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IN SILICO ANALYSIS OF THE STRUCTURE OF VARIABLE DOMAINS OF MOUSE SINGLE-CHAIN ANTIBODIES SPECIFIC TO THE HUMAN RECOMBINANT INTERFERON β1b

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In silico analysis of the DNA encoding single-chain Fv antibodies (ScFv) specific to the human recombinant interferon β1b and α2b (rhIFN-β1b, rhIFN-α2b) has been carried out. The V-, D- and J-gene segments, the complementarity-determining (CDR) and framework (FR) regions, n-nucleotides as well as mutation rates which take place during the affinity maturation of the examined sequences have been determined. For the panel of ScFv against rhIFN-β1b isolated from an immune combinatorial cDNA library uniqueness of the CDRH3 loop by the length and amino acid composition has been shown. Multiple alignments with the nearest homologies from the NCBI databases have revealed that the sequences of ScFv obtained are new.

Introduction. An important feature of antibodies is the extraordinary variability of their antigen-binding sites ensuring specific and high-affinity interaction with the target antigen. Due to their high binding ability antibodies are of great value as unique molecular probes for fundamental research, as well as in biotechnology and medicine.

Modern gene manipulation techniques enable generation of recombinant antibody fragments — single-chain antibodies (ScFv's), as well as their production in *Escherichia coli* cells. ScFv's are obtained by translating DNA sequences of immunoglobulin heavy-chain (V_H) and light-chain (V_L) variable domains joined in one gene [1]. The approaches to construction and selection of combinatorial cDNA libraries of *V*-genes *in vitro* allow isolation of ScFv with desired affinity and specificity, bypassing the stages of traditional hybridoma technology [2, 3].

The diversity of antigen-binding regions of antibody variable domains is defined by the structure of the underlying encoding gene elements. The genes of the light chain result from recombination of one variable (*V*) and joining (*J*) gene segments. Recombination events in the heavy chain are more complex due to the presence of an additional *D*-segment. One of the mechanisms of increasing diversity is incorporation of n-nucleotides (nontemplated nucleotides) at the gene segment joint site [4]. Later on, the DNA sequences of variable (V) domains are affected by the influence of somatic hypermutations taking place during the affinity maturation of B-cells. The highest rate of point mutations occurs at complementarity determining regions (CDR), as opposed to framework regions (FR) where the rate is relatively low.

Up to date, there have been created a number of databases of antibody germline gene segments, as well as software for identification of *V*-, *D*-, and *J*-gene segments, n-nucleotides, CDR and FR segments, mutation rates, etc. A detailed analysis of nucleotide and aminoacid sequences of antibody V-domains is needed for a number of reasons. First of all, it allows determining the degree of conformational uniqueness of the antigen binding site of the obtained antibodies, which can be achieved by comparing their aminoacid sequences with yet known immunoglobulin sequences. Second, while analyzing multiple antibody variants against one antigen (a panel of antibodies), determining *V*-, *D*-, and *J*-gene segments, n-nucleotides, and mutations rates enables evaluation of the panel's heterogene-

ity and proper choice of the most promising candidate molecules for further work. However, what makes most sense is that determining CDR and FR regions of antibody V-domains is an indispensable prerequisite for beginning manipulations to improve their functional characteristics, such as decreasing immunogeneity (in case of humanization), or DNA combinatorial mutagenesis — an *in vitro* technique used to increase affinity and stability of obtained antibodies.

The most widespread strategy for humanization is grafting of CDR regions of an antigen-specific monoclonal antibody with FR regions of selected V-domains of human antibodies [5]. An example of the practical value of the abovementioned technology is the fact that today out of 30 monoclonal antibody-based drugs approved by FDA (Food and Drug Administration, USA) for clinical application over one third are humanized antibodies [6].

The core of the combinatorial mutagenesis technique lies in directed randomization of certain regions of V-domains using a set of oligonucleotides with degenerate codons. Randomization targets may include CDR3 regions of V_H and V_L domains [7], all six CDRs [8], or certain aminoacids of V-domains [9].

The indicated method is particularly valuable while working with naïve and synthetic libraries of antibody fragments («single-pot» antibody libraries), as combined with the phage display it allows isolation of antibody molecule variants with improved characteristics of expression in bacteria, prolonged stability in blood stream, as well as exclusively high affinity to the target antigen [8].

Our previous work resulted in obtaining and characterization of a panel of mouse ScFv's against rhIFN-β1b. The goal of the present work was sequencing and *in silico* analysis of the primary structure of the ScFv's obtained.

Materials and methods. DNA sequencing was carried out using the automatic DNA sequencer IBI Prism 3130 (Applied Biosystems, USA). We sequenced DNA (approx. 1000 bp) obtained by polymerase chain reaction (PCR) using the following primers: pCANTAB-R1 5'-d [CCATGATTA-CGCCAAGCTTTGGAGCC]-3', pCANTAB-R2 5'-d [CGATCTAAAGTTTTGTCGTCTTTCC]-3' and preliminary obtained recombinant phagemids of the corresponding bacterial clones as matrices. For sequencing, we used the following primers:

pCANTAB 5-S3: 5'-d [GGTTCAGGCGGAGGTG-GCTCTGG] – 3';
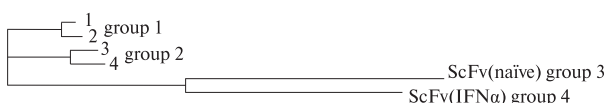pCANTAB 5-S4: 5'-d [CCAGAGCCACCTCCGCC-TAACC] – 3',
pCANTAB 5-S1: 5'-d [CAACGTGAAAAAATTAT-TATTCGC] – 3';
pCANTAB 5-S6: 5'-d [GTAAATGAATTTTCTGTAT-GAGG] – 3'.

Sequencing results were processed and interpreted with assistance of Vector NTI Advance 10 (Invitrogen, USA) software. The level of homology between ScFv nucleotide sequences was determined using ClustalW2 software (http://www.ebi.ac.uk / Tools/clustalw2/) [10]. Identification of *V-*, *D-*, and *J*-gene segments encoding ScFv specific to rhIFN-β1b and rhIFN-α2b, was assisted by Somatic Diversification Analysis (SoDA) software (http://dulci.org/soda/) [11]. All gene segment names are used in accordance with the standardization rules of the international information system IMGT [12]. Additional n-nucleotides and mutations that occurred during antibody affinity maturation were determined by comparing the target sequences with respective germline gene sequences of *V-*, *D-*, and *J*-gene segments from the IMGT database. CDR and FR regions were determined with the help of SoDA software in accordance with the criteria developed by IMGT. In view of this, we labeled the indicated regions as CDR-IMGT and FR-IMGT [13].

Search of closest homologs for the aminoacid sequences was performed using IgBLAST software (http://www.ncbi.nlm.nih.gov/igblast/) of the immunoglobulin V region sequences database of the National Center for Biotechnology Information (NCBI), USA. First, we looked for homologs to a whole ScFv sequence, and then separately for variable domains V_H and V_L. For sequences with highest homology, we built multiple alignments using Vector NTI Advance 10 software.

Results and Discussion. In previous publications, we described generation of an immune combinatorial cDNA library of mouse immunoglobulin *V*-genes and isolation of a panel of single-chain antibodies specific to rhIFN-β1b [14, 15]. For some isolated ScFv's such characteristics as binding specificity to native and denatured antigen, level of production in *E. coli* by secretion, stability etc. were determined. An interesting point was that some producers randomly selected out of more

A dendrogram reflecting the homology between nucleotide sequences of ScFv's carried out using ClustalW2 software. #1, #2, #4, #11 are ScFv's isolated from an immune cDNA library and specific to rhIFN-β1b; ScFv (naïve) — ScFv's isolated from a naïve cDNA library and specific to rhIFN-β1b; ScFv (IFNα) — ScFv's specific to rhIFN-α2b.

than 30 isolated positive clones invariably differed between each other in the production level of the ScFv's (0.6 to 3.5 mg/L) and their accumulation rate, ScFv's sensitivity to proteolysis, ScFv's affinity ($1.96 \cdot 10^{-8}$ M to $1.69 \cdot 10^{-9}$ M), and ScFv's binding specificity with native and denatured rhIFN-β1b [15].

In many experiments dedicated to study expression of ScFv in *E. coli*, the influence of the primary structure of variable domains on their production rate has been shown. It is also known that replacement of at least one aminoacid in CDR regions may have dramatic influence on antibody affinity and specificity [16]. The differences in production rates in bacterial cells, as well as various antigen-binding characteristics of some ScFv's from the obtained panel seem to suggest nonidentity of the primary structure of their variable domains. To test this suggestion we sequenced the DNA of all

ScFv's identified as positive in the reaction of binding to rhIFN-β1b. Among the 12 analyzed sequences isolated from different producers, we revealed four DNA variants that encode ScFv with different primary structure — #1, #2, #4, #11. These were the sequences we selected for further analysis.

DNA homology of ScFv's was estimated using ClustalW2 software [10]. For analysis, we used the four sequences of ScFv's against rhIFN-β1b isolated from the immune library, a sequence of ScFv against rhIFN-β1b preliminarily obtained from a naïve library [15], as well as a sequence of ScFv's against rhIFN-α2b [17]. According to the criterion of homology for their DNA, all six ScFv's were provisionally divided into four groups (Figure).

Mapping structurally and functionally valuable regions within antibody variable domains is an important task. Using SoDA software, we identified *V*-, *D*-, and *J*-gene segments, n-nucleotides, as well as mutations that occurred during affinity maturation of respective sequences *in vivo* [11]. Armed with this same software we identified the boundaries and lengths of all CDR-IMGT and FR-IMGT. The results are summarized in Table 1.

It was determined that the DNA sequences of V_H and V_L domains of the ScFv's from the first group (#1, #4) were formed by germline gene segments IGHV1−26 and IGKV3−2; the sequences

Table 1

ScFv's variable domain DNA analysis carried out using SoDA software

Group	ScFv	V-domain	*V*-gene segment	Number of V-mutations	Number of V-D n-nucleotides (V-J for V_L)	*D*-gene segment	Number of D-mutations	Number of D-J n-nucleotides	*J*-gene segment	Number of J-mutations	CDRH3 region length
1	1	H	IGHV1-26	23	3	IGHD4-1	0	2	IGHJ2	3	6
		L	IGKV3-2	5	0				IGKJ1	4	9
	4	H	IGHV1-26	24	3	IGHD4-1	0	4	IGHJ1	2	6
		L	IGKV3-2	9	0				IGKJ1	2	9
2	2	H	IGHV1-67	15	2	IGHD4-1	0	3	IGHJ4	4	6
		L	IGKV3-2	8	4				IGKJ5	2	9
	11	H	IGHV1-67	18	4	IGHD3-2	1	0	IGHJ1	2	6
		L	IGKV3-2	5	4				IGKJ5	2	9
3	ScFv (naive)	H	IGHV1S128	10	2	IGHD2-1	1	2	IGHJ2	4	13
		L	IGKV17-121	0	2				IGKJ1	2	9
4	ScFv (IFNα)	H	IGHV14-3	6	3	IGHD1-1	0	0	IGHJ4	4	10
		L	IGKV8-19	11	4				IGKJ1	2	9

N o t e . All *V*-, *D*-, and *J*-gene segments' names are in accordance with the IMGT standardization rules [12].

of the second group (#2, #11) were formed by the segments IGHV1−67 and IGKV3−2; those of the third group − by IGHV1S128 and IGKV17−121; and those of the fourth group − by IGHV14−3 and IGKV8−19, respectively. The results suggest that all the *V*-gene segment of the four ScFv's from the panel isolated from the immune combinatorial cDNA library belong to the first and the third IMGT subgroup, respectively [12]. The length of

CDRH3-IMGT and CDRL3-IMGT regions of the four ScFv's isolated from the immune library was 6 and 9 aminoacid residues, respectively. The CDRH3-IMGT region of ScFv isolated from the naïve library turned out to be longer and consisted of 13 aminoacid residues (Table 1).

One of *V*-genes' diversity sources are somatic hypermutations appearing *in vivo* during the processes known as B-cell affinity maturation. Random

Table 2

Alignment of CDR-IMGT regions for V$_H$ and V$_L$ of obtained ScFv's with nearest V-domain sequences [1]

Group	№ of sequence	CDR1	CDR2	CDR3
		Regions for V$_H$		
1	1	GYSFTGYP	INPYNGGT	ARF---------PA--Y
	4	********	********	***---------**--*
	AA085280[2]	*******T	********	**YPQFITTARRY*MD*
	AA085284[2]	*******T	********	**AAG---ILRLRDFD*
	AA085286[2]	*******T	********	**AAG---ILRLRDFD*
	ABK59911[2]	*******T	********	**DVRG------AWFA*
2	2	GYTFTDYA	ISTYSGDT	ARP---------PN--Y
	11	********	******N*	***---------*A--*
	AAF81417[2]	********	****N*N*	**YYG-------NYFD*
	CAA74917[2]	**K*****	****Y***	*LLRP---------FA*
	CAA74923[2]	**K*****	****Y***	*LLRP---------FA*
	AAA83267[2]	*****S*W	*NPSN*R*	**GGVYY-DLYYYALDY
3	ScFv(naïve)	GYTFTSYR	INPSNGRT	ARSYYG----NFYYFDY
	AAA83267[2]	*******W	********	**GGVYY-DLYY*AL**
4	ScFv(IFNa)	GFNIKDTF	IDPANGYT	ASR-------VDYAMDY
	AAA16583[2]	*******Y	******N*	*R*-------AS*****
		Regions for V$_L$		
1	1	ESVDNYGIS--F	AAS	QQSKEVPWT
	4	**********--*	***	**G******
	ABC55323[2]	**********--*	***	*******Y*
	1HOD_A[2]	**********--*	***	*******L*
	AAA39015[2]	**********--*	***	*********
	S09969[2]	**********--*	***	**G**I*Y*
2	2	ESVDKYGIS--F	AAS	QQSKEVPWT
	11	****N****--*	***	*********
	P01656[2]	****NS***--*	***	*********
	AAB30460[2]	****N****--*	***	*********
	CAA80107[2]	****N****--*	***	*******P*
	P01654[2]	****N****--*	***	*********
3	ScFv(naïve)	TDIDDD------	EGN	LQSDNLPLP
	AAZ50375[2]	******------	***	********T
4	ScFv(IFNa)	QSLLNSGNQKNY	WAS	QNDYSYPLT
	AAA38730[2]	************	***	*********

N o t e. [1] − search of closest homologs for the aminoacid sequences was performed using IgBLAST software of the immunoglobulin V-region sequences database of the NCBI; [2] − the sequence number corresponds to the number in GenBank; «_» indicated amino acids common for CDR-IMGT regions of ScFv's of groups 1 and 2; * indicated the same amino acids within each ScFv group; «-» − denotes the absence of an aminoacid at this position.

aminoacid replacements affect the antigen-binding properties of antibodies, which results from local changes in variable domain conformation as well as changes in quantity/quality of functional groups in aminoacid residues that directly contact with antigen [8]. For *V*-, *D*-, and *J*-gene segments of V-domains of the studied ScFv's, we have determined the respective mutation rates − the absolute quantity of nucleotide substitutions deduced from juxtaposing the sequences in question against the sequences of the identified germline gene segments (Table 1). It has been shown that the indicated rates for *V*-gene segments of ScFv's #1 and #4 (group 1) is higher compared to that of ScFv #2 and #11 (group 2). The fact that the absolute mutation rate for *V*-gene segments of ScFv isolated from the naïve library (ScFv (naïve)) is the lowest for all analyzed sequences was an expected result (Table 1).

Our search results for similar experimental works suggest that a panel of ScFv's against rhIFN-β1b has been obtained for the first time. Taking into account this fact, as well as the methodological novelty of the generation and selection scheme for the respective combinatorial library of *V*-genes [14], it was important to estimate the degree of uniqueness of the antigen-binding site of the isolated ScFv's. It is common knowledge that the specificity of the «antigen-antibody» interaction is, first of all, determined by the primary structure of the CDR regions which aminoacids are responsible for direct contact with the antigen. Exposed on the surface of V-domains, CDR regions are spatially accessible for interaction with the antigen and form canonical loop-like structures. Among other CDRs, CDRH3 is the most variable in length and aminoacid composition, and plays a key role in antigen recognition [16].

Using IgBLAST software of the NCBI immunologic sequence database we performed a search for closest homologs separately for the aminoacid sequences of both V-domains and the whole sequence of ScFv. Multiple alignment of the obtained ScFv's with their closest homologs from the database revealed differences in the primary structures of V_H and V_L in aminoacid substitutions at certain positions (results not presented), while the structure of CDRH3-IMGT turned out to be absolutely unique (Table 2). Uniqueness of the primary structure of CDRH3-IMGT as well as the absence of antibody sequences with homologous

V-domains in the database, suggests that the antigen-binding site of the four isolated ScFv is conformationally unique, and the sequences are novel.

Comparative analysis of the CDRH3-IMGT regions of these ScFv's has revealed a conservative motif **AR_P_Y** with substitutions at positions 120 (F/P) and 122 (A/N). Substantial differences were observed in CDRH1-IMGT and CDRH2-IMGT (Table 2). It is known from published data that only a slight part of the aminoacid residues of CDRs contribute significantly to the free energy (△G) of interaction with the antigen [18]. Some publications show a key role for aminoacids located in the central part of CDRH3, while the flanking aminoacids mostly stabilize the formed complex [8, 16, 19]. It is a matter of fact that single aminoacid substitutions in the central part of CDRH3 may dramatically affect antibody's affinity and specificity. The aforementioned substitutions in CDR-IMGT may explain the differences in the panel's ScFv's antigen-binding properties studied in the previous work [15]. We suppose the identified amino acid residues are promising targets for further ScFv's mutagenesis.

It seemed interesting to compare the structure of CDRH3-IMGT regions of ScFv's specific to antigens rhIFN-β1b and rhIFN-α2b that are structural and functional homologs. For comparison, we used the sequences of two highly specific ScFv's isolated from different independently generated immune libraries. For all six CDR-IMGT of ScFv's that were compared, we observed differences in the amino acid composition to the extant of individual substitutions, while CDRL1-IMGT and CDRH3-IMGT also differed by the length of the loop (Table 2, sequences #1 and ScFv (IFNα)). Taking into account the absence of crosslink binding between these ScFv's and the respective antigens, which we had shown before using ELISA, we may suppose their interaction with different epitopes.

Conclusion. Within a panel of ScFv's against rhIFN-β1b isolated from an immune combinatorial cDNA library of mouse genes, we have revealed four sequences with different primary structures. *In silico* analysis has identified structurally and functionally important regions in the variable domains of the obtained ScFv's. It has been shown that the structure of the antigen-binding site of the four isolated ScFv's is unique, and the sequences themselves are new.

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IN SILICO АНАЛИЗ СТРУКТУРЫ ВАРИАБЕЛЬНЫХ ДОМЕНОВ ОДНОЦЕПОЧЕЧНЫХ АНТИТЕЛ МЫШИ, СПЕЦИФИЧНЫХ К РЕКОМБИНАНТНОМУ ИНТЕРФЕРОНУ β1b ЧЕЛОВЕКА

Проведен *in silico* анализ структуры последовательностей ДНК, кодирующих специфические к интерферону β1b и α2b человека (rhIFN-β1b, rhIFN-α2b) одноцепочечные антитела (ScFv – single-chain Fv): определены *V, D* и *J* сегменты, границы антиген-связывающих (CDR) и каркасных (FR) участков, n-нуклеотиды, а также величина мутационных процессов, которые имели место при аффинном дозревании последовательностей *in vivo*. Для представителей панели ScFv против rhIFN-β1b, изолированных из иммунной комбинаторной библиотеки кДНК *V*-генов, показана уникальность участка CDRH3 как по длине, так и по аминокислотному составу. Множественное выравнивание с ближайшими гомологами базы данных NCBI показало, что полученные нами последовательности ScFv являются новыми.

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IN SILICO АНАЛІЗ СТРУКТУРИ ВАРІАБЕЛЬНИХ ДОМЕНІВ ОДНОЛАНЦЮГОВИХ АНТИТІЛ МИШІ, СПЕЦИФІЧНИХ ДО РЕКОМБІНАНТНОГО ІНТЕРФЕРОНУ β1b ЛЮДИНИ

Проведено *in silico* аналіз структури послідовностей ДНК, які кодують специфічні до рекомбінантного інтерферону β1b та α2b людини (rhIFN-β1b, rhIFN-α2b) одноланцюгові антитіла (ScFv – single-chain Fv): визначено *V*-, *D*- та *J*-генні сегменти, межі антиген-зв'язувальних (CDR) та каркасних (FR) ділянок, n-нуклеотиди, а також величину мутаційних процесів, що мали місце при афінному дозріванні даних послідовностей *in vivo*. Для представників панелі ScFv проти rhIFN-β1b, ізольованих з імунної комбінаторної бібліотеки кДНК *V*-генів, показано унікальність ділянки CDRH3 як за довжиною, так і за амінокислотним складом. Множинне вирівнювання з найближчими гомологами бази даних NCBI показало, що одержані нами послідовності ScFv є новими.

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