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A New System for TNF-α Quantification in Human Blood Samples

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Abstract—Full-length human TNF-α and its truncated derivative in which 18 N-terminal amino acid residues were replaced with T7-tag were obtained as inclusion bodies as a result of expression of artificial genes in *E. coli* cells. The purification and refolding procedures for the recombinant proteins were developed, and biological activity of the resultant proteins was demonstrated. Polyclonal antibodies against TNF-α were obtained. A sandwich ELISA test system for TNF-α quantification (sensitivity, 100 pg/mL) was constructed. It was shown that this system is applicable for detecting TNF-α elevation in human blood.

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Tumor necrosis factor α (TNF-α), an antigenic protein, was initially characterized as a protein that causes necrosis of experimental tumors in mice [1]. Data accumulated so far indicate that TNF-α is one of the key protein immune mediators produced primarily by activated macrophages. TNF-α is biologically active in the form of a trimer, which is believed to be necessary for fulfilling its numerous functions [2]. As an antiinflammatory cytokine, TNF-α plays the key role in pathogenesis of some diseases and conditions, such as rheumatoid arthritis, psoriasis, and septic shock [3, 4]. Some diseases of both infectious (tuberculosis) and autoimmune (scleroses and sarcoidosis) etiology are accompanied by elevation in the TNF-α level [5]. The dynamics of TNF-α in patient's blood may serve as an indicator of development of infection and subsequent stages of disease. This fact can be used in clinical practice for diagnosing various infectious diseases.

Although a number of commercial systems for TNF-α quantification are available, only a few papers describe their use in clinical practice. Possibly, this is due to an increased sensitivity of these systems, which may reach 0.5 pg/mL, while the content of TNF-α in patient's blood amounts to hundreds of picograms. The goal of this study was to design a new immunochemical system for detecting TNF-α in blood samples of patients and healthy subjects, which can be used in clinical practice.

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MATERIALS AND METHODS

Expression in *E. coli* was performed using T7 promoter-based vector systems (pET23a, Novagen). Plasmid DNA was isolated as described in [6], *E. coli* was cultured in standard LB nutrient medium. Protein expression and primary purification were performed as described in [7]. During protein denaturation, the content of moist pellet of inclusion bodies was 0.5 g per 10 mL of a buffer containing 50 mM Tris-HCl and 7 M urea. Protein was reduced in the presence of 10 mM DDT (pH 8.0). Sulfitolysis was performed in the presence of 50 mM sodium tetrathionate and 50 mM sodium sulfite (pH 8.8). Undissolved residue was removed by centrifugation. Before loading on a DEAE–Toyopearl column, protein solution was diluted five to ten times with the chromatographic buffer containing 10 mM Tris-HCI (pH 8.0) and 4 M urea. Protein was eluted with a NaCl concentration gradient (0–1 M) in the chromatographic buffer. The content of the product in fractions was determined by denaturing PAGE. Protein concentration was determined by a modified Lowry protein assay using the bicinchoninic reagent [8].

Refolding was performed by one-step 17-fold dilution of denatured protein. Refolding buffers were prepared according to the protocol [http://www.athenaenvironmental.com/]. The total concentration of protein remained in solution after separation of aggregates was determined by a modified Lowry method.

Immunization of rabbits with the recombinant TNF-α is described in [7]. Immobilization of the recombinant TNF-α on cyanogen bromide-activated agarose (Amersham Pharmacia Biotech) was performed as recommended by the manufacturer. Polyclonal antibodies were affinity purified as described in [9]; the concentration of RA-TNF antibodies was 1 mg/mL. The antibodies were biotinylated with biotin hydroxysuccinimide

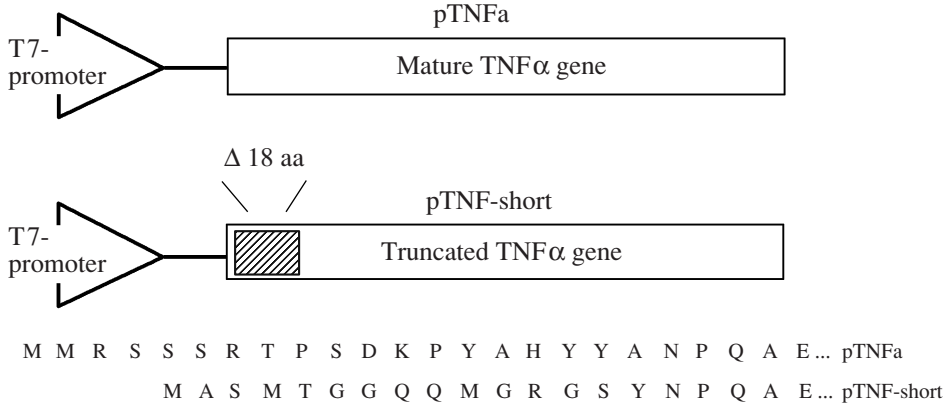

Fig. 1. Schematics of pET23a-based expression constructs pTNFa and pTNF-short, encoding the full-length and truncated variants of human TNF-α. The N-terminal sequences of the products of both constructs are shown.

(Amersham Pharmacia Biotech) according to the protocol provided by the manufacturer.

The biological activity of TNF-α was assayed by the standard test for apoptosis induction in L929 mouse fibrosarcoma cells treated with actinomycin D [10]. The cells were plated in two flat-bottom 12-well Costar plates (30 thousand per well) filled with a medium containing 50% DMEM, 50% F12, and 1% serum (PanEco, Russia). The cells were cultured at 37°C in a moist atmosphere containing 5% CO_2 until a monolayer on the bottom of wells formed. Then, the culture liquid was replaced with a fresh one (2 mL) supplemented with actinomycin D (10 μg/mL) and tested TNF-α preparations (final concentration, 50 ng/mL). In parallel experiments, the same TNF-α preparations preliminarily neutralized with RA-TNF antibodies were used. To neutralize TNF-α preparations, they were incubated at a final concentration of 10 μg/mL in the presence of antibodies (100 μg/mL) and PBS (20 mM sodium phosphate (pH 7.5) and 100 mM NaCl) at 4°C for 20 h. The plates were incubated at 37°C for 16 h in a moist atmosphere containing 5% CO_2. Cells grown in the culture medium supplemented with PBS as well as cells with free RA-TNF antibodies served as a control. After incubation, the culture medium was removed and the cells were stained with 0.1% crystal violet prepared in 2% ethanol. The optical density was measured at 595 nm.

Calibration of Sensitivity of the Sandwich System

Before experiments, 96-well polystyrene Costar plates were activated by overnight incubation at 4°C with RA-TNF antibodies (10 μg/mL) in 50 mM sodium carbonate–bicarbonate buffer, pH 9.6 (NCA buffer). Nonspecific binding was blocked by incubation at room temperature for 1.5–2 h with 1% bovine serum albumin (BSA) in NCA buffer. Then, a series of dilutions of the full-length TNF-α (six points with a fivefold step, from 20000 to 6 pg/mL) was prepared in the presence of a healthy donor blood diluted 5 to 20 times with PBS. Incubation was performed at room temperature for 2 h. Thereafter, biotinylated antibodies B-RA-TNF were added at a concentration of 2 μg/mL. After incubation of the mixture at room temperature for 1 h, horseradish peroxidase conjugated with streptavidin (IMTEK, Moscow, cat. no. P-S Avs) was added at a concentration of 1 μg/mL, and the resulting mixture was incubated at room temperature for another 1 h. The color reaction for peroxidase was performed in the substrate buffer (400 μg/mL *o*-phenylenediamine, 0.015% H_2O_2, and 100 mM sodium citrate (pH 5.0)). The reaction was stopped by addition of 30 μL of 10% H_2SO_4. The optical density was measured at 492 nm.

The resolving capacity of the constructed test system with respect to clinical blood samples was studied by the same procedure that was used for determination of the sensitivity of this system (see above). Experiments were performed with sera of 100 healthy subjects; each serum was diluted five times with PBS.

RESULTS AND DISCUSSION

The antigenic protein for the immunological test system intended for TNF-α detection was obtained using the pET23a-based constructs described earlier, which encode the full-length mature TNF-α (construct pTNFa) and its N-terminally truncated variant lacking 18 amino acid residues (construct pTNF-short) [7]. In the product of the pTNF-short construct, 18 N-terminal amino acid residues were replaced with 15 amino acid residues of T7 protein 10 (Fig. 1).

The recombinant proteins were accumulated in the form of inclusion bodies with a yield of 200 mg/mL. The inclusion bodies were solubilized under denaturing conditions either by the conventional procedure by breaking disulfide bonds as a result of reduction with DTT or by oxidative sulfitolysis with formation of the sulfonated derivative of the protein [11]. It was assumed that the reduced and sulfonated forms of the

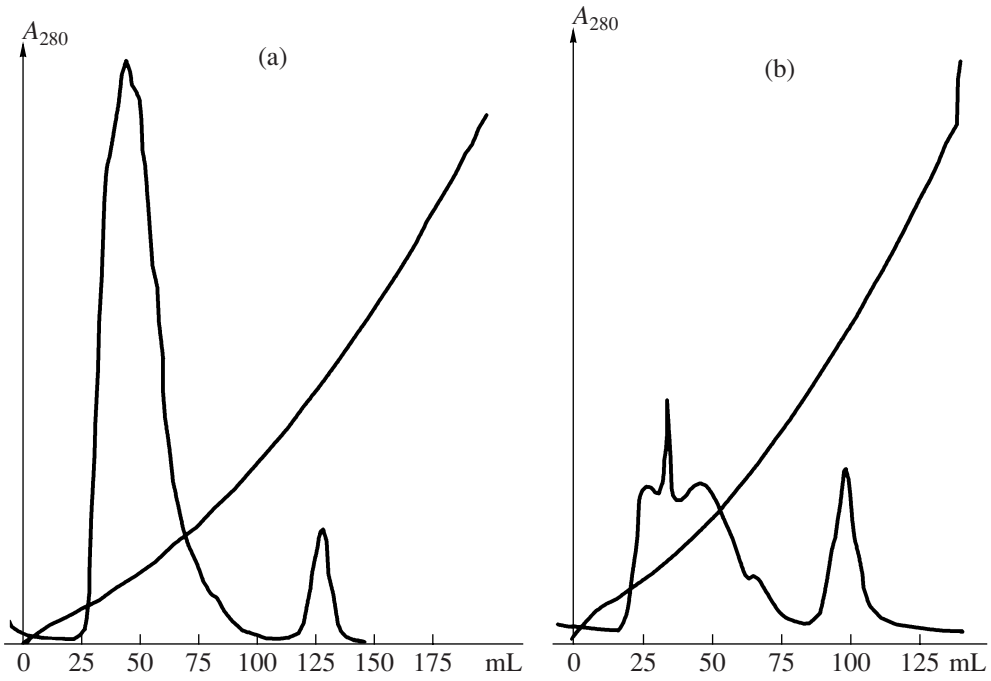

Fig. 2. TNF-α chromatographic profile. Chromatography was performed on DEAE-Toyopearl under denaturing conditions; before loading onto the column, the inclusion bodies were solubilized in urea simultaneously with (a) reduction with DTT and (b) sulfitolysis.

denatured protein will differ in purification efficiency under denaturing conditions and subsequent refolding. Clarified preparations of denatured protein were subjected to anion-exchange chromatography under denaturing conditions. Chromatography was performed in a buffer containing 4 M urea. The advantage of reducing the concentration of the denaturing agent is that it is sufficient to dilute the initial preparation only twice during subsequent refolding by dilution (to a final urea concentration of 2 M). As a result, the refolded protein solution is more concentrated.

After the treatment with DTT, the full-length TNF-α was chromatographed on DEAE-Toyopearl (pH 8.2) and elution with a NaCl concentration gradient. The protein and DNA were desorbed from the resin at 120 mM and 450 mM NaCl, respectively. Anion-exchange chromatography of the full-length TNF-α after sulfonation was performed under the same conditions. However, in this case, three peaks on the chromatogram were observed, which presumably corresponded to different products of sulfitolysis of two disulfide bonds in the TNF-α molecule. The protein and DNA were desorbed from the column at 80–160 and 420 mM NaCl, respectively (Fig. 2). Since the obtained preparation was not sufficiently homogeneous, in further experiments we used reduction with DTT. The product of the pTNF-short construct was purified by the same techniques.

Proteins were refolded using the set of buffers [http://www.athenaenvironmental.com/]. The efficiency of TNF-α refolding was assessed by the yield of the soluble protein and by the results of the cytotoxicity test performed with L929 mouse fibrosarcoma cells [10]. The highest specific biological activity was detected in the proteins refolded in buffers nos. 7 and 13 (Fig. 3).

The specificity of effect of the TNF-α forms was assessed in the test for neutralization of TNF-α activity by purified rabbit antibodies raised against human TNF-α. The results shown in Fig. 3 show that the cytotoxic effect of renatured TNF-α preparations is not associated with contaminants, because it was completely suppressed by purified antibodies that are unable to interact with these contaminants. Regarding the presence of the cytotoxic activity in the truncated TNF-short protein, it should be noted that the truncated TNF-α contained no Lys-11, which is involved in stabilization of the TNF-α trimer [12]. Reed et al. [2] assumed that the trimeric structure is necessary for TNF-α binding to receptors, and the presence of biological activity in the TNF-short protein is quite unexpected. It cannot be ruled out that the complex formed by the truncated protein with the receptor may have an abnormal structure as compared to the natural ligand and that, possibly, this protein has an unusual spectrum of physiological activity, which may be important for fundamental studies of the biological role of TNF-α and the mechanisms of information transmission by ligand–receptor complexes. It may be also useful in diagnosing and treating some diseases caused by immune system malfunction.

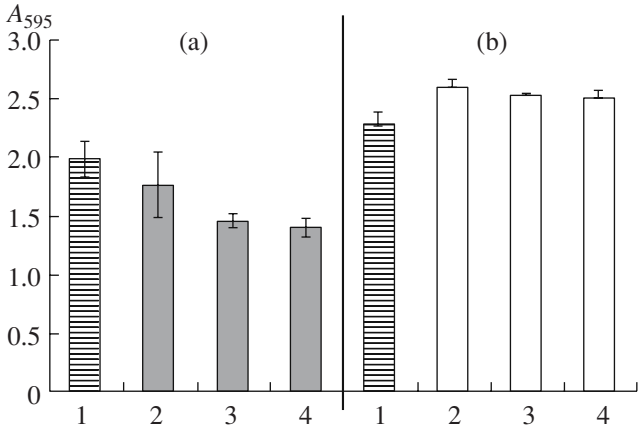

Fig. 3. Cytotoxic effect of refolded TNF-α on mouse fibrosarcoma L929 cells in the (a) absence and (b) presence of antibodies to TNF-α: (*1*) negative control, (*2*) TNF-α refolded in buffer no. 7, (*3*) TNF-α refolded in buffer no. 13, and (*4*) TNF-α-short refolded in buffer no. 13.

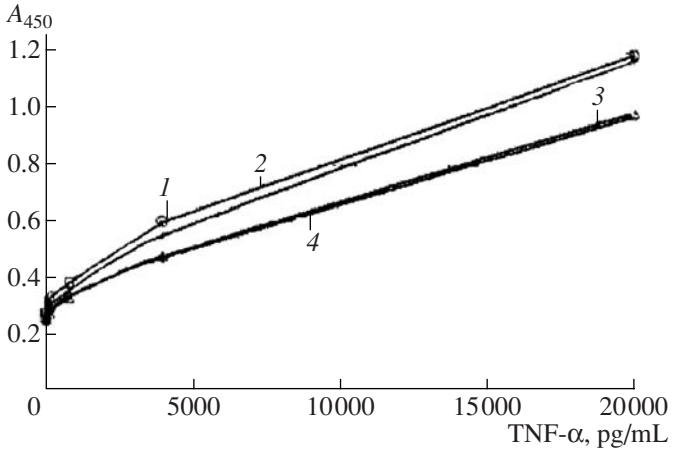

Fig. 4. Calibration of sensitivity of the sandwich ELISA test system for determination of TNF-α content. The content of donor serum in the sample was (*1*) 5, (*2*) 7, (*3*) 10, and (*4*) 20%.

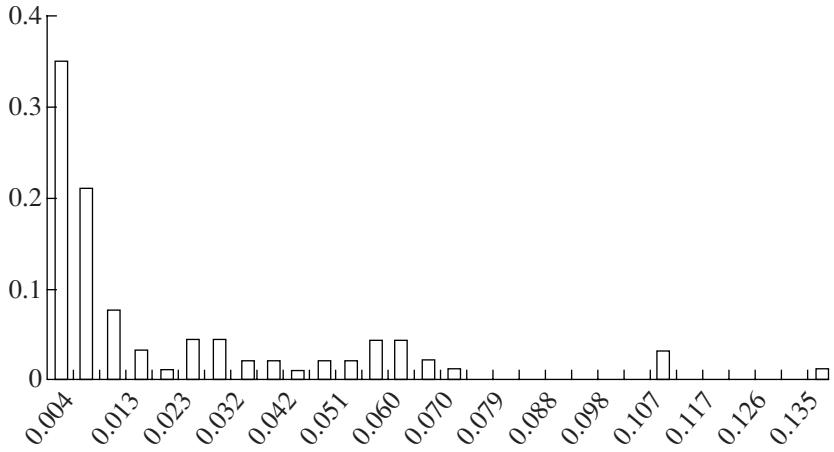

Fig. 5. Distribution of TNF-α level in sera of healthy donors determined by the sandwich ELISA test system.

The full-length TNF-α was used for immunization of rabbits to obtain monoclonal antibodies. After purification on a sorbent containing immobilized full-length TNF-α, part of antibodies was biotinylated. The scheme of the sandwich ELISA test included adsorption of nonbiotinylated antibodies at a saturating concentration on the surface of an immunological plate, blocking of nonspecific binding with BSA, addition of a source of antigen (donor sera), marking the bound antigen with biotinylated antibodies, and visualization with horseradish peroxidase-conjugated streptavidin. To determine the sensitivity and specificity of this system, we calibrated it with the use of a series of dilutions of the full-length TNF-α in the presence of blood serum of a healthy subject which was diluted 5–20 times and served as a competitor (Fig. 4). As can be seen from the calibration curve, the resultant signal was proportional to TNF-α concentrations in the initial sample varying from 100 to 5000 pg/mL and did not depend on the amount of the competitor.

The level of the signal detected by the test system was verified using a sample of healthy donors (100 persons). When serum was diluted five times, approximately half of A_{492} values was below 0.01. The remaining A_{492} values varied in the range from 0.01 to 0.08, and several values were as high as 0.12. Thus, our system can detect weak signals that significantly differ from zero. This sensitivity makes it possible to reliably detect TNF-α elevation in human blood in any pathologies.

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