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Novel Mutants of Human Tumor Necrosis Factor with Dominant-Negative Properties

L. N. Shingarova[1]*, E. F. Boldyreva[1], S. A. Yakimov[1], S. V. Guryanova[1], D. A. Dolgikh[1,2], S. A. Nedospasov[2,3], and M. P. Kirpichnikov[1,2]

[1]Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia; fax: (495) 330-6983; E-mail: lshing@mx.ibch.ru
[2]Biological Faculty, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 330-6983; E-mail: dolgikh@nmr.ru
[3]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, ul. Vavilova 32, 19991 Moscow, Russia; fax: (499) 135-1405; E-mail: sergei.nedospasov@gmail.com

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Abstract—Tumor necrosis factor (TNF) is a polyfunctional cytokine, one of the key mediators of inflammation and innate immunity. On the other hand, systemic or local TNF overexpression is typical of such pathological states as rheumatoid arthritis, psoriasis, Crohn's disease, septic shock, and multiple sclerosis. Neutralization of TNF activity has a marked curative effect for some diseases; therefore, the search for various TNF blockers is a promising field of protein engineering and biotechnology. According to the previously developed concept concerning the possibility of designing dominant-negative mutants, the following TNF variants have been studied: TNFY87H + A145R, TNFY87H + A96S + A145R, and TNFV91N + A145R. All of these form inactive TNF heterotrimers with the native protein. The ability of mutants to neutralize the effect of TNF was investigated. The addition of mutants to the native protein was shown to provide a concentration-dependent suppression of TNF cytotoxicity against the mouse fibroblast cell line L929. Thus, novel inhibitors of human TNF can be engineered on the basis of these muteins.

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Key words: tumor necrosis factor (TNF), mutants, TNF inhibitors

Human tumor necrosis factor-α (TNF) is a polyfunctional cytokine, one of the key mediators of inflammation, innate immunity, protective functions of the organism, and structural organization of lymphoid organs [1, 2]. In humans TNF is synthesized mainly by activated macrophages, B- and T-lymphocytes, and NK cells as a transmembrane homotrimer with monomer molecular weight of 26 kDa. This protein is cleaved by a specific metalloprotease. As a result of processing, a trimeric form of soluble TNF is obtained; its monomer has a molecular weight of 17 kDa and consists of 157 amino acid residues [3, 4]. Diversity of biological properties of TNF is mediated by two high-affinity receptors, TNFRI (p55) and TNFRII (p75), which bind TNF with approximately equal affinity [5]; moreover, both soluble and membrane protein forms are bound to the receptors and transmit intracellular signal. There is experimental evidence of that the soluble TNF form is associated with chronic inflammatory processes [6]. Many autoimmune and inflammatory human diseases are accompanied by local or systemic TNF overproduction, which results in pathological states including septic shock. Enhanced TNF content in blood was found for various human diseases such as cerebral malaria, meningococcal meningitis, rheumatoid arthritis, psoriasis, Crohn's disease, septic shock, multiple sclerosis, and others. The studies of recent years have shown that inhibition of TNF protein binding with the receptors has a pronounced clinical effect in patients with inflammatory diseases of noninfectious etiology. TNF-specific antibodies and recombinant polypeptides, comprising the antigen-specific domains of antibodies specific to the receptors of this cytokine, proved to be the most efficient inhibitors. Infliximab, Etanercept, Lenercept, and other

Abbreviations: DN-TNF, dominant-negative TNF inhibitors; TNF, tumor necrosis factor; TNFRI and TNFRII, TNF receptors.

* To whom correspondence should be addressed.

preparations obtained on the basis of such polypeptides have successfully passed clinical trials [7]; however, application of these preparations is rather expensive due to high production costs and may be accompanied by a number of side effects. Thus, further search for inexpensive TNF antagonists is a prospective trend of protein engineering and biotechnology. Previously, a group of US researchers showed that some TNF mutants can interact with the native protein, forming inactive heterotrimers and thereby neutralizing its activity [8]. When developing this concept, we obtained novel TNF mutants with enhanced ability for heterotrimerization, which are potential blockers of a new type—autoblockers.

MATERIALS AND METHODS

The strains *Escherichia coli* XL-1 Blue (Stratagene, USA), *E. coli* BL21(DE3) (Novagen, USA), and *E. coli* SG20050 [9] were used in this work. Oligonucleotides were synthesized by Eurogen (Russia).

Recombinant DNA cloning was carried out in *E. coli* XL-1 Blue cells by standard methods [10] using restrictases, DNA-ligase, and *Taq*- and *Pfu*-polymerases (Fermentas, Lithuania). The genes of mutant TNF variants were constructed by the method of splice overlap extension PCR (SOE-PCR). Amplification was performed using *Pfu*-polymerase under conditions recommended by the manufacturer. PCR parameters were as follows: 3 min of template denaturation at 95°C; 25 cycles (denaturing, 95°C, 45 sec; annealing of primers, 52-55°C, 45 sec; elongation, 72°C, 45 sec); and completion at 72°C for 5 min. The plasmid pmut1 encoding the TNFY87H + A145R protein was obtained by amplification of the DNA of plasmid pTNF331 carrying the TNF gene [9] using mutagenizing primers CATCGCCGTCTCCCACCAGACCAAGGTC (87f), GACCTTGGTCTGGTGGGAGACGGCGATG (87r), CGACTATCTCGACTTTCGCGAGTCTGGGCAG (145f), and CTGCCCAGACTCGCGAAAGTCGAGATAGTCG (145r). The second stage of PCR was performed with primers TCGATAAATTCGGTACCTAA and TTCATTAAGCTTCACAGGGCAATGATCCC to the 5′- and 3′-ends of the TNF gene. After the treatment with restrictases *Cla*I and *Hind*III and purification in 1% agarose gel, the amplification product was cloned in a vector obtained by treating the DNA of plasmid pTNF331 with the same restrictases. For obtaining plasmid pmut2 encoding TNFY87H + A96S + A145R, amplification was performed in a similar way on the pmut1 template with primers CTCCTCTCTTCCATCAAGAGC (96f) and GCTCTTGATGGAGAGAGGAG (96r) at the first stage of PCR. The TNFV91N + A145R-encoding plasmid pmut3 was obtained by pTNF331 amplification in the presence of mutagenizing primers CGACTATCTCGACTTTCGCGAGTCTGGGCAG (145f), CTGCCCAGACTCGCGAAAGTCGAGATAGTCG (145r), CAGACCAAGAACAACCTCCTCTCT (91Nf), and GAGGAGGTTGTTCTTGGTCTGGTA (91Nr). The cloning was performed in plasmid pTNF331 as described above. For obtaining plasmid pETTNF encoding TNF with N-terminal hexahistidine sequence, the TNF gene was amplified in the presence of primers TTCATTGCTAGCTCGAGCCGAACCCCG and TTCATTAAGCTTCACAGGGCAATGATCCC and cloned in plasmid pET28a (Novagen), cut by restrictases *Nhe*I and *Hind*III. The structure of plasmids was confirmed by restriction analysis and sequencing (Russian Genome Center).

Isolation of proteins. Cells of the strain *E. coli* SG20050 transformed by pTNF331, pmut1, pmut2, or pmut3 were grown in 200 ml of LB (Luria–Bertani medium) with ampicillin (100 μg/ml) at 37°C for 18 h. The biomass after centrifugation was suspended in buffer 1 containing 20 mM potassium phosphate, pH 7.0, 15 mM NaCl, 10% sucrose, and 10 μg/ml phenylmethylsulfonyl fluoride (PMSF), and the cells were destroyed by sonication. The suspension after sonication was centrifuged, the precipitate was removed, and the supernatant was dialyzed against buffer 2 (50 mM sodium phosphate, pH 7.5, and 10 μg/ml of PMSF) and applied to a hydroxyapatite column (20 ml) (hydroxyapatite Bio-Gel HTP; Bio-Rad, USA). The proteins were separated in a linear concentration gradient of sodium phosphate (50-300 mM). The fractions containing recombinant protein were collected, dialyzed against buffer 3 (20 mM Tris-HCl, pH 7.5 or pH 8.0 for mutant proteins), and applied to a column (10 ml) with DEAE cellulose (DEAE-Toyopearl 650M; Toyo Soda, Japan). The target protein was eluted with the NaCl linear gradient (0-0.15 M) in buffer 3 and sterilized by filtration through 0.22 mm MILLEX filters (Millipore, USA). $(His)_6$TNF was isolated from cells of strain *E. coli* BL21(DE3) (Novagen) transformed by plasmid pETTNF. The cells were grown in 200 ml of LB with ampicillin (100 μg/ml) at 37°C to A_{560} 0.5-0.7 and induced by adding 0.2 mM IPTG, followed by cultivation for 3 h. The biomass after centrifugation was suspended in buffer 4 (50 mM Tris-HCl, pH 8.0, 1 mM PMSF, and 200 mM NaCl), and the cells were destroyed by sonication. Imidazole was added to the supernatant to 10 mM and it was applied to the Ni-Sepharose column (1 ml) (Ni-Sepharose FastFlow, GE Healthcare) equilibrated with the same buffer. Column was washed with buffer 4 with 20 mM imidazole, and proteins were eluted with buffer 4 containing 300 mM imidazole. The fractions containing hybrid protein were combined and then dialyzed against buffer 5 (20 mM Tris-HCl, pH 7.5, 100 mM NaCl) and sterilized. Concentrations of hybrid proteins were determined using Protein Assay reagent (Bio-Rad). The yield of purified TNF and the mutants from 1 g of biomass was 8-10 mg; $(His)_6$TNF was 12 mg.

Preparation of TNF heterotrimers with mutant proteins. $(His)_6$TNF (20 μg) was mixed with 20 μg of the

mutant protein (TNFY87H + A145R, TNFY87H + A96S + A145R, TNFV91N + A145R) or TNF and incubated in buffer containing 100 mM NaCl and 20 mM Tris-HCl, pH 7.5, for 1 h at 37°C plus 18 h at 4°C. Interchange products were isolated on Ni-NTA Spin Columns (Qiagen) according to the manufacturer's protocol. Isolated heterotrimers were analyzed by protein electrophoresis according to Laemmli [11].

Determination of biological activity of recombinant human TNF and its derivatives. Biological activity of TNF was estimated from the cytotoxic effect of TNF on L929 mouse fibroblast cells by the method of Kramer [12] with some modifications. L929 cells ($2 \cdot 10^4$) were placed into the wells of 96-well plates (Nunc, Denmark) in 100 μl of complete medium 199 (Biolot, Russia) containing 10% embryonic calf serum (Biolot) and incubated for 18 h. Then the medium was removed, and 100 μl of complete medium containing double dilutions of TNF (from 1 μg/ml to 0.1 pg/ml) with actinomycin D (1 μg/ml) (Reanal, Hungary) was added to the cell monolayer and incubated at 37°C in a CO_2-incubator (KEBO Biomed, Sweden). After 18 h, 10 μl of MTT solution (Sigma, USA) in PBS (5 mg/ml) was added into each well and incubated for 2.5 h. Then the supernatant was removed, 100 μl of DMSO (Biolot) was added to the remaining cells, and absorption was measured at 540 nm in a Titertek Multiskan MCC plate reader (Flow Laboratories, Finland). Viability was calculated by the formula: C = (B : A) × 100%, where A is light absorption in the control well (without TNF) and B is light absorption in the tested well. Measurements were made in four repeats. The activities of mutants or TNF in the mixture with mutants were determined in a similar way. Plots were constructed using the averaged data from four measurements. The spread of values at each point was no more than 10%.

RESULTS AND DISCUSSION

The diversity of TNF biological functions is mediated by two high-affinity receptors: TNFRI (p55) and TNFRII (p75). Though the related protein—lymphotoxin—is characterized by formation of active heterotrimers between the α and β subunits, it is considered that the biological activity of TNF is provided only by homotrimers interacting with the receptors and thereby transmitting the signal into a cell. Three-dimensional structures of TNF and the p55 receptor have been established. TNF regions adjacent to amino acids 30, 80, and 140 are involved in the contact with the receptors; therefore, mutations in these regions lead to a change in protein activity [13, 14]. So, it has been shown [8] that some mutants (the authors call them dominant-negative TNF inhibitors, DN-TNF) have no biological activity because they do not bind to the receptors, but can interact with the native protein with formation of inactive heterotrimers (Fig. 1) and, thus, reduce the efficient concentration of active TNF homotrimer. In particular, such mutants include the point mutants A145R and Y87H with the substitutions of Ala for Arg and Tyr for His in TNF amino acid residues 145 and 87, respectively, and a double mutant containing both substitutions. On the other hand, based on computer analysis (unpublished data), we have revealed new potential mutations in the TNF molecule that might result in formation of additional hydrogen bonds between TNF monomer molecules in the trimer without disturbing the tertiary structure of the protein. Such mutations include point substitutions of V91N and A96S. Based on these data, we planned to obtain two novel TNF mutants: a double mutant with V91N + A145R substitutions, a triple mutant with Y87H + A96S + A145R substitutions, and the already described Y87H + A145R mutant [8] for comparison. We assumed that the new proteins would preserve the properties of DN-TNF but possess enhanced ability for trimerization and, consequently, higher blocking activity.

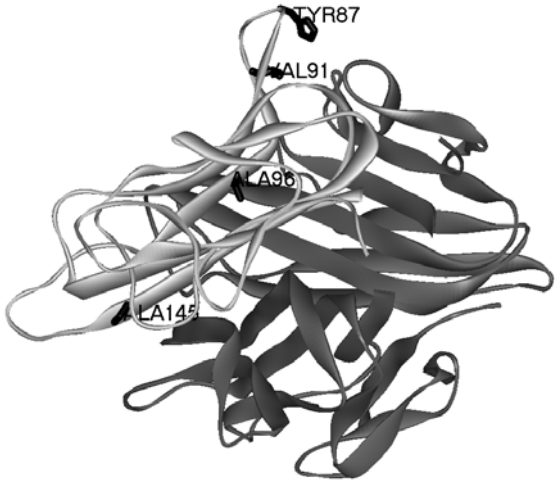

Fig. 1. Schematic representation of the spatial structure of TNF heterotrimer. The monomer of the mutant analog of the protein is shown in gray. Positions of mutations are indicated.

Previously, we constructed plasmid pTNF331 [9] carrying the human TNF gene (3-157) truncated at the N-terminus by two amino acid residues. It was established that removal of several N-terminal residues (up to seven) did not affect the cytotoxic activity of TNF [16]. The gene in the plasmid is placed under the control of two constitutive promoters, A2 and A3, from the early region of bacteriophage T7 and the synthetic Shine–Dalgarno (SD) sequence. This expression system provided a high level of biosynthesis of recombinant TNF by *E. coli* cells. The pTNF331-based constructions encoding mutant TNF forms were obtained. Point mutations were introduced by the method of polymerase chain reaction (PCR) with two mutagenizing and two terminal primers using

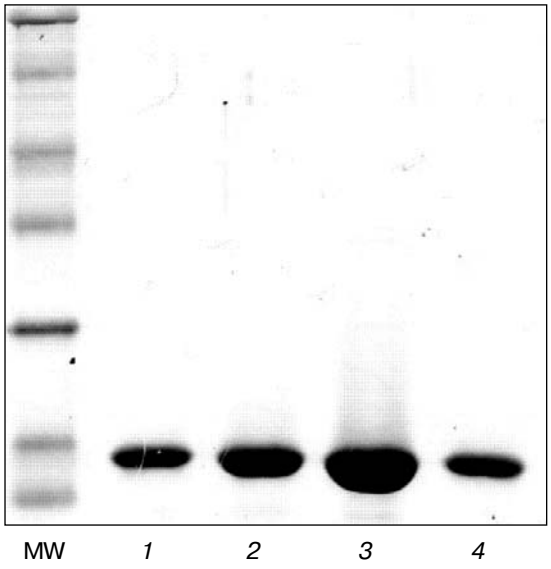

Fig. 2. Laemmli electrophoresis of purified TNF and its mutant analogs: *1*) TNF; *2*) mutant TNFY87H + A145R; *3*) mutant TNFY87H + A96S + A145R; *4*) TNFV91N + A145R; MW, molecular weight standards (Fermentas).

pTNF331 as a template. The resulting plasmids pmut1, pmut2, and pmut3 were used for transformation of *E. coli* SG20050 cells. All of the constructed genes are characterized by a high level of expression in bacterial cells (up to 20% of total cell protein); the recombinant proteins are present in soluble form.

The next stage of the study was elaboration of laboratory conditions for isolation of recombinant proteins in preparative quantities. It was based on the method of two-stage chromatography on hydroxyapatite and DEAE Toyopearl 650M columns previously developed for wild type TNF isolation, with certain modifications. The modifications consisted in optimization of buffer solution compositions and chromatographic separation parameters. In the case of the mutants, the protein was isolated at pH 8.0 because the introduced Arg residue resulted in a change in the isoelectric point of the mutants, and optimal separation was observed at this pH value. The selected scheme of isolation proved to be equally effective both for purification of wild type TNF as well as the mutant proteins. The degree of purification was more than 95% (Fig. 2). The yield of purified proteins was 40 to 55 mg protein in equivalent of 1 liter of overnight culture.

The mechanism of DN-TNF blocking effect is based on formation of inactive heterotrimers with the native protein. For the study of their formation processes, we obtained a strain-producer of wild type TNF containing a hexahistidine sequence at the N-terminus of the native protein. It is known that addition of up to nine amino acid residues to the N-terminal region of TNF neither disturbs its spatial structure nor affects its biological activity [8, 16]. The TNF gene was amplified on the pTNF331 template and cloned into plasmid pET28a by the recognition sites of restrictases *Nhe*I and *Hind*III. The level of $(His)_6$TNF biosynthesis by the cells of *E. coli* strain BL21(DE3) transformed by the obtained plasmid pETTNF reached 25% of total cell proteins. It was purified from the soluble cell lysate fraction by Ni-affinity chromatography.

Heterotrimers were formed by mixing $(His)_6$TNF and the mutants Y87H + A145R, V91N + A145R, and Y87H + A96S + A145R in the ratio of 1 : 1 followed by incubation for 1 h at 37°C and then for 18 h at 4°C. $(His)_6$TNF with the native TNF was incubated under the same conditions as the control. Interchange products were isolated on analytical columns with Ni-NTA agarose and analyzed by SDS-PAGE (Fig. 3). Ni-NTA agarose interacts with the proteins containing a hexahistidine linker; consequently, the isolated proteins could be theoretically either $(His)_6$TNF homotrimers or $(His)_6$TNF/mutant heterotrimers. Since the TNF mutants have no $(His)_6$, their molecular weight is less and the heterotrimer under denaturing conditions of electrophoresis yields two bands: for $(His)_6$TNF and for the mutant component of the heterotrimer.

It has been shown that the products isolated by affinity chromatography contain both $(His)_6$TNF and the proteins without hexahistidine sequence, i.e. all of the mutants form heterotrimers with wild type TNF. Heterotrimer formation was also observed in the case of $(His)_6$TNF/TNF, but to a much lesser extent than with the mutants.

The biological properties of the mutants were studied in the standard test for TNF cytotoxicity against the cells of L929 mouse fibroblasts. Different dilutions of the proteins Y87H + A145R, V91N + A145R, and Y87H +

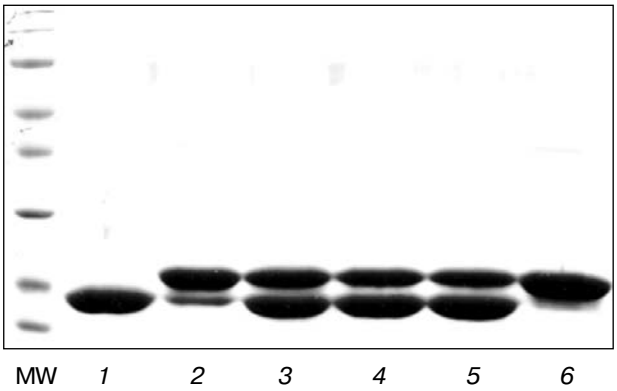

Fig. 3. Laemmli electrophoresis of TNF homo- and heterotrimers isolated by affinity chromatography on Ni-NTA Sepharose: *1*) TNF; *2*) TNF + $(His)_6$TNF; *3*) TNFY87H + A145R + $(His)_6$TNF; *4*) TNFY87H + A96S + A145R + $(His)_6$TNF; *5*) TNFV91N + A145R + $(His)_6$TNF; *6*) $(His)_6$TNF. MW, molecular weight standards (Fermentas).

A96S + A145R (0-100 ng/ml) were added to the cells, and viability of the cells was measured by the degree of binding of MTT dye. Recombinant TNF was used as a control. This experiment showed that the mutants had no cytotoxic activity typical of the native protein (data not shown). Further, the ability of the isolated proteins to neutralize cytotoxicity induced by the native TNF was investigated. The excess of mutant analogs was added to TNF in ratio 10 : 1, the mixture was incubated for 1 h at 37°C, and cytotoxic activity of the mixture was measured in line L929 as described above. It was shown that all of the mutants significantly reduced the activity of the added native protein (Fig. 4). The mutant TNFV91N + A145R proved to be the most active inhibitor; its inhibitory activity depended on mutein concentration. So, when TNFV91N + A145R was added to TNF in the ratios of 5 : 1 and 10 : 1, the TNF concentration causing a 50% decrease in cell survival was observed to be increased from 0.07 to 1 or 2 ng/ml, i.e. 14- or 28-fold (Fig. 5). With 20-fold excess of the mutant, this concentration reached 6.5 ng/ml (i.e. increased 93-fold) (Fig. 5). In other words, the effective TNF concentration decreased nearly 100 times due to formation of inactive heterotrimers. These data lead to the conclusion that the two novel mutants, especially the mutant TNFV91N + A145R, are more efficient TNF blockers *in vitro* than the previously studied Y87H + A145R. It is interesting that complete neutralization of TNF does not take place even with a 20-fold excess of DN-TNF, though theoretically all native TNF should have become incorporated in heterotrimers. This fact seems to be due to continuous exchange of monomers between the trimer molecules; as a result, a certain background quantity of homotrimer is always present statistically. It should be emphasized that DN-TNF form inactive heterotrimers only with the soluble form of TNF (sTNF) and do not interact with the membrane form of the protein (tmTNF). This very fact demonstrates their advantage over the inhibitors based on antibodies or soluble receptors that block both forms. The studies of recent years have shown that the signals transmitted by sTNF are associated with chronic inflammation, whereas tmTNF plays a key role in suppression of inflammation and formation of resistance to various pathogens. On the other hand, the function of TNF concerning preservation of the structure of lymphoid organs is provided primarily by soluble TNF produced by B cells [17]. Long-term therapy with antibodies can result in immunosuppression and enhanced susceptibility to infections, in particular, to *Mycobacterium tuberculosis* and *Listeria monocytogenes* [7, 18, 19]. It should be noted that, although DN-TNF can be free from these drawbacks, these proteins are small and quickly excreted from the body. In particular, such mutants have no sufficient blocking activity in the model of septic shock because of insufficient lifetime *in vivo* (data not shown), so they must be stabilized. A known method of modification is introduction of polyethyleneglycol (PEG) residues into a protein molecule without changing its activity. It is important to emphasize that additional mutations must be introduced into the TNF molecule for this kind of stabilization of the mutant proteins. The properties of PEGylated DN-TNF are actively studied by many research teams. Thus, experiments in mice have shown that sTNF inhibition by the PEG derivatives of DN mutants XENP345 and XENP1595 (mutations A145R + I97T and Y87H + A145R) prevents amyloid-associated neuropathology in

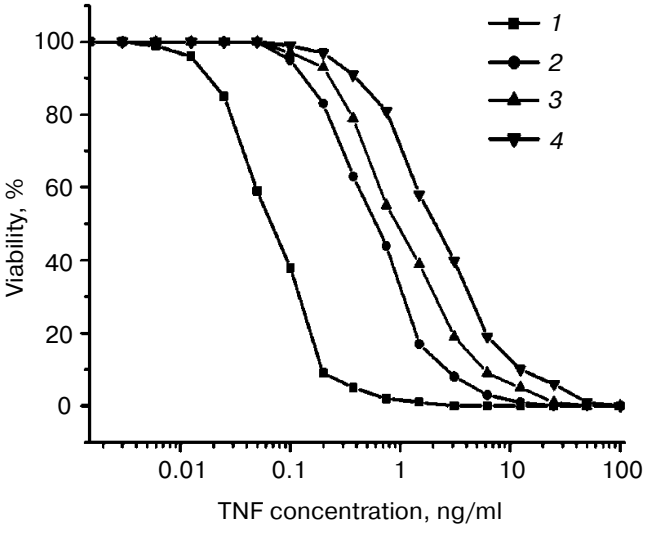

Fig. 4. Cytotoxic effects of TNF and TNF/mutant mixtures on L929 mouse fibroblasts: *1*) TNF; *2*) TNF + mut1; *3*) TNF + mut2; *4*) TNF + mut3. The ratios of TNF/mutant concentrations, 1 : 10; mut1, TNFY87H + A145R; mut2, TNFY87H + A96S + A145R; mut3, TNFV91N + A145R.

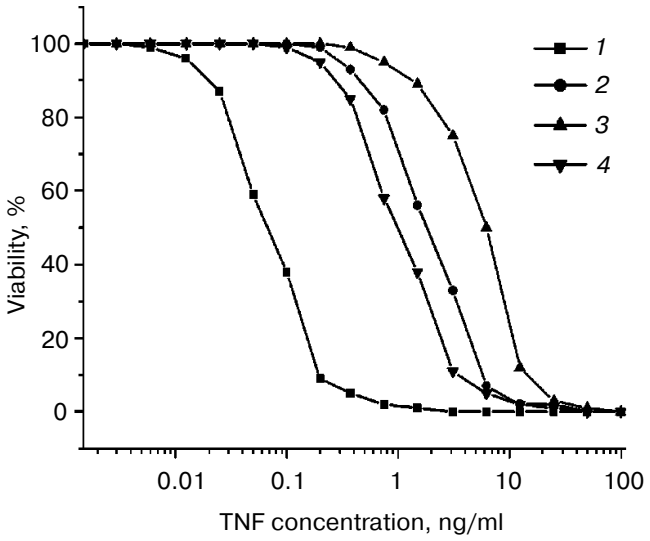

Fig. 5. Effect of mutant TNFV91N + A145R concentration on cytotoxic activity of native TNF in the standard test in L929 mouse fibroblasts: *1*) TNF; *2*) TNF + mut3 (1 : 10); *3*) TNF + mut3 (1 : 20); *4*) TNF + mut3 (1 : 5).

the model of Alzheimer's disease [20], prevents endotoxin-induced inflammation of liver [21], and slows the degradation of neurons in a model of Parkinson disease [22], though they still cannot protect animals from septic shock (data not shown).

Thus, the mutants TNFY87H + A96S + A145R and TNFV91N + A145R that we have obtained effectively inhibit TNF activity *in vitro*, possess higher blocking activity compared to the mutant TNFY87H + A145R described in literature, and, after appropriate modification, might serve as a basis for creation of novel "soft" blockers of human TNF.

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