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ENGINEERING OF A RECOMBINANT FAB ANTIBODY FRAGMENT AGAINST CORTISOL

The paper dwells upon the molecular cloning of genes coding the structure of mouse immunoglobulins, which can specifically bind steroid hormone cortisol. For these purposes primers covering all families of genes, which code the structure of immunoglobulins G. Molecular-genetic constructions for effective expression of recombinant antibodies in cells of *Escherichia coli* were constructed. Heterologous expression of Fab-fragments was performed. Recombinant protein has been isolated in homogenous state.

Introduction. Cortisol (Fig. 1) is one of the major steroid hormones and is required to maintenance many functions of the human organism. As well as other glucocorticosteroids, cortisol is synthesized by the zona fasciculata of adrenal cortex from the common predecessor cholesterol [1]. Measuring of the cortisol level in blood is of great importance for monitoring of functions of pituitary and adrenal glands. There is a great set of diagnostic systems for quantitative determination of cortisol in biological fluids. The majority of them anyway use the principle of affine recognition of cortisol by the appropriate antibodies (immunoglobulins). The modern task is to improve functional properties of the recognition module of the diagnostic systems, and the reduction of their prime cost either. One of the possible solutions of this task lies in the using of recombinant antibodies possessing the wider range of properties in comparison with full-scale antibodies.

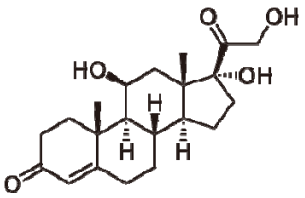

Fig. 1. Structural formula of cortisol

Immunoglobulins are a family of the glycoproteins presented in blood plasma and tissue fluids of the vertebrata, capable to distinguish and bind the foreign for organism substances (anti-genes). The antigene surface, with which the antibody interacts, is called an epitope, and the site of antibody by means of which it interacts with the antigens called a paratope [2].

There are five classes of immunoglobulins in highest mammals: IgG, IgM, IgA, IgE and IgD. The greatest practical interest is represented by the most widespread class – IgG, which is presented in a human body by four subclasses (isotypes): IgG1, IgG2, IgG3 and IgG4.

Immunoglobulin is the multimeric protein consisting of two identical heavy chains and two light chains (Fig. 2).

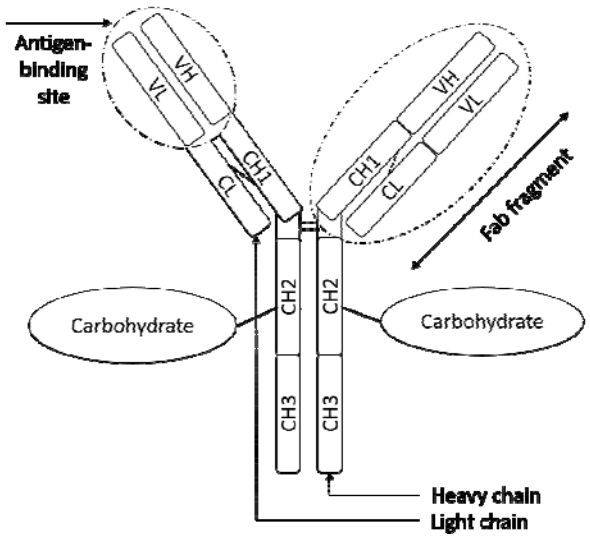

Fig. 2. Molecular structure of immunoglobulin

The molecule of immunoglobulin can be conditionally divided into two functional subunits – Fab-fragment (fragment-antigen binding) and Fc-fragment (fragment crystallizable). The Fc-fragment consists of two pairs of constant domains (CH2 and CH3) and is responsible for interaction with cellular receptors at the activation of the immune response, and with compliment system proteins. The Fab-fragment participates in recognition of an antigene and consists of one variable (VL and VH) and one constant (CL and CH1) domain. Polypeptide chains are connected with each other by disulfide bonds and non-covalent interactions [3]. Besides, the CH2 site a molecule is glycosylated.

With the development of the molecular genetic technologies mini-antibodies became a common practice: Fab-(fragment antigen binding) and scFv-(single chain fragment variable) fragments capable to bind antigene specifically, but possessing bigger penetration capability due to their small size than

full-size antibodies [4]. The Fab-fragment is a protein with a molecular weight of 50 kDa, consisting of a heavy chain fragment (VH and CH1) and the light chain connected together by disulfide bonds and non-covalent interactions. The fragment consisting only of variable fragments (VH and VL), connected among themselves with a peptidy linker, is a scFv-fragment and it has a molecular weight of 15 kDa. Recombinant mini-antibodies can be produced in the bacterial expression system, unlike the full-size immunoglobulins Fc-domain of which is glycosylated and, thus, requires the systems of post-transmitting modification [5]. It is easy to manipulate the structure and, respectively, the physical and chemical properties of fragments of antibodies at the gene level, and then they can be turned into the full-size antibodies to acquire the efector functions [5, 6].

Main part. The main objective was the receiving of high-affinity antibody Fab-fragments against steroid hormone cortisol.

The monoclonal antibodies produced by hybridomas 5G-H2 (are received in Laboratory of protein hormone chemistry IBOH NAS of Belarus) are capable to specifically bind steroid hormone cortisol. However, the use of the hybridoma cell lines producing monoclonal antibodies has a number of essential shortcomings such as instability of cellular lines, the high price of cultivation, impossibility to carry out genetically engineered manipulations with genes of immunoglobulins [5]. Besides, the antibodies received by hybridomic technology may contain impurity of mouse immunoglobulins that increase probability of false positive results when using in diagnostic systems. All these restrictions may be evaded by using of recombinant antibodies received by means of genetically engineered methods and expressed in bacterial system.

Allocation of total RNA from the 5G-H2 hybridomas producing specific antibodies to a steroid hormone cortisol was the first stage. Total RNA was allocated by means of SV Total RNA Isolation kit (Promega) and was used at once for conducting reaction of the reverse transcription with oligo-$(dT)_{18}$ primers for receiving cDNA (Reverta-L kit reagents of Central Research Institute of Epidemiology of Rospotrebnadzor was used).

Synthesized cDNA was used as a matrix for amplification of the genes coding the structures of the heavy and light chains of immunoglobulin. On the basis of the analysis of literary data [7, 8] and the nucleotide sequences of immunoglobulins from the *IMGT.org* database the specific degenerate primers covering all variety of genes of cDNA, coding light and heavy chains of mouse immunoglobulins were designed. Amplification was carried out in BioRad T100 amplifier under following conditions: denaturation of 94ºC 4 min; 30 cycles – denaturation of 94ºC 1 min, annealing of 55ºC 1 min, elongation of 72ºC 1 min. The final stage of elongation – 72ºC 10 min.

Amplified fragments were divided by the gel electrophoresis method in 2% agarose gel with the use of TAE-buffer at the presence of ethidium bromide (results were registered by means of Gel Imager 2 ("Vilber Lourmat") videosystem). In every PCR reaction the positive and negative control of amplification was carred out.

After PCR products of amplification were cloned in a pXcmkn12 plasmid vector and sequenced for the confirmation of correctness of sequence of cloned DNA (nucleotide sequence reading was carried out with the use of sets of "ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycler Sequencing Kit", "Big Dye X Terminator Purification Kit" and the Applied Biosystems 3130 sequencer). Sequence revealed that the cloned heavy chain belongs to the G2a type that is corresponds to the isotype of antibodies produced by hybridoma, and the easy chain belongs to the κ-type.

For expression of Fab-fragments of antibodies specially designed plasmid vector pCW-Fab was used, which provides the high level expression of the recombinant antibodies in the periplasmic space of *Escherichia coli* [3, 9].

For carrying out preparative expression *E. coli* DH5α cells, transformed by the bicistronic vector of pCW-Fab containing cloned genes of the Fab-domain, were used.

Cultivation of *E. coli* DH5α cells was carried out on the TB medium. Induction of cellular culture to synthesis of recombinant protein was carried out by means of IPTG (isopropyl-β-D-1-thiogalactopyranoside), starting the transcription by means of induction of lactose operon.

Cells were destroyed with the use of a homogenizer of Emulsiflex-C5 with the addition of detergent (CHAPS), inhibitor of serine proteases (PMSF) and β-mercaptoethanol. The destroyed cells were precipitated by centrifugation. Supernatant passed through a chromatographic column with ProteinA sepharose. ProteinA is protein, expressing in the cells of golden staphylococcus *Staphylococcus aureus* and capable to specifically bind the CH1 domain of the immunoglobulins heavy chain. It allows to use a method of an affinity chromatography for purification of recombinant Fab-fragments (the system of allocation and purification of Bio-Rad "Bio-Logic" proteins was used).

Homogeneity of the received protein was controlled by means of PAAG electrophoresis at reducing conditions, which showed the existence of two polypeptide chains with the expected molecular weight, this fact confirms the efficiency of the created structures for recombinant Fab-fragments expression.

Conclusion. The molecular cloning of the genes coding the structure of mouse immunoglobulin specific to cortisol was carried out. The results of the sequence confirmed the belonging of the cloned fragments to a class of immunoglobulins. The created molecular and genetic structures allowed to carry out a heterological expression of recombinant Fab-fragments in *E. coli* cells. Recombinant protein in a homogeneous state with the correspondent to our expectations molecular weight is received. Properties of the recombinant Fab-fragments of antibodies require the further studying.

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