INTRODUCTION

Passive immunotherapy with antibodies targeting viral capsid components is a promising strategy in the design of new drugs against influenza viruses [1, 2]. This approach is of particular importance because of the high antigenic variation of surface influenza A virus (IAV) proteins that decreases the efficacy of vaccines and low-molecular therapeutic agents. In recent years, neutralization of monoclonal antibodies (mABs) cross-protective against diverse IAV serotypes have been pursued in the design of broad spectrum antivirals [3–7]. A large-scale screening of more than 100,000 individual, cultured antibody-producing B cells selected from several donors with significant heterosubtypic immunity against several IAV subtypes, has been a great success [8]. A unique antibody FI6 that targets the recombinant and natural hemagglutinins of phylogenetic groups I and II was found. The broad specificity of this antibody appears to be associated with the targeting of a conserved epitope in the F subdomain of hemagglutinin, which is less mutation-prone than the HA1 domain. Transfer of this antibody at a dose of 2–20 mg/kg into mice and ferrets after lethal H1N1 and H5N1 challenge conferred full protection. The discovery of this broad-spectrum antibody opens up myriad opportunities for the creation of different recombinant immunoglobulins on its basis.

The respiratory tract is the major route for IAV entry into host cells; hence, intranasal administration of neutralizing antibodies can significantly enhance the effect of passive immunotherapy [9, 10]. The intranasal delivery of recombinant immunoglobulin A, the most
prevalent antibody at human mucosal sites, appears as a very intriguing option [11]. Class A immunoglobulins come in various isoforms (monomer, dimer, secretory form) and, thus, employ different mechanisms for virus neutralization. IgA-isotype antibodies can block virus interaction with the surface of human cells, neutralize the viral particles inside cells, and facilitate the destruction of infected cells by attracting and activating neutrophils [12].

The aim of this study was to produce recombinant variants of antibody FI6 of IgA-isotype and to compare its immunological properties with those of IgG-isotype.

**EXPERIMENTAL**

**Construction of the bi-promoter vector for the expression of recombinant FI6 IgG1-isotype antibodies**

We had previously synthesized [13] cDNA sequences of the variable domain of the heavy FI6 VHv3 and light FI6 VKv2 chains of antibody FI6 [8].

A fragment with a nucleotide sequence encoding the leader peptide MAWVWTLLFMAAAQSAQA and the untranslated regulatory region were fused to the 5’-end region of the previously synthesized cDNA of the heavy chain variable domain FI6 VHv3 through splicing by overlap extension polymerase chain reaction (SOE-PCR). To create the bi-promoter expression cassette, the SOE-PCR DNA fragment was treated with NheI and Bsp120I restriction enzymes and cloned into the pSK+/hEF1- HTLV-BGH vector pretreated with MluI restriction enzyme.

**Construction of bi-promoter plasmids for the expression of recombinant FI6 IgA1- and IgA2m1-isotype antibodies**

The constant heavy-chain domains of IgG1- and IgA2m1-isotypes were obtained as follows. Exons of the corresponding genes were amplified using the human chromosomal DNA template and specific oligonucleotide primers and cloned into an intermediate vector, pAL-TA (Eurogen, Russia). Exons of the constant domains of the same isotypes were spliced by SOE-PCR. The obtained fragments were treated with SacI and Sfr274I restriction enzymes; each of these fragments, the NheI-SacI fragment from pSK+/hEF1-HTLV-FI6HG1-BGH containing cDNA of the leader peptide MAWVWTLLFMAAAQSAQA and cDNA of the variable heavy-chain region of antibody FI6, was cloned into the pSK+/hEF1-HTLV-BGH vector pretreated with NheI and Sfr274I restriction enzymes. Thus, the plasmids pSK+/hEF1-HTLV-FI6HA1-BGH and pSK+/hEF1-HTLV-FI6HA2m1-BGH were obtained that contained the hEF1-HTLV promoter, cDNA of the leader peptide MAWVWTLLFMAAAQSAQA, the variable heavy-chain region of antibody FI6, cDNA of the constant domain of human IgA1- or IgA2m1-isotype (respectively), and an untranslated region which includes the polyadenylation site BGH flanked by MluI restriction sites.

At the final stage of creation of pBiPr-ABIgA2m1FI6 bi-promoter vectors, fragments MluI-MluI (2500 bp) from the plasmids pSK+/hEF1-HTLV-FI6HA1-BGH and pSK+/hEF1-HTLV-FI6HA2m1-BGH, respectively, were inserted into the pOpti-FI6L dephosphorylated vector pretreated with MluI restriction enzyme.

**Preparation of cell lines producing recombinant antibodies**

CHO DG44 cells (Invitrogen, USA) were transfected with linearized pBiPr-ABIgG1FI6, pBiPr-ABIgA1FI6, and pBiPr-ABIgA2m1FI6 plasmids using the Lipofectamine 3000 reagent (Invitrogen, USA) according to the standard protocol. Primary selection of transfected cells was performed using the CD OptiCHO medium (Invitrogen, USA) with addition of 8 mM L-glutamine (Gibco, USA), 0.1% Pluronic F-68 (Gibco, USA), and a 1X antibiotic/antimycotic solution (Gibco, USA). Fluorescent screening and selection of the producer clones were performed to obtain a stable cell line. The cells were seeded on a semi-solid CloneMedia medium (Molecular Devices, USA) with the addition of mouse an-
tibodies to the constant domains of human immunoglobulins G (Molecular Devices, USA) or A (Russian Research Center for Molecular Diagnostics and Therapy, Russia) depending on the isotype of the FITC-labeled recombinant antibodies. After 14 days of cell cultivation, some producing clones were selected using the ClonePix FL device (Molecular Devices, USA) based on fluorescence intensity. The selected clones were cultured in the presence of increasing methotrexate concentrations from 20 to 500 nM to enhance productivity.

**Extraction and purification of recombinant antibodies**

The culture of cells producing recombinant antibodies was grown in spinner flasks with a 500 mL working volume. For this, 2.5–3.0 × 10⁵ cells/mL were seeded in a 300 mL CD OptiCHO medium and grown for 14–18 days in a CO₂-incubator at 37°C, 8% CO₂ and a stirring rate of the spinner of 50–70 rpm.

The culture fluid was centrifuged at 4000 g; 50 mM 2-(N-morpholino)ethanosulfonic acid (MES) and 150 mM NaCl were added to the supernatant, pH 5.7.

The culture fluid containing FI6-IgG was loaded on the Protein G-Sepharose 4B Fast Flow column (diameter 2.5 cm, gel height 3.5 cm, volume 17 mL), pre-equilibrated with a MES solution pH 5.7, at a recirculation rate of 42 mL/h (8.6 mL/h × cm²) for 21 h at 4°C. Antibodies were eluted with 0.1 M glycine buffer at pH 2.7 and an elution rate of 70 mL/h. Immediately after the eluate was obtained, pH was adjusted to ~7.5 with 2 M Tris and concentrated using the 30000 NMWL ultrafiltration membrane to a volume of ≈1.5–2 mL and dialyzed against a phosphate buffer (200X volume) at pH 7.4 overnight.

For affinity chromatography of FI6-IgA1 and FI6-IgA2m1, an immunosorbent based on FabH A₃ mouse monoclonal antibodies (mABs) (Russian Research Center for Molecular Diagnostics and Therapy, Russia) to human immunoglobulin kappa-chain was obtained. Antibodies were immobilized on activated BrCN-sepharose according to Kavran et al. [15]. The immobilization degree of FabH A₃ antibodies was 5 mg per 1 mL of sepharose. The pH of the culture fluid containing FI6-IgA antibodies was adjusted to 8.0 with a 1 M Tris solution and loaded on the column via recirculation for 18 h at a rate of 15 mL/h. For the elution of the FI6-IgA1 and FI6-IgA2m1 antibodies, 0.1 M sodium-acetate buffer, pH 3.0; 0.5 M NaCl; 0.1 M glycine buffer, pH 2.5; 0.5 M NaCl; 0.1 M glycine buffer, pH 2.0; 0.5 M NaCl were consecutively used. All eluates were neutralized with a 1 M Tris solution.

**Immunochemical analysis of recombinant antibodies**

In this work, we used highly purified relic and current strains of IAV produced by Hytest Ltd. (Turku, Finland) and the Research Institute of Influenza RAMS (St. Petersburg, Russia) obtained from infected chicken embryos by successive ultracentrifugation in a sucrose density gradient and inactivation with merthiolate for 24 hours (Table 1). Virus inactivation was confirmed on a MDCK cell culture.

Recombinant antibody titration was performed by indirect, enhanced light immunosorbent assay (ELISA). The inactivated IAV strains were sorbed at a concentration of 5 µg/mL at 4°C overnight in 50 µL of a 0.1 M carbonate buffer at pH 9.2–9.4 in the wells of a 96-well plate with high binding capacity (Corning-Costar, Netherlands). FabH A₃ mABs conjugated to horseradish peroxidase were used as the secondary antibody for detection.

For Western blot, electrophoretic separation of influenza A virus strain A/Solomon Islands/03/06 in 10% Table 1. Characterization of the viral samples used in the work

| Manufacturer | Serotype | Strain/year of isolation |
|--------------|----------|-------------------------|
| Hytest Ltd 8IN73 | Influenza A (H1N1) | A/Taiwan/1/86 |
| Hytest Ltd 8IN73-2 | Influenza A (H1N1) | A/Beijing/262/95 |
| Hytest Ltd 8IN73-3 | Influenza A (H1N1) | A/New Caledonia/20/99 |
| Hytest Ltd 8IN73-4 | Influenza A (H1N1) | A/Solomon Islands/03/06 |
| Research Institute of Influenza | Influenza A (H1N1) | A/California/07/09 |
| Hytest Ltd 8IN74 | Influenza A (H3N2) | A/Samara/222/99=A/Shangdong/9/93 |
| Hytest Ltd 8IN74-1 | Influenza A (H3N2) | A/Panama/2007/99 |
| Hytest Ltd 8IN74-2 | Influenza A (H3N2) | A/Kiev/301/94 |
| Hytest Ltd 8IN74-3 | Influenza A (H3N2) | A/Wisconsin/67/05 |
| Hytest Ltd 8IN74-4 | Influenza A (H3N2) | A/Brisbane/10/07 |
| Research Institute of Influenza | Influenza A (H3N2) | A/Sydney/5/97 |
| Hytest Ltd 8IN75-2 | Influenza B | B/Tokio/53/99 |
polyacrylamide gel upon non-reducing conditions was performed. Electrophoretic transfer (electroblotting) of proteins from the gel to the nitrocellulose membrane S045A330R (Advantec MFS, Inc., USA) was conducted. Transferred proteins were detected on the nitrocellulose membrane by indirect ELISA. The membrane was blocked with a 5% casein solution for 1 h at room temperature on a shaker, rinsed three times with PBS-T (10 mM K$_2$HPO$_4$, pH 7.5, 0.145 M NaCl, 0.1% Tween 20), and incubated for 1 h on a shaker at room temperature. After three times rinsing with PBS-T, the membrane was incubated with a solution of corresponding recombinant antibodies at a concentration of 1 µg/mL in a phosphate-salt buffer for 1 h at 37°C. Western blots were stained by adding a substrate (3,3-diaminobenzidine, 4-chlorine-1-naphthol and hydrogen peroxide).

$K_a$ of the antigen–antibody complex was estimated according to Friguet et al. [16]. At the first stage, mABs at a constant concentration of 1 nM (150 ng/mL) were incubated with an inactivated antigen of the influenza A(H1N1)/Solomon Islands/03/06 strain in a concentration range of 0.1–10 nM (10–1000 ng/mL) for 2 h at room temperature with constant stirring on a shaker to achieve a thermodynamic equilibrium in a three-component system: free antigen, free antibody, and a antigen–antibody complex. At the second stage, the concentrations of free antibodies were measured by solid–phase ELISA with an antigen immobilized on the wellplate. At the final stage, the $K_a$ value was estimated using the Klotz equation [17] from the values of the total antigen concentration and free recombinant antibody concentration.

RESULTS AND DISCUSSION

Recombinant immunoglobulins were generated using nucleotide sequences encoding the variable domains of the heavy Fl6VHv3 and light Fl6VKv2 chains of a broad-spectrum neutralizing antibody Fl6 [8]. Such modified sequences differ from the sequences encoding the heavy and light chains of immunoglobulin Fl6 by the fact that they contain less somatic mutations and correspond more to the variable domain germ-line sequences of human immunoglobulin.

Recombinant IgA1- and IgA2m1-isotype antibodies were obtained to study the ability of the Fl6 antibody to interact with IAV of IgA-isotype. The IgG1-isotype antibody Fl6 was obtained as a positive control.

Human immunoglobulin A comes in two isotypes – IgA1 and IgA2. IgA1 dominates in the serum, while the proportion of IgA2 is higher in secretions [18]. The most significant structural differences between these isotypes are associated with the hinge region. IgA1 has a 13-amino-acid-longer hinge than IgA2, resulting in more flexible antigen-binding sites for the IgA1-isotype antibodies. This advantage renders IgA1 more susceptible to proteolytic cleavage at the hinge region compared to IgA2 [12]. IgA2-isotype antibody comes in two allotypes: IgA2m1 and IgA2m2, which differ in the number of glycosylation sites and, most significantly, the location of inter-chain disulfide bonds [19, 20]. IgA2m1 lacks disulfide bonds between the constant domain of the light chain and the constant domain of the heavy chain (CH1), which are typical for the structure of immunoglobulins. In this case, the disulfide bond forms between the constant domains of light chains and the interaction between the light and heavy chain is non-covalent.

For the expression of recombinant antibodies in CHO cells, we had previously developed a bi-promoter vector which was effective in producing antibodies. This expression vector contains two transcription units, and pCMV and hEF1-HTLV promoters that control the transcription of heavy and light antibody chains in one plasmid. The plasmid also contains the dihydropholate reductase gene (DHFR), which is translated with an independent ribosomal binding site. During amplification of the DHFR gene copies in the chromosome of producer lines by means of methotrexate (MTX) selective pressure, this vector allows simultaneous increase in the light- and heavy-chain gene copy numbers. Three expression plasmids different in the constant domains of immunoglobulin heavy chains were obtained (Fig. 1).

Stable cell lines based on CHO DG44 cells were generated for the production of recombinant immunoglobulins. Recombinant IgG and IgA antibodies were isolated from a serum-free culture medium. After affinity chromatography, recombinant IgG and IgA-isotype antibodies were analyzed using polyacrylamide gel electrophoresis upon reducing and non-reducing conditions (Fig. 2).

An analysis of gel electrophoresis showed that the size of the detected protein fragments reflects the features of the location of inter-chain disulfide bonds in each of the studied isotopes. Thus, two bands corresponding to the light and heavy chains of the immunoglobulins appear on the electrophoregrams of IgG- and IgA1-isotype antibodies upon reducing conditions. The IgA2m1-isotype antibody was found to have a unique location of the inter-chain disulfide bonds characteristic of this isotype. As mentioned previously, IgA2m1-isotype antibodies lack an inter-chain disulfide bond between the constant domain of the light chain and the CH1-domain of the heavy chain, which is common to most immunoglobulins. Herewith, the constant domains of the light chains are interconnected by a di-
sulfide bond. Dimers of the light (~46 kDa) and heavy chains (~105 kDa) are found on gel electrophoregrams (Fig. 2B) upon non-reducing conditions.

The antigen-binding activity of the recombinant proteins was studied by Western blotting with an inactivated A/Solomon Islands/03/06 H1N1 influenza strain (Fig. 3).

Western blot data confirm the ability of the recombinant antibodies to recognize the native hemagglutinin of IAV. Western blotting confirmed previous results on the Fab-fragment of the FL6 IgG1-isotype antibody [13] indicating that antibody FL6 can interact with both whole HA0 hemagglutinin and the HA1 and HA2 fragments formed during hydrolysis of the whole protein in
Table 2. Comparison of $K_d$ values for FI6 recombinant anti-bodies of IgG and IgA isotypes

| Antibody | $K_d$, nM |
|----------|-----------|
| IgG1     | 1.2–1.8   |
| IgA1     | 0.7–1.5   |
| IgA2m1   | 3.3–3.9   |
ated with the unique IgA1 hinge site structure. Overall, our study shows that production of the IgA isotype FI6 antibody does not deteriorate its antigen-binding properties. It is noted that retaining high degrees of antigen-binding and neutralizing properties when reformatting an antibody isotype is not an obvious result as indicated by the data in [22]. It was shown that the chimeric (mouse-human) antibody 9F4 of IgA1 isotype to H5N1 subtype hemagglutinin exerts a lower neutralizing activity compared to parental mouse antibody and the chimeric version of the IgG-isotype.

CONCLUSION
Recombinant monomer antibodies of IgA1- and IgA2m1-isotypes on the basis of variable domains of the broad-spectrum antibody FI6 to influenza A virus hemagglutinin were obtained. These antibodies recognize 10 relic and current IAV strains in indirect ELISA and are characterized by a $K_d$ value of the antigen–antibody complex no higher than 4 nM. The affinity of the studied antibody samples to the IAV strains of the H1N1 subtype is higher than the affinity to the H3N2 subtype strain. Our data show that production of antibody FI6 of monomer IgA form does not change its antigen-binding properties, which is an important prerequisite for the use of IgA-isotype antibodies for therapeutic purposes.

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