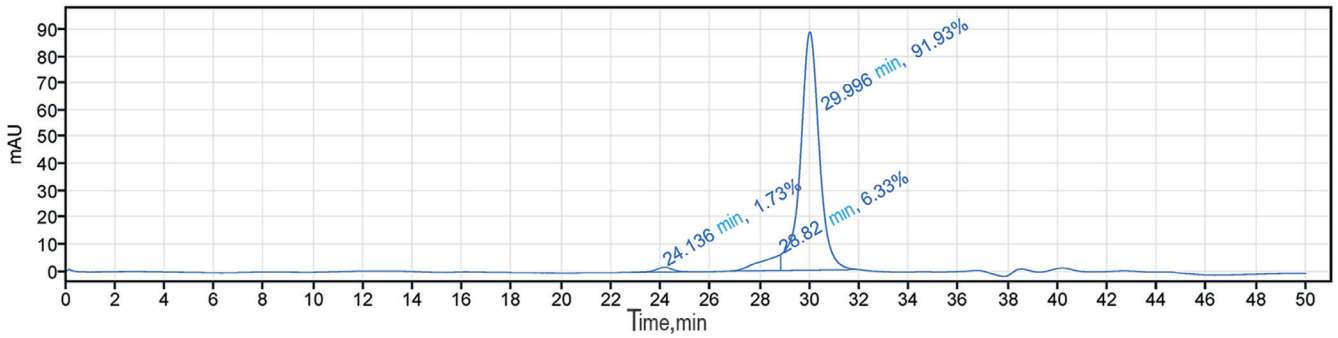
At the next stage of the work, the r27F3 and rCR9114 rAB preparations were accumulated in preparative amounts and purified from the culture liquid using chromatography on a column with the MabSelect™ SuRe™ affinity sorbent (*Cytiva*, USA). The final rAB purification and buffer replacement were performed by gel filtration using Superdex® 200 pg sorbent (*Cytiva*, USA). Earlier, we used this approach to purify rAB [24] and obtain rAB preparations of a high degree of purification sufficient for further *in vitro* and *in vivo* laboratory studies. In order to level the potential risk of contamination of preparations with recombinant adenoviruses used for cell transduction, preparations were additionally filtered following final purification to remove enveloped and non-enveloped viruses using Viresolve® Pro virus filters (*Millipore*, USA) having a pore size of 20 nm.

As a result, 12 mg of r27F3 rAB and 10 mg of rCR9114 rAB were obtained from 4 L of culture liquid. The purified r27F3 rAB preparation had a concentration of 1.12 mg/mL, and the purified rCR9114 rAB preparation had a concentration of 0.56 mg/mL.

HPLC analysis of purified rAB preparations r27F3 and rCR9114 yielded the following results, which are presented in Figs. 2 and 3.

As a result, a high degree of purity of the preparations obtained (more than 90%) was demonstrated. The difference in height and area of the r27F3 and rCR9114 rAB peaks is associated with their different concentrations in the preparation. In addition, different retention times of the main peaks in preparations of r27F3 and rCR9114 were demonstrated, which indicates a different hydrodynamic radius for these two molecules. Apparently, rCR9114 rAB has a much more voluminous spatial structure and appears on the chromatogram with an earlier peak compared to r27F3.

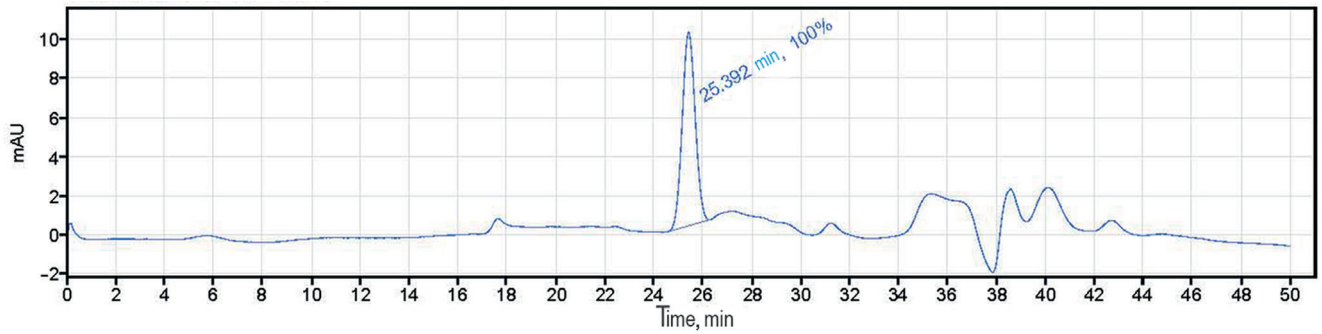
Analysis of the polypeptide spectrum of preparatively purified antibodies was performed by protein electrophoresis in SDS-PAGE. To assess the ability of antibodies to form a complex of heavy and light chains (H+L), a plus–minus modification of sample processing was used, i.e., denaturing (+) and non-denaturing (−) conditions. This modification makes it possible to identify the molecular weight of both the heavy and light



Results for peak (Area percentage 1%)

Retention time, min	Signal description	Width, min	Area	Height	Area, %
24.136		2.985	88.1	1.8	1.73
28.820	DAD1A, Sig = 280.4, Ref = 360.1	2.046	322.1	5.9	6.33
29.996		3.138	4674.8	88.8	91.93

Fig. 2. HPLC chromatogram of the purified r27F3 antibody, as well as the percentage of the target and impurity peaks.



Results for peak (Area percentage 1%)

Retention time, min	Signal description	Width, min	Area	Height	Area, %
25.392	DAD1A, Sig = 280.4, Ref = 360.1	1.713	366.2	9.9	100.0

Fig. 3. HPLC chromatogram of the purified rCR9114 antibody, as well as the percentage of the target and impurity peaks.

chains separately and of the secreted IgG (H+L) functional complex.

The results of electrophoresis in SDS-PAGE are shown in Fig. 4. Under denaturing conditions, two fragments weighing ~55 kDa and 25 kDa were observed, which correspond to the molecular weight of the H and L chains of the antibody. Under non-denaturing conditions, one fragment of 160 kDa was observed, which corresponds to the full IgG complex. It was also shown that the resulting rAB preparations have a high degree of electrophoretic purity.

Study of activity and specificity of r27F3 and rCR9114 rAB

The Fab specificity of the recombinant influenza antibodies was analyzed by Western blotting. A panel of purified influenza A and B viruses was used as antigens. Western blot samples were processed under non-denaturing conditions.

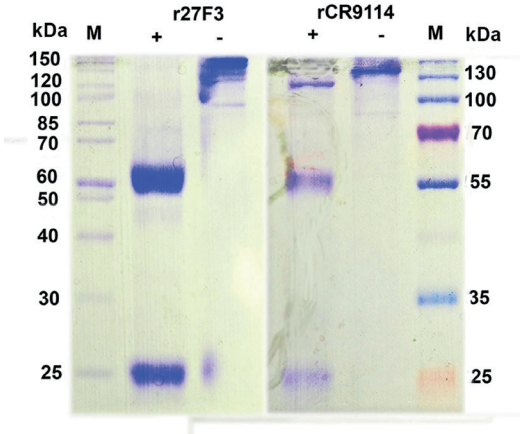


Fig. 4. Electropherogram of purified r27F3 and rCR9114 antibodies. M are markers of molecular weight; "+" are denaturing conditions; "–" are non-denaturing conditions.

The results of Western blotting with r27F3 rAB is demonstrated in Fig. 5; with rCR9114 rAB—in Fig. 6.

According to immunoblotting data (Fig. 5), the r27F3 antibody has a wide heterosubtypic specificity, interacts with hemagglutinins of influenza A viruses of H1, H3, H5, H7, H9 subtypes and does not interact with hemagglutinin of influenza A virus of H2 subtype and influenza B viruses. Obtained specificity for the r27F3 antibody completely corresponds to the literature data [17].

According to the immunoblotting data (Fig. 6), the rCR9114 antibody also has a wide specificity,

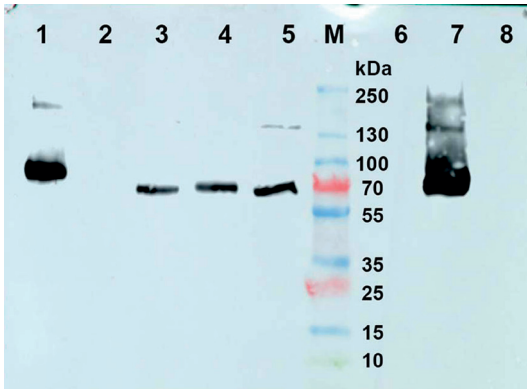


Fig. 5. Western blot analysis of the Fab specificity of the purified r27F3 antibody. M is marker of molecular weight; the numbers indicate influenza viruses:
1 – A/California/04/09 (H1N1);
2 – A/BlackDuck/NewJersey/1580/78 (H2N3);
3 – A/Aichi/2/68 (H3N2);
4 – A/Duck/Pensylvania/10218/84 (H5N2);
5 – A/Chicken/NJ/294598-12/2004 (H7N2);
6 – B/Phuket/3073/13;
7 – A/Swine/Hong Kong/9A-1/98 (H9N2);
8 – B/Washington/20/2019.

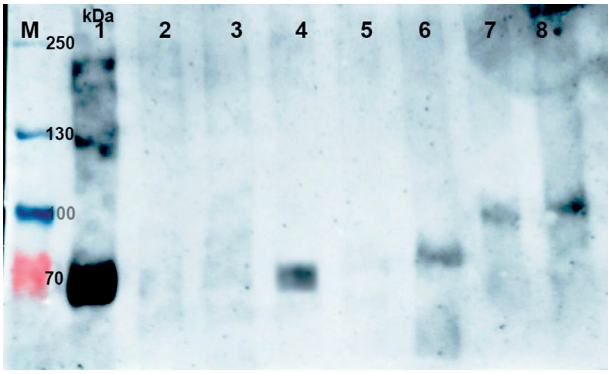


Fig. 6. Western blot analysis of the Fab specificity of the purified rCR9114 antibody. M is marker of molecular weight; the numbers indicate influenza viruses:
1 – A/California/04/09 (H1N1);
2 – A/BlackDuck/NewJersey/1580/78 (H2N3);
3 – A/Aichi/2/68 (H3N2);
4 – A/Duck/Pensylvania/10218/84 (H5N2);
5 – A/Chicken/Moscow/NJ294598-12/2004 (H7N2);
6 – A/Swine/Hong Kong/9A-1 /98 (H9N2);
7 – B/Phuket/3073/13; 8 – B/Washington/20/2019.

interacts with hemagglutinins of influenza A viruses of H1, H5, and H9 subtypes belonging to subgroup 1 [18] (with the exception of influenza virus H2 subtype) and influenza B viruses both Victoria and Yamagata lines, but does not interact with influenza viruses of the H3 and H7 subtypes belonging to subgroup 2 [25]. According to the literature data, the CR9114 antibody has the ability to bind to hemagglutinins of influenza A viruses belonging to subgroup 2. The dissociation constants K_d of antigen-antibody complexes (1.1–2.2) are comparable to K_d when interacting with hemagglutinins of influenza B viruses of the Yamagata line (1.3.–1.8), and less than the K_d of influenza B viruses of the Victoria line (1.5–4.8) [18]. A possible explanation for the lack of interaction of the rCR9114 antibody we obtained with influenza viruses belonging to subgroup 2 is that this antibody had a light chain of the 27F3 antibody. The light chain of 27F3 belongs to the kappa type of subgroup 3, while the light chain of CR9114 belongs to the lambda type of subgroup 1. Although antigen binding for CR9114 occurs only through the heavy chain, the light chain may be involved in energy redistribution within heavy chains, which contributes to the conformation of individual sections of the heavy chain and interaction with the antigen. It is possible that the presence of a light chain of another antibody did not allow the rCR9114 antibody to bind effectively enough to influenza A viruses belonging to subgroup 2.

The specificity of the r27F3 and rCR9114 antibodies was also assessed using a virus neutralization assay. The results obtained are presented in Table 2.

According to the literature data, the 27F3 antibody in the microneutralization reaction is able to neutralize H1N1, H5N1, and H6N1 influenza viruses belonging to subgroup 1, and H3N2, H7N9, and H10N8 influenza viruses belonging to subgroup 2 [17]. According to Table 2, r27F3 antibody neutralizes H1N1, H5N2, and H9N2 influenza

Table 2. Results of the virus neutralization assay of r27F3 and rCR9114 antibodies with influenza A and B viruses. N.D. – neutralization is not detected

Influenza virus	IK_{50} (μg/mL)	
	r27F3	rCR9114
A/California/04/09 (H1N1)	2.8	5.6
A/Aichi/2/68 (H3N2)	N.D.	N.D.
A/Duck/Pensylvania/10218/84 (H5N2)	1.4	1.4
A/Chicken/Moscow/NJ294598-12/2004 (H7N2)	11.2	N.D.
A/Swine/Hong Kong/9A-1/98 (H9N2)	2.8	2.8
B/Phuket/3073/13	N.D.	N.D.
B/Washington/20/2019	N.D.	N.D.

A viruses from subgroup 1 and H7N2 influenza A virus from subgroup 2. It was not possible to detect neutralization of the influenza A/Aichi/2/68 (H3N2) virus of subgroup 2, although the antibody was able to interact with it according to immunoblotting data.

The ability of the rCR9114 antibody to bind antigens through the virus neutralization reaction is fully consistent with the literature data [18]. The rCR9114 antibody neutralizes influenza A viruses belonging to subgroup 1; neutralization of influenza A viruses from subgroup 2 and influenza B viruses could not be detected.

Thus, it was shown that the r27F3 antibody obtained using adenoviral vectors demonstrates the ability to bind to influenza A viruses of H1N1, H3N2, H5N2, H9N2 subtypes, but does not demonstrate the ability to bind to influenza A virus of H2N3 subtype and influenza B viruses, as it was described in the literature [18].

The rCR9114 antibody also showed the ability to interact with influenza A viruses belonging to subgroup 1 (H1N1, H5N2, H9N2, but not H2N3), but not subgroup 2 (H3N2, H7N2), and in addition, was able to bind to influenza viruses B both the Victoria and Yamagata lines. It is possible that the replacement of the own light chain of the rCR9114 antibody with the light chain of the r27F3 antibody affected the functional activity of the antibody and caused the loss of the ability to bind to subgroup 2 influenza A viruses.

CONCLUSIONS

We obtained recombinant human adenoviruses of the fifth serotype carrying the heavy chain genes of anti-influenza 27F3 and CR9114 antibodies, as well as recombinant human adenovirus carrying the light chain gene of the 27F3 antibody. After determining the conditions for infection of a suspension HEK293 cell line obtained with recombinant adenoviruses, it was shown that the optimal conditions for obtaining the maximum yield of antibodies comprise a dose of 200 PFU/cell for each rAd5 carrying genes of the rAB heavy and light chains followed by co-incubation of viruses and cells for 3 days prior to the onset of 50–60% CPE. The rABs obtained as a result of transduction of the cell suspension with adenoviruses were purified from the culture liquid using the chromatographic method with a yield of 12 mg from 4 L of the culture liquid for r27F3 and 10 mg for rCR9114. The obtained rABs corresponded to native varieties in terms of molecular weight and the possibility of reacting with secondary antibodies to the human IgG Fc fragment. The functional activity of the obtained rAB was assessed using Western blot analysis and virus neutralization assay using influenza A and B viruses of various subtypes. The functional activity of the r27F3 antibody corresponded to the functional activity of the native 27F3 antibody. The loss of the rCR9114 antibody's ability to interact with influenza A viruses belonging to subgroup 2 (H3N2 and H7N2) may be due to the replacement of the light chain of the CR9114 antibody with the light chain of the 27F3 antibody.

Thus, using the described technology, two broad-spectrum anti-influenza antibodies were obtained in preparative quantities and purified. This technology can be used to obtain purified preparations of human rABs in cases where antibody glycosylation is a key issue.

Authors' contributions

M.M. Shmarov, D.N. Shcherbinin – idea, concept, and design of the study;

M.M. Shmarov – approval of the final version of the article for publication;

E.S. Sedova – interpretation of the research results, writing the text of the article;

A.A. Lysenko, I.B. Esmagambetov – consultation on the issues of conducting individual stages of experimental work;

D.N. Shcherbinin, K.A. Pervoyakina, E.A. Bogacheva – recombinant adenovirus production;

N.Yu. Viskova – production of preparative amounts of recombinant antibodies in the culture fluid;

I.B. Esmagambetov, V.V. Prokofiev, E.I. Ryabova – preparative isolation of recombinant antibodies from the culture liquid and HPLC–gel-filtration;

L.V. Verkhovskaya, E.D. Avdonina, A.S. Bandelyuk, A.A. Lysenko – dot blot, protein electrophoresis in polyacrylamide gel, Western blot analysis;

A.S. Bandelyuk, E.S. Sedova – accumulation and concentration of influenza viruses and conducting virus neutralization assay.

The authors declare no conflicts of interest.

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