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Construction of TNF-Binding Proteins by Grafting Hypervariable Regions of F10 Antibody on Human Fibronectin Domain Scaffold

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Abstract—Tumor necrosis factor (TNF) plays a key role in the pathogenesis of various diseases. To study the possibility of constructing TNF-binding proteins by grafting hypervariable regions of immunoglobulins (CDR), we have replaced amino acid sequences of loops from the tenth type III domain of human fibronectin (^{10}Fn3) by amino acid sequences of CDR from the light and heavy chains of the anti-TNF antibody F10. The assessment of TNF-binding properties of the resulting proteins by ELISA has revealed the highest activity of Hd3 containing sequences CDR-H1 and CDR-H2 of the antibody F10 and of Hd2 containing sequences CDR-H1 and CDR-H3. The proteins constructed by us on the fibronectin domain scaffold specifically bound TNF during Western blotting and also weakened its cytotoxic effect on L929 line cells. The highest neutralizing activity was demonstrated by the proteins Hd2 and Hd3, which induced, respectively, 10- and 50-fold increase in the EC_{50} of TNF.

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Key words: tumor necrosis factor, TNF-binding proteins, fibronectin domain, CDR grafting, ELISA, cytotoxicity test

Cytokines represent a family of mediators of protein nature synthesized by immune system cells. Cytokines are involved in various biological processes including proliferation, inflammation, immune response, regeneration, etc. [1]. At present, attention is attracted to the role of cytokines in development of chronic diseases associated with an increase in their concentration in the organism such as autoimmune diseases, asthma, psoriasis, multiple sclerosis, etc. Tumor necrosis factor (TNF) is the best-known proinflammatory cytokine, which plays a key role in pathogenesis of rheumatoid arthritis and Crohn's disease [2].

The use of binding proteins, especially immunoglobulins, to neutralize pathological consequences of increased cytokine concentration in the human organism seems to be a promising approach for treatment of such diseases [3]. Thus, two of three preparations capable of blocking TNF and approved for clinical use in rheumatoid arthritis and Crohn's disease are recombinant antibodies (infliximab and adalimumab) [2]. Well-known advantages of antibodies are their high affinity and selectivity and also their long-term circulation in the organism. However, such antibodies are produced using eukaryotic expression systems, and this makes the treatment very expensive. Moreover, structural features of full-size antibodies prevent their penetration into tissues and binding to some targets [4]. Use of alternative scaffold proteins (ASP) is an approach for solution of this problem. Cytokine-binding preparations based on ASPs can be prepared using expression in bacterial cells. The small size of the molecules of such proteins allows modifying their structure for addition of different functional modules and is favorable for their penetration into tissues and interaction with intracellular antigens [5].

The purpose of this work was to study the possibility of constructing TNF-binding proteins based on the tenth type III domain of human fibronectin (^{10}Fn3) by grafting

Abbreviations: ASP, alternative scaffold proteins; bioTNF, biotinylated TNF; CDR, complementarity-determining regions; ^{10}Fn3, tenth type III domain of human fibronectin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IPTG, isopropyl-β-*D*-thiogalactopyranoside; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF, tumor necrosis factor.

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sequences of the complementarity determining regions (CDR) from the high affinity TNF-binding antibody F10 [6], and also to assess the contribution of individual CDR loops to formation of complexes with the antigen. Using PCR, we have replaced the coding sequences of the fibronectin domain by synthetic DNA fragments encoding hypervariable loops of the light and heavy chains of antibody F10. The proteins prepared by expression in *Escherichia coli* cells have been characterized by their ability to bind TNF and also to neutralize its cytotoxic effect on mouse fibroblasts.

MATERIALS AND METHODS

In this work we used reagents from BioRad (USA), Sigma (USA), Merck (USA), Panreac (Spain), and components for bacteria culture media from Difco (USA); complete 199 medium, fetal calf serum, and dimethylsulfoxide (DMSO) were from Biolot (Russia). Solutions were prepared in deionized Milli Q water. Recombinant human TNF was prepared as described in work [7], and biotinylated TNF (bioTNF) was prepared as described in work [8]. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) was isolated as described in work [9].

Construction of plasmids. Recombinant DNAs were cloned by standard methods [10] in *E. coli* XL-1 Blue cells (Stratagene, USA) using enzymes from Fermentas (Lithuania). Oligonucleotides (Table 1) and the gene encoding ^{10}Fn3 were synthesized by Evrogen (Russia). Nucleotide sequences of the gene and oligonucleotide primers were designed using the Reverse Translate program from the Sequence Manipulation Suite program package (http://www.bioinformatics.org/sms2).

The genes were amplified by PCR using *Pfu*-polymerase under conditions recommended by the supplier of the enzyme. The PCR parameters were as follows: template denaturation for 3 min at 95°C; 25 cycles (denaturation at 95°C for 45 sec, annealing of primers at 52-55°C for 45 sec, elongation at 72°C for 45 sec); final extension at 72°C for 5 min.

In the first stage of assembling of the gene *FNH* four PCR-fragments were obtained on the template of plasmid pETFN3 DNA (pET32a vector with inserted ^{10}Fn3 gene) using primers T7prom + FN3H1 (fragment H1), FN3H2 + FN3H3 (fragment H2), FN3H4 + FN3H5 (fragment H3), and FN3H6 + T7term (fragment H4). The PCR products were analyzed and purified by electrophoresis in 1.5% agarose gel. DNA fragments H1-H4 with lengths of 199, 126, 121, and 200 bp, respectively, were prepared. In the next stage the fragments were joined in pairs (H1 + H2 and H3 + H4) by PCR with primers T7prom + FN3H3 and FN3H4 + T7term, respectively. The resulting fragments were purified similarly and combined by PCR with primers T7prom + T7term. The *FNL* gene was constructed similarly.

To construct genes encoding variants FNHd1-3 and FNLd1-3, PCR was conducted in the same way as described above except for the reaction with the pair of primers encoding the replaced loop at the first stage. Thus, to prepare the gene *Hd3* at the first stage three PCR fragments were obtained on the template of plasmid pETFN3 DNA using primers T7prom + FN3H1 (fragment H1), FN3H2 + FN3H3 (fragment H2), and FN3H4 + T7term (fragment H3). At the next stage these fragments were joined by PCR with primers T7prom + T7term.

The final PCR products were isolated from 1.5% agarose using a MinElute Gel Extraction Kit (Qiagen,

Table 1. Nucleotide sequences of primers used for constructing genes *FNH* and *FNL*

Primer	Nucleotide sequence, 5′-3′	Length
FN3H1	ATGCCATAATGGGTAAAGGTATACCAAGAGATCAGCAG	38
FN3H2	ACCTTTACCCATTATGGCATGAACCGTTACTACCGTATC	39
FN3H3	CGCATAGGTCGGTTCGCCGGTATAGGTGTTAATCCACGGAACGGTGAATTC	51
FN3H4	GAACCGACCTATGCGGATGATTTTAAAGGCCGCTTTACCGCGACCATCTCT	51
FN3H5	AATCGCCGGTGCCGCTAAATTCGGTAACCGCGTAAAC	37
FN3H6	GGCACCGGCGATTTTGATTATCCGATCTCTATCAAC	36
FN3L1	GTTGTTCACGCTCTGGCTCGCTTTCCAAGAGATCAGCAG	39
FN3L2	CAGAGCGTGAACAACGATGTGACCCGTTACTACCGTATC	39
FN3L3	ATAGCGGTTAAACGCATGCGGAACGGTGAATTC	33
FN3L4	CATGCGTTTAACCGCTATACCGCGACCATCTC	32
FN3L5	CGGGCTGCGATAATCCTGCTGGGTAACCGCGTAAAC	36
FN3L6	GATTATCGCAGCCCGTGGACCCCGATCTCTATCAAC	36

Germany), treated jointly with restrictases *Nde*I and *Xho*I and cloned into a vector prepared by cleavage of plasmid pET32a by the same restrictases and isolation of a large fragment. As a result, plasmids pETH and pETL and their derivatives pETHd1-d3 and pETLd1-d3 were constructed. The plasmid structure was confirmed by restriction analysis and sequencing (Genom, Russia).

Isolation of proteins. The *E. coli* strain BL21(DE3) cells (Novagen, USA) transformed by one of the resulting plasmids were grown in 200 ml of LB medium supplemented with ampicillin (100 μg/ml) at 37°C to A_{560} = 0.5-0.7 and induced by addition of 0.2 mM isopropyl-β-*D*-thiogalactopyranoside (IPTG), and the cultivation was continued for 3 h at 30°C. To isolate recombinant proteins from the soluble cell fraction, the biomass after centrifugation was suspended in buffer A (50 mM Tris-HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 200 mM NaCl), and the cells were broken by ultrasonication. The solution was centrifuged, and the supernatant was supplemented with imidazole to 10 mM and placed onto a Ni-Sepharose Fast Flow column (GE Healthcare, USA) equilibrated with the same buffer, washed with buffer B (20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 20 mM imidazole), and eluted with buffer C (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 300 mM imidazole).

To isolate proteins from inclusion bodies, urea and Triton X-100 were added to the ultrasound-treated suspension, respectively, to 6 M and to 1%. Washing was performed in buffer B gradually decreasing the urea concentration to 1 M, then in the same buffer without urea, and elution was performed with buffer C.

Fractions containing the purified protein were combined and dialyzed twice against buffer D (20 mM Tris-HCl (pH 8.0), 50 mM NaCl). The protein concentration was calculated using individual molar absorption coefficients by determination of the absorption at 280 nm.

Enzyme immunoassay (ELISA). TNF (1 μg in 100 μl of PBS) was placed into wells of a 96-well plate (Costar, USA) and incubated at 37°C for 1 h. After three 5-min washings in buffer PBST (PBS + 0.1% Tween-20) the wells were supplemented with 200 μl of 3% BSA solution in PBS and maintained for 30 min at 37°C. The solution was removed, and the wells were washed in PBST as described above. Then serial dilutions of the fibronectin domain protein derivatives in 100 μl of 1% BSA in PBS were introduced, incubation was performed overnight at 4°C, and then five 5-min washings were performed with PBST. The wild type recombinant ^{10}Fn3 was used as a control, and the antigen on the plate (TNF) was replaced with the control protein (GM-CSF). All experiments were performed in three repeats.

Immune complexes were visualized using an anti-His–HRP conjugate (Invitrogen) and 3,3′,5,5′-tetramethylbenzidine (TMB) (Immunotek, Russia) as a substrate. The reaction was stopped by addition of 100 μl of 10% H_2SO_4. The staining intensity was measured at 450 nm with a Model 680 reader (BioRad).

Enzyme immunoassay on HisGrab Nickel Coated Plates (Pierce, USA) was performed according to the manufacturer's recommendations. Protein Hd3 (1 μg in 100 μl of PBS) was placed into the plate wells and maintained for 1 h at room temperature. After washing, serial dilutions of bioTNF in 100 μl 1% BSA in PBS were incubated overnight at 4°C and then washed again in PBST. The bound bioTNF was detected using a NeutrAvidin–HRP conjugate (Pierce), and the staining and measurement were performed as described above.

To analyze by Western blotting, the proteins separated by Laemmli gel electrophoresis (2 μg TNF, GM-CSF, and lysate of the TNF producer strain cells) were transferred onto a nitrocellulose membrane (BioRad) as described in work [11]. Upon incubation in 3% BSA in TBS for 1 h at 37°C to prevent nonspecific interactions, the membrane was incubated overnight in solution of one of recombinant proteins (10 μg/ml) in 1% BSA in TBS at 4°C. The membrane was stained with an anti-His–HRP conjugate according to recommendations of the manufacturer. As substrate, a precipitating TMB (Clinical Science Products, USA) was used.

Analytical gel filtration. Chromatography was performed on a Superdex 200 10/300 GL column (GE Healthcare) at the flow rate of 0.4 ml/min in 100 mM Tris-HCl (pH 8.0) buffer supplemented with 150 mM NaCl. The detection was realized by measuring the absorption at 230 nm. For preparative gel filtration the protein (2 mg/ml) was placed onto a 75-ml column with Sephadex G-100, and elution was conducted at the flow rate of 2 ml/h in 20 mM Tris-HCl (pH 8.0) buffer supplemented with 50 mM NaCl. The fractions were collected with a BioFrac fraction collector (BioRad).

Determination of cytotoxic activity of TNF. The standard test was conducted on mouse fibroblast cell line L929 as described in work [12]. The L929 cells ($2 \cdot 10^4$) were placed into wells of 96-well plates (Nunc, USA) in 100 μl of complete 199 medium supplemented with 10% fetal calf serum and incubated for 18 h. Then the medium was removed, and to the cell monolayer 100 μl of the complete medium containing successive twofold dilutions of TNF (from 1 μg/ml to 0.1 pg/ml) were added, or TNF mixtures with the proteins under study (at the molar ratio of 1 : 10) supplemented with actinomycin D (1 μg/ml), and incubation was performed at 37°C in a CO_2 incubator. After 18 h every well was treated with 10 μl of MTT solution in PBS (5 mg/ml) and incubated for 2.5 h. Then the supernatant was removed, and to the remaining cells 100 μl of DMSO was added, and the optical absorption was measured at 540 nm with a Titertek Multiskan MCC (Flow Laboratories, USA). Cell survival was calculated by the formula $C = (B/A) \times 100\%$, where A and B were light absorption in the control and tested wells, respectively. The measurements were performed in four repeats.

RESULTS

Construction and expression of the synthetic ^{10}Fn3 gene. Among various modern ASPs used for preparation of binding proteins we have chosen the tenth type III domain of fibronectin (^{10}Fn3). This protein is small (94 amino acid residues) and has a high thermostability (T_m = 90°C) and good solubility. The presence of Fn3 in normal human serum provides for low immunogenicity and, thus, is promising for its use as a therapeutic agent. The spatial structure of ^{10}Fn3 is β-sandwich-like consisting of six antiparallel strands and resembles the structure of the immunoglobulin domain VH [13]. To construct artificial binding proteins, researchers usually vary sequences of two or three loops that connect the β-structure regions and are structural analogs of hypervariable regions (CDR) of antibodies [14]. A number of high affinity proteins capable of binding various polypeptide ligands have been prepared on the fibronectin domain scaffold [4].

Based on the known primary structure of the fibronectin domain, we have designed a nucleotide sequence of the corresponding gene taking into account the frequency of using the codons in the *E. coli* cells. This gene was prepared by assemblage of synthetic oligonucleotides and cloned into the vector based on pET32a (Novagen) under the control of a strong regulated promoter T7*lac*. On the 3′-terminus of the artificial gene there is a sequence encoding a hexahistidine fragment to promote a subsequent purification by metal affinity chromatography. Upon the induction the expression level of the resulting gene in the strain BL21(DE3) cells reached 20% of the total cell protein, and the bulk of the expression product was soluble. The recombinant ^{10}Fn3 was isolated from the soluble fraction of cell proteins by chromatography on Ni-Sepharose Fast Flow (GE Healthcare), and as a result a highly purified protein (Fig. 1) was prepared with the yield of ~20 mg/liter of the bacterial culture.

Construction of TNF-binding proteins on the ^{10}Fn3 scaffold. To design the primary structure of artificial TNF-binding proteins, we have used data on structural homology of the BC, DE, and FG loops of the fibronectin domain and of CDRs of immunoglobulin molecules [14]. We supposed that CDR grafting from the TNF-binding antibody molecule into the homologous region of an immunoglobulin-like protein should allow us to retain the loop conformation and ensure the ability of the resulting artificial variant ^{10}Fn3 to bind cytokine. The nucleotide sequence of cDNA encoding the heavy and light chains of the high affinity ($K_d = 5 \cdot 10^{10}$ M^{-1}) anti-TNF antibody F10 was determined earlier [6]. Note that limits of CDR from the F10 antibody in this work were determined according to the Kabat scheme [15]. Other algorithms used for analyzing the amino acid sequence of the antibody heavy chain, e.g. IMGT [16], resulted in a shorter sequence CDR-H2 (WINTYTGEPT instead of WINTYTGEPTYADDFKGRF).

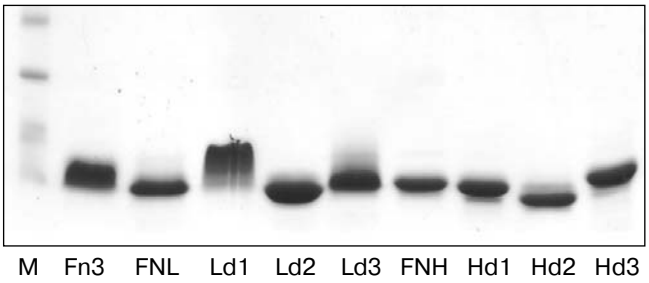

Fig. 1. Electrophoresis of the fibronectin domain purified variants in 14% SDS-polyacrylamide gel. M, markers from Fermentas (Lithuania).

In the first stage we designed two variants of artificial binding proteins: we wanted to replace in the FNH protein the sequences of the BC, DE, and FG loops of the fibronectin domain by the CDR1-3 sequences of the heavy chain of antibody F10 and in the FNL protein – by CDR of F10 light chain (Table 2). The genes for these variants were prepared by a two-stage PCR with three pairs of overlapped oligonucleotide primers encoding the sequences of each CDR on the synthetic gene ^{10}Fn3 template.

In the next stage it was planned to successively replace every loop corresponding to CDRs in the proteins FNH and FNL by natural loops of the fibronectin domain in order to determine the relative contribution of each CDR to the functional activity of the binding proteins. To construct the corresponding genes encoding the hybrid variants Hd1-3 and Ld1-3 (Table 2), PCR was performed similarly, removing from the reaction mixture the pair of primers encoding the loop to be replaced. All resulting genes were cloned into the same vector as for the ^{10}Fn3 gene.

Protein electrophoresis of lysates from the *E. coli* BL21(DE3) strain cells transformed by the corresponding plasmids revealed that IPTG induced in the cells effective synthesis of all mutant variants of the fibronectin domain. In the majority of cases the solubility of the resulting proteins (especially of FNH) was somewhat lower than the solubility of the natural ^{10}Fn3, but addition of urea and nonionic detergent Triton X-100 ensured the solubility of ~80% of the recombinant proteins. By metal affinity chromatography, all variants were isolated with high purity (no less than 90%). The yield of the proteins was 20-30 mg/liter.

Analytical gel filtration and binding activity. Monodispersity of isolated proteins was studied by analytical gel filtration using a precalibrated Superdex 200 column. All preparations were found to contain both the monomer (elution time ~45 min) and the dimer (elution time 41-43 min) and also of high molecular weight aggregates in some cases (e.g. in FNH) (Fig. 2). In proteins prepared from the soluble fractions of the bacterial cells

Table 2. Amino acid sequences of loops of ^{10}Fn3 and of its variants

Protein	Sequence of BC loop	Sequence of DE loop	Sequence of FG loop
^{10}Fn3	DAPAVTV	GSKS	GRGDSPASSK
FNL	KASQSVNNDVT	HAFNRY	QQDYRSPWT
Ld1	DAPAVTV	HAFNRY	QQDYRSPWT
Ld2	KASQSVNNDVT	GSKS	QQDYRSPWT
Ld3	KASQSVNNDVT	HAFNRY	GRGDSPASSK
FNH	YTFTHYGMN	WINTYTGEPTYADDFKGRF	EFSGTGDFDY
Hd1	DAPAVTV	WINTYTGEPTYADDFKGRF	EFSGTGDFDY
Hd2	YTFTHYGMN	GSKS	EFSGTGDFDY
Hd3	YTFTHYGMN	WINTYTGEPTYADDFKGRF	GRGDSPASSK

the monomer was prevalent, and the use of urea and detergent during the isolation promoted the accumulation of soluble protein aggregates.

To determine the cause of this accumulation, amino acid sequences of different variants of the fibronectin domain were studied using the TANGO program (http://tango.crg.es [17]), which allowed us to determine the aggregation potential of protein regions possessing β-structure. The *C*-terminal part of the ^{10}Fn3 molecule (residues 71-77, TITVYAV) and also the corresponding region of proteins FNH and FNL were shown to contain an aggregation site with a potential of 75-95%. Moreover, an additional region with a potential of 95% is present in the *N*-terminal part of the FNH molecule at the boundary with the first CDR (residues 20-27, LLISWYTF), whereas such region is absent in the other variants. The presence of this region seems to be an explanation for the tendency of FNH to produce aggregates.

To compare the functional activity of different forms of proteins FNH and FNL, they were separated by preparative gel filtration. Note that analytical gel filtration of the purified fraction of the FNH monomer after its storage for two weeks at 4°C revealed the presence of ~15% of the dimer; therefore, the ability of the purified proteins to bind TNF was studied immediately upon their isolation.

To determine the TNF-binding activity of different fractions of the FNH and FNL proteins by ELISA, human recombinant TNF was adsorbed on the plate surface and the isolated proteins were used as primary antibodies. The complexes were visualized with peroxidase-conjugated antibodies against the hexahistidine fragment. The binding of the wild type fibronectin domain with TNF and also the binding of all resulting variants with GM-CSF (negative control) were at the background level. The efficiency of interaction with TNF of different FNH fractions within the range of the studied protein concentrations depended on the protein aggregation: at the same concentration the staining of FNH dimer was 1.6-fold more intense than the staining of FNH monomer, and the staining of the multimeric fraction was 2.5-fold more intense (data not presented). Similar results were obtained in comparison of the binding activities of monomers and dimers of FNL.

All variants studied bound TNF depending on the concentration, and the binding level was significantly higher than in the control wells. However, the binding curves were not saturated until the FNH and FNL concentrations were 7.5 μM, and this prevented determination of the binding constants for these proteins by this method.

Role of individual loops of FNH and FNL in binding of TNF. Studies on interactions with TNF of the variants FNHd1-3 and FNLd1-3 revealed that the replacement of amino acid sequences of each of three CDRs from the light chain by the corresponding sequences from the wild type fibronectin loops decreased the binding efficiency (Fig. 3a). The maximal decrease (by 81%) was observed on replacement of the second CDR sequence (CDR-L2). Hybrid variants Ld1 and Ld3 bound TNF at approximately the same efficiency, which was 50-55% of the FNL binding efficiency.

Replacement of the amino acid sequences CDR-H1-H3, as discriminated from such for FNL variants, in two of three cases increased the binding (Fig. 3a). Whereas protein Hd1 had virtually the same binding efficiency with the antigen as FNH, the binding efficiency of the Hd2 variant was 1.8-fold and of the Hd3 variant 3.7-fold higher. Thus, hybrid variant Hd3 prepared by us and containing the sequences CDR-H1 and CDR-H2 from the F10 antibody displayed the highest TNF-binding activity.

Interaction constants of the fibronectin domain variants with TNF were assessed by ELISA using plates capable of binding proteins with the hexahistidine fragment. The protein Hd3 was adsorbed on the plate surface and then incubated with serial dilutions of biotinylated TNF.

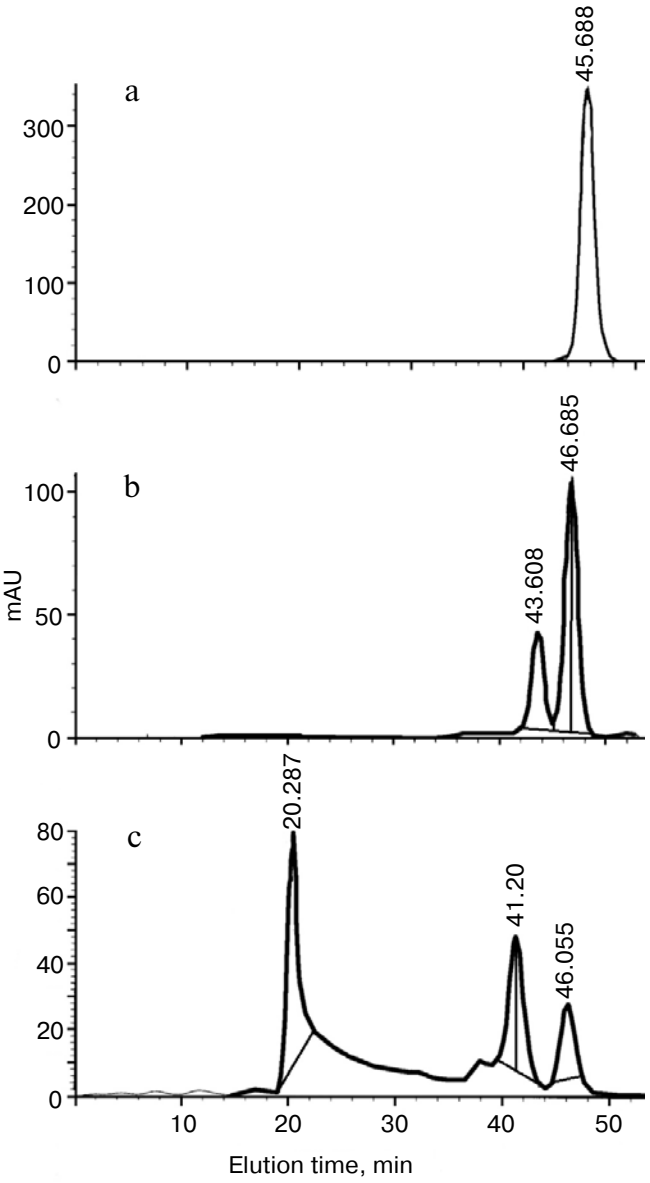

Fig. 2. Analytical gel filtration on a Superdex 200 column of proteins ^{10}Fn3 (a), FNL (b), and FNH (c).

The binding of bioTNF was detected using NeutrAvidin–HRP conjugate, and the interaction constant of protein Hd3 with TNF was found to be ~$3.5 \cdot 10^7$ M^{-1} (Fig. 3b).

To confirm these results and also to additionally test the specificity of interaction of the prepared proteins with TNF, we used Western blotting. The antigens separated by SDS-PAGE (recombinant TNF, lysate of recombinant TNF producer strain cells, and recombinant GM-CSF) were transferred onto a nitrocellulose membrane and maintained in solution of either proteins FNL, FNH, or their variants. The formation of complexes was detected using antibodies against the hexahistidine fragment conjugated with peroxidase. The results confirmed the presence of a specific interaction between the fibronectin variants and TNF monomer (Fig. 4). No nonspecific binding with GM-CSF and with bacterial proteins was detected.

Influence of TNF-binding proteins on cytotoxic activity of TNF. To study the ability of the prepared proteins to inhibit TNF-induced cytotoxicity, L929 cells were cultured in the presence of a mixture of different dilutions of TNF with purified preparations of TNF-binding proteins taken in 10-fold molar excess. The mixtures were preincubated for 2 h at room temperature. The variants Hd2 and Hd3 displayed the highest TNF-neutralizing activity (Fig. 5). Addition of Hd2 to TNF in the molar ratio of 10 : 1 decreased 10-fold the cytotoxic effect of TNF, and addition of Hd3 in the same ratio decreased the cytotoxicity 50-fold. Thus, in this test the prepared proteins displayed properties of TNF antagonists. However, their activity as TNF antagonists was significantly lower than the activity of the monoclonal antibody F10: the addition of F10 in the same ratio completely inhibited the effect of TNF within the range of concentrations studied (data not presented).

DISCUSSION

Antibody molecules ensure specific high affinity binding of antigens due to multiple noncovalent interactions. The antibody region interacting with the antigen epitope (a paratope) is formed by hypervariable regions (CDR) of the hypervariable domain of immunoglobulins. Every light and heavy chain of the antibody contains three CDRs with a loop conformation stabilized by the scaffold structure of variable domains.

Regions that determine complementarity are the most variable and relatively isolated fragments of antibody molecules [18]. Synthetic peptides corresponding to CDR are able to specifically bind the target molecules [19]. The strategy of CDR grafting is widely used for decreasing the immunogenicity of recombinant antibodies (humanization). In many cases the high binding ability of the resulting hybrid molecules can be retained [20]. Grafting of CDR loops was also used for varying the amino acid sequence of artificial binding ASP-based proteins, such as GFP, neocarzinostatin, and plasminogen activator [21-23].

In our work this strategy was used for the first time to prepare TNF-binding proteins on the fibronectin domain scaffold. To construct genes of such proteins, we used as a template the synthetic gene ^{10}Fn3 optimized for expression in *E. coli* cells. The replacements of amino acid sequences in the loops of the fibronectin domain by CDRs from the light and heavy chains of antibody F10 did not negatively influence the expression levels of the recombinant proteins. All resulting variants were characterized by high level of synthesis – up to 20% of total cell protein. However, solubility of some variants, especially

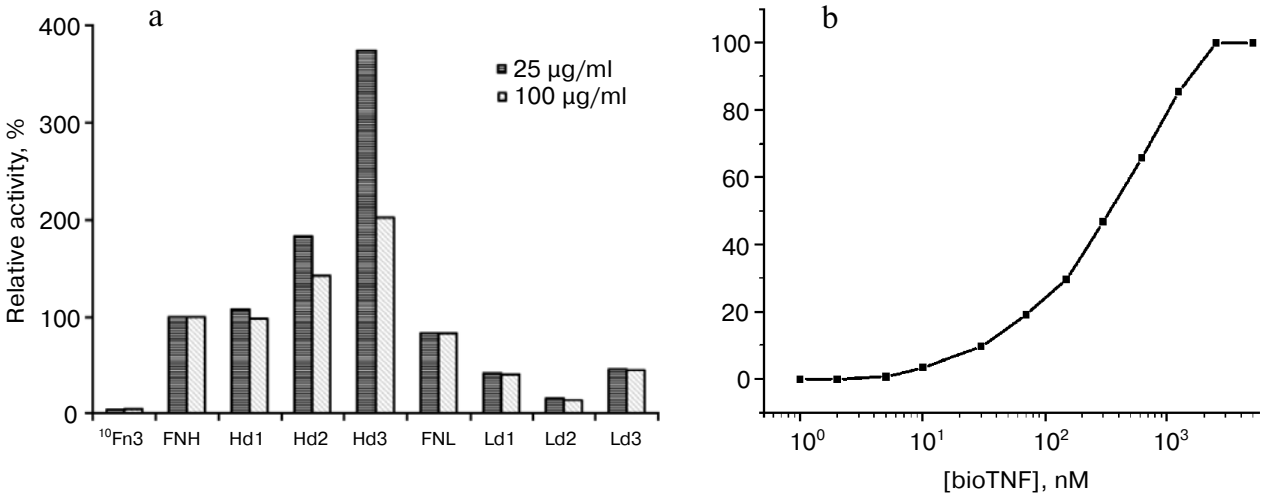

Fig. 3. a) Analysis by ELISA of TNF-binding properties of proteins FNL, FNH, and their hybrid variants on plates with TNF. The mean value of OD_{450} in the wells with the FNH protein is taken as 100%. b) Assessment by ELISA of affinity for the interaction of protein Hd3 with TNF on HisGrab Nickel Coated Plates (Pierce).

FNH, was decreased, possibly because of destabilization of the domain structure caused by the elongation of the loop. Thus, in the FNH variant the sequence of the DE loop (4 amino acid residues) was replaced by the sequence of CDR-H2 (19 amino acid residues). The structure of just the DE loop of the three fibronectin domain loops has the highest rigidity [12], and therefore the elongation of this loop is accompanied by the maximal destabilization of the molecule [24].

The use during the isolation of a buffer containing urea and detergent and the subsequent renaturation allowed us to prepare purified soluble preparations of FNH and its derivatives, but analytical gel filtration revealed that the resulting proteins contained a rather high content of aggregates with molecular weight >600 kDa. Soluble aggregates of protein molecules can often be produced when molecular bases of biological processes underlying some diseases are studied [25] and also on constructing artificial proteins [26]. The spatial structure of such aggregates is characterized by the presence of β-folded packages with β-folds arranged perpendicularly to the polymer axis [27]. Aggregates are often observed in preparations of shortened antibodies, especially in those of isolated VH-domains [28]. Note that the presence of a potential region of β-aggregation in the natural ^{10}Fn3 molecule and also in molecules of FNL and its variants does not lead to formation of multimers. It seems that the decreased solubility of FNH and the presence in its sequence of an additional aggregation region promote formation of soluble polymers in the preparation of this protein.

The comparison of TNF-binding activity of preparations containing isolated monomers or dimers of FNH with the activity of the fraction of soluble aggregates of this protein has shown the higher affinity of the aggregates. This can be explained by an increased avidity of aggregates responsible for multipoint binding of the target antigen. To provide for the avidity, an artificial multimerization of monomeric binding proteins is often performed by addition of certain amino acid sequences [29], treatment with enzymes [30], or using gene engineering approaches [31]. In our work the possibility of formation of multimeric preparations due to β-aggregation during bacterial synthesis and isolation of mutant variants of fibronectin domain has been observed for the first time.

The comparative determination by ELISA of the binding activities of the FNL and FNH proteins and also of their variants allows us to characterize the relative

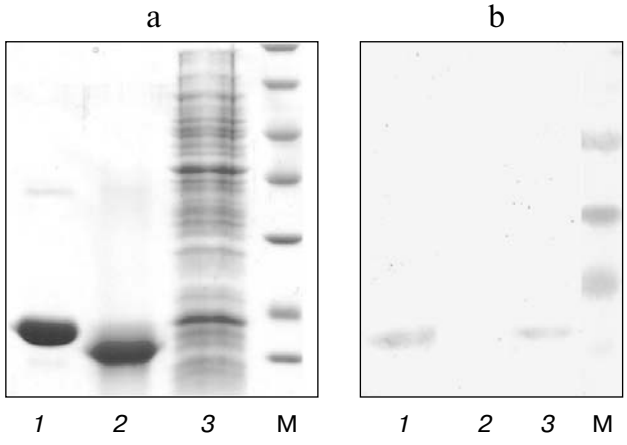

Fig. 4. Gel electrophoresis in 14% SDS-polyacrylamide gel (a) and Western blotting with protein Hd3 used as an antibody (b) of TNF (*1*), of GM-CSF (*2*), and of the SG20050 strain biomass with plasmid pTNF31d (*3*). M, markers from Fermentas (Lithuania).

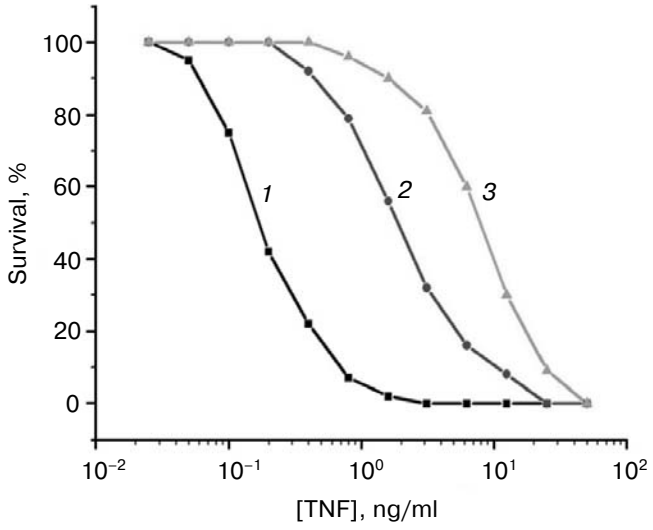

Fig. 5. Cytotoxic effect on L929 cells of TNF and of TNF in mixture with binding proteins: *1*) TNF; *2*) Hd2 + TNF; *3*) Hd3 + TNF.

contribution of individual CDRs to the interaction with the antigen epitope. Thus, all ^{10}Fn3 variants containing the CDR sequences from the heavy chain of the F10 antibody provide for more effective binding of TNF than the FNL protein and its variants. These results are in agreement with the literature data on the dominating role of CDR-H loops in formation of the binding surface (paratope) [32]. Replacement of amino acid sequences of each of three CDRs of the FNL protein by sequences of the corresponding loops from ^{10}Fn3 is accompanied by a decrease in the binding function of the FNLd1-3 variants, which is most pronounced in the case of the FNLd2 variant. Thus, all three CDRs of the F10 antibody light chain seem to be involved in the interaction with the target ligand. Also, CDR-L2 within the resulting binding protein is more important than CDR-L1 and CDR-L3.

The structure of CDR-H3 is the most variable of all CDR sequences [33]. This loop is also believed to be the main contributor to the interaction with the antigen epitope. Varying the amino acid sequence of CDR-H3 is a conventional approach for increasing the affinity of artificial antibodies and their shortened variants [34]. We have compared affinities for TNF of the FNHd1-3 proteins prepared by us, and the highest binding activity was displayed by the Hd3 variant with the CDR-H3 corresponding sequence replaced by the sequence of the ^{10}Fn3 loop FG. Its activity was 3.7-fold higher than the activity of the FNH protein and 2-fold higher than the activity of the Hd2 protein containing a similarly replaced CDR-H2. Thus, our results indicate that the CDR-H2 loop plays the most important role in the binding of the target ligand with the resulting artificial protein.

It has been mentioned that the determination of CDR limits of the F10 antibody heavy chain using different algorithms gives different results. The sequence CDR-H2 used by us for grafting and presented in work [14] consists of 19 amino acid residues. It perhaps contains some of the scaffold residues of the variable domain of the F10 heavy chain that stabilize the loop conformation and promote strengthening of the antigen binding. However, an increase in the binding activity of the Hd2 and Hd3 variants compared to that of the FNH protein seems to be due to structural changes caused by increase in the mobility of loop DE and the associated destabilization of the loops of the FNH molecule. Calculations of the spatial conformation of the FNH molecule based on molecular dynamics have confirmed these hypotheses (data not presented).

The biological activity of the prepared proteins was assessed using a standard test for TNF cytotoxity in mouse fibroblasts of the L929 strain. The interaction of TNF with the binding proteins prevented its binding with the type I mouse TNF receptor and cell death. Consequently, the increase in the cell survival is an index of efficiency of the studied proteins as TNF antagonists. Results of these studies correlate with the ELISA data on the binding. The highest binding activity was demonstrated by the Hd2 and Hd3 proteins, which induced, respectively, a 10- and 50-fold increase in the effective concentration of TNF necessary for 50% cell death.

The findings have shown that TNF-binding proteins can be prepared by grafting amino acid sequences of CDRs from the high affinity F10 antibody against TNF using the tenth type III domain of human fibronectin as a scaffold protein. The interaction of proteins we constructed with TNF has been proved by ELISA, Western-blotting, and also by neutralizing the cytotoxic effect of TNF on L929 cells. Thus, the prepared proteins are shown to interact with both TNF monomer and native trimer acting as antagonists of this cytokine.

Note that the TNF-binding activity of Hd3, which was the best of the prepared recombinant proteins, was three orders lower than the activity of the F10 antibody, the antigen-binding region of which was used as a source of amino acid sequences of the loops. The antibody paratope conformation responsible for the high affinity binding of the ligand is stabilized by amino acid residues of the scaffold regions [35]. We think that using an alternative scaffold has resulted in changes in the spatial structure of CDR that in turn caused a decrease in the affinity to the target ligand. It is known that combinatory approaches are promising for preparation on fibronectin domain scaffold of TNF-binding proteins with binding constant to 20 pM [36]. The further increase in the binding activity of the prepared proteins requires a complex approach including modeling of the structure of the antigen-binding region, mutagenesis of individual residues, constructing of combinatory libraries, and selection of the best variants.

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