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METHODS

Development and Properties of Recombinant Proteins Based on the Broadly Neutralizing Antibody to Influenza A Virus

T. K. Aliev[a]*, I. G. Dement'yeva[b], V. A. Toporova[c], M. N. Bokov[b], L. P. Pozdnyakova[b], V. S. Rybchenko[d], D. A. Dolgikh[d], P. G. Sveshnikov[b], and M. P. Kirpichnikov[d]

[a] *Department of Chemistry, Moscow State University, Moscow, 119234 Russia*
[b] *Russian Research Center for Molecular Diagnostics and Therapy, Simferopol bul'v. 8, Moscow, 117638 Russia*
[c] *Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, GSP-7, Moscow, 117997 Russia*
[d] *Department of of Biology, Moscow State University, Moscow, 119234 Russia*
**e-mail: ta12345@list.ru*

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Abstract—We studied the possibility of using a broadly neutralizing anti-influenza A antibody as a module for the development of different protein constructs for diagnostics. For this purpose, we constructed two recombinant proteins—a Fab-fragment of the antibody and Fab-mCherry, which is a hybrid of the Fab-fragment and the mCherry fluorescent protein. Both proteins were expressed in *Escherichia coli* cells and purified in a functionally active state from culture medium. The antibody Fab-fragment was shown to bind all 11 tested strains of the influenza A H1N1 and H3N2 subtypes. A stronger binding was observed for group I hemagglutinins; this correlates with the immunochemical profile of the parental antibody. Comparison of the dissociation constants of complexes of the antibody Fab-fragment and Fab-mCherry with A(H1N1)/Solomon Islands/03/06 virus particles demonstrated that the attachment of the mCherry protein did not interfere with the antigen-binding properties of the antibody Fab-fragment.

Keywords: influenza A virus, broadly neutralizing antibodies, mCherry protein, Fab-fragment, expression of recombinant proteins, *Escherichia coli*.

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INTRODUCTION

Annually, 5 to 15% of the world population comes down with influenza and, for 250000–500000 people, this infection is lethal [1]. The most virulent of three types of influenza viruses is the type A virus, which can infect humans and animals. Several of its subtypes caused epidemics and pandemics. The reason for such a high virulence of the type A influenza virus (IVA) is variability of surface antigens of the viral capsid, which are hemagglutinin and neuraminidase.

For many years, preparation of an immune serum with a broad specificity to different IVA subtypes has been one of the highest priorities of modern pharmacology. Several successful examples of the preparation of antibodies heterospecific to several of the 16 subtypes known at the time have been described [2]. Until recently, however, it has not been possible to obtain an antibody with specificity to all the known IVA subtypes. A significant breakthrough in this field was achieved as a result of a large-scale screening of more than 100000 individual cultured antibody-producing B-cells of several donors, whose immune response against a number of IVA subtypes was significantly heterotypical [3]. As a result, a unique neutralizing antibody FI6 was found that can bind recombinant and natural hemagglutinins of subgroups 1 and 2 with the EC_{50} values of 10 to 270 ng/mL. It has been demonstrated in mice and ferrets infected by a lethal dose of IVA strains H1N1 and H5N1 that the full protection is achieved by administering the antibody in dosages of 2 to 20 mg/kg body weight.

The discovery of the antibody FI6 opens up new possibilities to develop vaccines, therapeutic preparations, and diagnostic tools for IVA of various subtypes. Moreover, different protein molecules that are created by bioengineering and contain antigen-binding regions of the desired antibody can be used as therapeutic or diagnostic agents [4]. Many of such constructions are based on the use of single-chain antibodies and Fab-fragments. These protein modules retain antigen-binding properties of variable domains of the parent antibody but, in contrast to the full-sized antibodies, can be efficiently expressed in various cells, particularly in yeast and *E. coli* cells.

The aims of this work were to obtain and study a recombinant Fab-fragment of the broadly specific

antibody FI6 and Fab-mCherry, which is a hybrid of the Fab-fragment and the mCherry fluorescent protein [5]. The objectives of this study were to elucidate the possibility to express these recombinant proteins in *E. coli* cells in active form and to examine their capacity for high-affinity interaction with IVA of different subtypes.

MATERIALS AND METHODS

Nucleotide sequences of variable domains of the FI6 antibody were constructed by chemical-enzymatic synthesis using polymerase chain reaction and a set of oligonucleotide primers with mutually overlapping terminal regions. The assembly of a bicystronic expression plasmid for producing a Fab-fragment of the FI6 antibody in *E. coli* cells and the biosynthesis and purification of the protein were performed according to the previously described scheme [6, 7]. The constructed nucleotide sequences of variable domains were combined with constant domains of human immunoglobulin G (IgG1, kappa). The DNA sequence encoding the light chain of the Fab-fragment was inserted into the vector pTrc99A that contained precloned gene of the stII signal peptide of the thermostable *E. coli* endotoxin II. The DNA sequence encoding the heavy chain of the Fab-fragment was inserted into the auxiliary vector pBluescript SK(+) containing the sequence of the signal peptide stII, the Shine-Dalgarno sequence, and a dodecahistidine tag. In the next step, the expression cassette comprising the heavy chain of the Fab-fragment and the abovementioned components of the auxiliary vector was inserted into the pTrc99A vector containing the light chain. Consequently, the bicystronic expression vector pTrcLHFI6 was produced, in which the light and heavy chains of Fab-fragment were transcribed from the single trc-promoter and the translation initiation of each chain occurred independently on the regions located before each encoding sequence in the bicystronic vector.

To create a hybrid protein with FabFI6, the gene of the mCherry fluorescent protein was produced by PCR using as a matrix the pmCherry-C1 plasmid (Clonetech, United States) and specific oligonucleotide primers encoding XhoI and SalI restriction sites. The produced fragment, which did not encode an N-terminal methionine amino acid residue, was treated with restriction endonucleases XhoI and SalI and inserted, using the XhoI site, into the auxiliary vector containing a dodecahistidine tag. The obtained plasmid was treated with restriction enzymes XhoI and HindIII, and the resulting 757-bp fragment containing the gene of the pCherry protein with a dodecahistidine tag was ligated with the 5583-bp fragment obtained by treating the pTrcLHFI6 plasmid with the same pair of restriction enzymes. The vector pTrcLHFI6-Ch, in which the Cherry protein was located on the C-terminus of the constant C_H1 domain of the heavy chain of the FI6 antibody, was obtained as the result.

To produce the recombinant Fab-fragments of the FI6 antibody and the recombinant protein FabFI6-mCherry in the BL-21 (DE-3) *E. coli* cells, we used the autoinduction method [8], according to which cells were grown for 48 hours at 32°C and 220 rpm on a shaker in the 2ZY nutrient medium, which contained 2 mM $MgSO_4$, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 0.5% glycerin, 0.05% glucose, 0.2% α-lactose monohydrate, and ampicillin. After cultivation, the cells were pelleted by centrifugation; recombinant proteins were isolated from the culture medium by metal affinity chromatography on a Co^{2+}-iminodiacetate-sepharose carrier (GE Healthcare, United States) as previously described [7].

For immunochemical characterization of Fab-fragment of FI6, we used a set of highly purified relic and current strains of IVA (Fig. 1) produced by Hytest Ltd. (Turku, Finland) and Research Institute of Influenza, Russian Academy of Medical Sciences (St. Petersburg, Russia). The strains were obtained from infected chick embryos by sequential ultracentrifugation in sucrose density gradient and subsequent inactivation with merthiolate for 24 hours. The inactivation of viruses was confirmed on the MDCK cell culture. For indirect enzyme immunoassay, we used mouse monoclonal antibodies (MoAb) F8 to nucleoprotein and C102 to hemagglutinin H1 IVA (both antibodies were previously obtained in the Russian Research Center for Molecular Diagnostics and Therapy). The specificity of the recombinant proteins was confirmed by immunoblotting. For the immunoblotting, antigens of different IVA strains of H1N1 and H3N2 subtypes were first subjected to electrophoretic separation in 12% polyacrylamide gel under reducing conditions and then transferred to a nitrocellulose membrane. The transferred proteins were detected using the antispecies peroxidase conjugate of goat antibodies against mouse IgG (for antibodies C102 and F8) and the peroxidase conjugate of MoAb 4G7 against human kappa chain (for Fab-fragment of FI6) for 1 hour at 37°C.

To determine the affinity of the Fab-fragment and the hybrid protein FabFI6-Cherry, we used indirect enzyme immunoassay (EIA), which makes it possible to determine the concentration of free antibodies in an antigen-antibody mixture upon reaching equilibrium at room temperature [9]. The A(H1N1)/Solomon Islands/03/06 strain was used as the antigen. The dissociation constant was calculated by the Klotz equation [10].

RESULTS AND DISCUSSION

To synthesize the genes of variable domains of the heavy and light chains of the FI6 antibody broadly

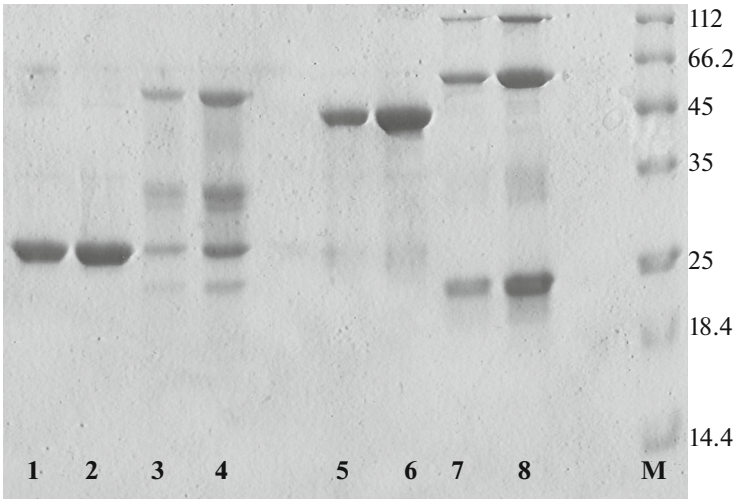

Fig. 1. Gel-electrophoretic analysis (12% polyacrylamide gel with sodium dodecyl sulfate) of the proteins FabFI6 (lanes 1, 2, 5, and 6) and FabFI6-mCherry (lanes 3, 4, 7, and 8) obtained after metal-affinity chromatography. M—protein molecular weight markers. The samples on the lanes 1–4 were reduced by β-mercaptoethanol. The amount of protein in the samples: lanes 1 and 5—2 μg protein; lanes 2 and 6—1 μg protein; lanes 4 and 8—2.5 μg protein; and lanes 3 and 7—1.25 μg protein.

specific against known IVA subtypes, we used the FI6VHv3 amino acid sequence for the heavy chain and the FI6VKv2 amino acid sequence for the light chain [3]. These sequences are a modified version of the initial antibody FI6; this version is more similar to the sequence of variable domains of the human immunoglobulin germlines. Based on the amino acid sequence, nucleotide sequences of the variable domains of light and heavy chains were compiled taking into account the frequency of codon occurrence in *E. coli* genes; the chemical-enzymatic synthesis of the respective DNA fragments was carried out.

A bicistronic plasmid pTrcLHFI6 was constructed to express the recombinant Fab-fragment of the FI6 antibody. The presence of the thermostable *E. coli* enterotoxin signal peptide stII on the N-termini of the translated light and heavy chains made it possible to transfer the mature Fab-fragment of the antibody directly into the culture medium.

In order to assess whether the Fab-fragment of the FI6 antibody can be used as the main component of recombinant constructs without significantly altering its antigen-binding properties, we proposed to obtain and study the affinity of the FabFI6-mCherry hybrid protein. The mCherry protein is a mutant form of the DsRed fluorescent protein from *Discosoma sp.* [5].

Based on the nucleotide sequence encoding mCherry, we constructed an expression plasmid pTrcLHFI6-Ch, which ensured biosynthesis of the hybrid protein FabFI6-mCherry in *E. coli* cells.

The FabFI6 and FabFI6-mCherry recombinant proteins were expressed by autoinduction of BL-21 (DE-3) *E. coli* cells transformed with the plasmids pTrcLHFI6 and pTrcLHFI6-Ch. The proteins were isolated from the culture medium by metal-affinity chromatography. The results of the gel-electrophoretic analysis of the purified proteins under reducing and nonreducing conditions are given in Fig. 1.

It turned out that the mobility of the FabFI6 protein in nonreducing conditions corresponds to the theoretically calculated molecular mass of 49.2 kDa, whereas, in the presence of β-mercaptoethanol, the interchain disulfide bond dissociates to form the heavy and light chains of the Fab-fragment. The molecular weight of the FabFI6-mCherry protein is 77.9 kDa. The molecular weight of its hybrid heavy chain carrying the mCherry protein on the C-terminus is ~54 kDa. The analysis of the FabFI6-mCherry protein after electrophoresis in reducing and nonreducing conditions indicates the presence of an ~23 kDa band that differs by its electrophoretic mobility from the light chain. The emergence of this fragment is described in Gross's study [11], in which it has been shown that a labile acylimine bond between residues Phe65 and Gln66 in the chromophore of the DsRed protein hydrolyzes upon boiling of the electrophoretic samples.

We studied the antigen specificity of the recombinant protein FabFI6 against several strains of the subtypes H1N1 and H3N2, which belong to different subgroups of IVA (Table 1).

It should be noted that an interaction study of a number of these strains with the full-length antibody FI6 was carried out previously [3]. For the immunochemical analysis of FabFI6, we also used other strains of H1N1 and H3N2 (given in bold in Table 2) that had not been used in Corti's study [3].

It follows from the results of indirect EIA of FabFI6 with different IVA strains (Table 2) that the recombinant Fab-fragment of the FI6 antibody retains

Table 1. Information on the virus preparations used for immunochemical characteristics of the recombinant proteins obtained in this study

Supplier	Subtype	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, RAMS	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, RAMS	Influenza A (H3N2)	**A/Sydney/5/97**

Table 2. Results of the FabFI6 titration in the indirect EIA (optical density at the wavelength of 450 nm) obtained using four IVA/H1N1 strains and five IVA/H3N2 strains for the immobilization; strain B/Tokyo/53/99 of the influenza virus B was used as a negative control

Antigen FabFI6, μg/mL	A(H1N1)/				A(H3N2)/					B/Tokyo/ 53/99
	Taiwan/ 1/86	*Beijing/ 262/95*	*New Caledoni/ 20/99*	*Solomon Islands/ 03/06*	*Samara/ 222/99*	*Panama/ 2007/99*	*Kiev/ 301/94*	*Wisconsi/ 67/05*	*Brisbane/ 10/07*	
1	*	*	*	*	0.776	2.264	0.887	1.123	1.185	0.394
0.3	*	*	*	*	0.331	1.419	0.213	0.261	0.316	0.176
0.1	*	*	*	*	0.297	1.340	0.117	0.122	0.116	0.109
0.03	2.638	2.669	2.444	2.953	0.163	1.004	0.086	0.084	0.068	0.092
0.01	1.359	1.140	0.767	1.567	0.109	0.572	0.050	0.074	0.044	0.097
0.003	0.625	0.319	0.255	0.422	0.055	0.247	0.036	0.060	0.034	0.138
0.001	0.337	0.120	0.163	0.170	0.030	0.123	0.026	0.050	0.031	0.178

the ability of the "parent antibody" to be specific against the strains of subtypes of the hemagglutinin subgroups 1 and 2. Just as the full-sized human antibody, FabFI6 has a higher affinity to strains of the H1N1 subtype than to H3N2.

Figure 2 shows the result of comparing the cross-reactivity of the recombinant FabFI6 with MoAbs F8 and C102. The results of indirect EIA show that the MoAb F8 directed against a conserved IVA epitope interacts with all the studied strains of the subtypes H1N1 and H3N2. The MoAb C102 recognizes only hemagglutinin molecules of the strain H1N1. FabFI6 binds with hemagglutinins of all the strains; notably, the subtype H1N1 is better recognized than the subtype H3N2.

Specificity of FabFI6 to hemagglutinins from IVA of different strains was confirmed by immunoblotting (Fig. 3). It follows from the immunoblotting results that FabFI6 interacts with full-sized hemagglutinins HA0 of all the studied strains and also, preferably, with fragments of hemagglutinins HA1 and HA2, which are formed during the electrophoresis of IVA antigens under reducing conditions. Hydrolysis of the full-sized hemagglutinins during gel-electrophoresis under reducing conditions was observed when analyzing recombinant hemagglutinins [12]. The ability of FabFI6 to interact with HA1 and HA2 is explained by the fact that the FI6 MoAb is targeted against the hemagglutinin F-subdomain, which is located at the junction of the domains H1 and H2 [3]. In this process, the heavy chain of the antibody interacts with the H1 domain and the light chain interacts with an alpha-helix of the H2 domain.

Combined data from EIA and immunoblotting indicate that the FI6 antibody Fab-fragment

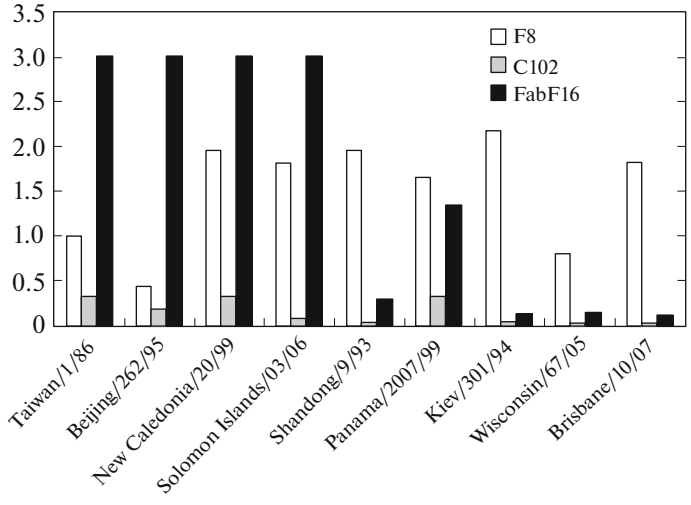

Fig. 2. Cross-reactivity diagram of MoAbs F8, C102, and FabFI6 based on the data of indirect EIA with four immobilized IVA/H1N1 strains and five immobilized IVA/H3N2 strains at the antibody concentration of 100 ng/mL. The Y-axis indicates optical density at the wavelength of 450 nm at the MoAb concentration of 0.1 μg/mL.

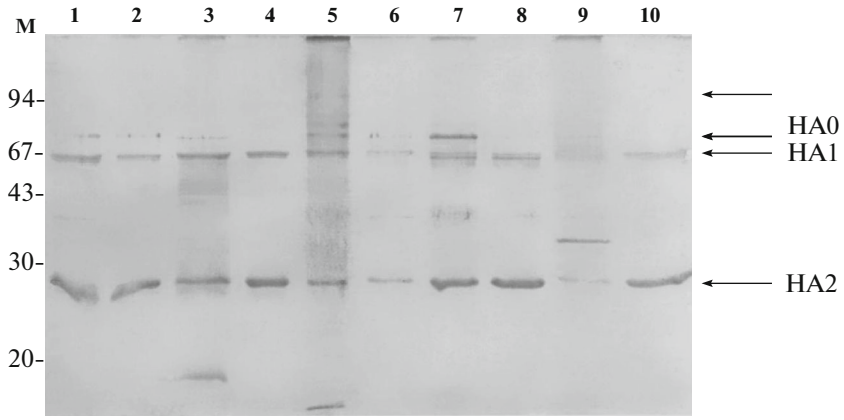

Fig. 3. Immunoblot of the IVA antigens from different strains with FabFI6 under reducing conditions after the separation in 12% polyacrylamide gel in the presence of sodium dodecyl sulfate. Lanes 1 to 5—IVA/H1N1; lanes 6 to 10—IVA/H3N2; lane M—molecular weight standards, kDa; lane 1—A/Taiwan/1/86; lane 2—A/Beijing/262/95; lane 3—A/New Caledonia/20/99; lane 4—A/Solomon Islands/03/06; lane 5—A/California/07/09; lane 6—A/Samara/222/99; lane 7—A/Panama/2007/99; lane 8—A/Kiev/301/94; lane 9—A/Wisconsin/67/05; and lane 10—A/Brisbane/10/07.

expressed in *E. coli* is functionally active and has specificity to all the studied IVA strains.

To assess the effect of the addition of the mCherry protein to the C-terminal part of the FabFI6 heavy chain on the antigen-binding activity of the antibody, we determined the dissociation constants (K_d) of the antigen-antibody complex for FabFI6 and FabFI6-mCherry. Averaged values of K_d were of 3.8×10^{-9} M for FabFI6 and of 3.1×10^{-9} M for FabFI6-mCherry. This result indicates that the antigen-binding activity of the hybrid protein does not decrease in comparison with the respective parameter for the parent Fab-fragment.

CONCLUSIONS

The studies showed that the recombinant protein FabFI6 constructed on the basis of variable domains of the FI6 antibody, which is broadly selective to hemagglutinins of IVA, is expressed in *E. coli* in a functional state. According to the data of immunochemical assay performed using 11 IVA strains of subtypes H1N1 and H3N2, FabFI6 has a cross-specificity comparable with the respective parameter of the parent antibody [3]. Comparison of the affinity of proteins FabFI6 and FabFI6-mCherry demonstrated that the recombinant protein FabFI6 can be used successfully as a basic module to produce recombinant constructs on its basis without affecting the antigen-binding parameters.

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