Recombinant TNF-Binding Protein from Variola Virus as a Novel Potential TNF Antagonist

I. P. Gileva*, T. S. Nepomnyashchikh, I. A. Ryazankin, and S. N. Shchelkunov

FGUN State Research Center of Virology and Biotechnology Vector, 630559 Koltsovo, Novosibirsk Region, Russia; fax: (383) 336-7409; E-mail: gileva@vector.nsc.ru

Abstract—Gel-filtration chromatographic separation of the lysate of Sf21 insect cells infected with recombinant baculovirus BVi67 containing the gene for TNF-binding protein (CrmB) of variola virus (VARV) revealed that hTNF-cytotoxicity neutralization activity is associated with a fraction corresponding mainly to high molecular weight proteins (above 500 kDa) and less with fractions corresponding to proteins of 270 or 90 kDa. The recombinant VARV-CrmB protein has been purified by affinity chromatography. Difference in the experimentally determined and estimated (according to amino acid composition) VARV-CrmB molecular weight is due to glycosylation of the recombinant protein expressed in the insect cells. VARV-CrmB neutralizes *in vitro* the cytotoxic effect of hTNF and hLTα, and its TNF-neutralizing activity is two to three orders of magnitude higher compared to the analogous effects of type I and II soluble TNF receptors, comparable with the activity of mAb MAK195, and somewhat lower than the effect of the commercial drug Remicade.

Tumor necrosis factor (TNF) and lymphotoxin (LT) play key roles in the development of inflammatory and immune reactions in the human body. They initiate a cascade of intracellular reactions by interacting with cell receptors TNFRI (p55) and TNFRII (p75) [1, 2]. However, overproduction of these cytokines and subsequent hyperactivation of corresponding receptors leads to the development of chronic inflammation and tissue destruction [3]. Immunocorrection of such diseases includes application of drugs that inhibit synthesis and processing of the abovementioned cytokines and also preventing interaction of cytokines with excitatory cells [4]. Among the latter group, we can distinguish drugs in which effectors are represented by monoclonal antibodies (infliximab, adalimumab) [5-7] or recombinant chimeric proteins consisting of TNF receptor and immunoglobulin

domains (etanercept) [8, 9]. Although some TNF blockers, e.g. Remicade (Centocor Inc, USA), Enbrel (Immunex Corp, USA), and Humira (Abbott Laboratories, USA), in which the active ingredients are infliximab, etanercept, and adalimumab, correspondently, have passed clinical testing and are allowed for use in medical practice, there are contraindications for their application (cardiovascular diseases [10], tuberculosis [11], latent viral infections [12]). Also, the indicated drugs are not universal. For example, etanercept is effective for rheumatoid arthritis therapy [13], but not for septic shock [14]. Therefore, the search for novel TNF antagonists is still an important task.

Variola virus (VARV) is a very dangerous human pathogen. During evolution, this virus mastered different mechanisms to overcome the immune response [15]. Specifically, the viral genome determines the synthesis of secreted proteins − viroceptors that are structurally similar to cytokine cell receptors [16]. The viral proteins function as soluble cytokine-binding receptors, therefore blocking their activity. Earlier we obtained recombinant baculovirus BVi67 containing the gene of TNF-binding VARV protein (VARV-CrmB), demonstrated that culture medium of Sf21 cells infected with this recombinant baculovirus has TNF-neutralizing activity [17, 18], and

---

Abbreviations: aAG, α_1-acid glycoprotein; CPXV, cowpox virus; Crm, cytokine response modifier; F_c, constant fragment of immunoglobulin molecule; LPS, lipopolysaccharide; LT, lymphotoxin; mAb, monoclonal antibody; MPXV, monkeypox virus; TNF, tumor necrosis factor; TNFRI(II), tumor necrosis factor receptor type I(II); TNFsR, soluble tumor necrosis factor receptor; VARV, variola virus.
* To whom correspondence should be addressed.

developed a method for recombinant viral protein separation [19]. TNF-binding VARV protein has substantially higher TNF-neutralizing activity in experimental systems *in vitro* and *in vivo* compared with analogous viral proteins of monkeypox virus (MPXV) and cowpox virus (CPXV) [20].

The goal of this study was to determine detailed characteristics of the recombinant protein VARV-CrmB produced in insect cells as a potential TNF antagonist. For that purpose, we analyzed in what form this protein is synthesized in insect cells and estimated the efficiency of neutralization of hTNF cytotoxic activity *in vitro* against known TNF antagonists − monoclonal TNF-neutralizing antibody MAK195 [21], soluble cell receptors TNFRI and TNFRII, and the commercial drug Remicade [22].

MATERIALS AND METHODS

Materials. For cell culture, we used 96-well plates from Orange Scientific (USA), Grace's medium, antibiotics, and L-glutamine from Gibco/BRL (USA), DMEM produced by FGUN State Research Center of Virology and Biotechnology Vector (Russia), and fetal bovine serum produced by BIOLOT (Russia). We also used BrCN-Sepharose 4B (Amersham Pharmacia Biotech, USA), soluble cell receptors TNFsRI and TNFsRII (R&D Systems, USA), Coomassie Blue R250 dye (Sigma, USA), and GelCode Glycoprotein Staining kit and Glycoprotein Carbohydrate Estimation kit (Pierce, USA). Preparations of the recombinant cytokines hTNF ($6 \cdot 10^7$ U/mg) and hLTα (10^7 U/mg) were kindly furnished by N. M. Pustoshilova (Engineering Immunology, Russia). TNF-neutralizing monoclonal antibodies MAK195 and Remicade drug were kindly furnished by G. M. Ignat'ev (Tarasevich NISC, Russia). The Remicade capsule contains infliximab (100 mg), sucrose (500 mg), Tween 80 (0.5 mg), NaH_2PO_4 (2.2 mg), and Na_2HPO_4 (6.1 mg).

Cell cultures. Cell lines from the bollworm *Spodoptera frugiperda* Sf21 and mouse fibroblasts L929 were obtained from the culture collection of FGUN State Research Center of Virology and Biotechnology Vector. Cells of Sf21 line were cultivated at 27°C in Grace's medium with the addition of 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells of line L929 were cultivated in an atmosphere with 5% CO_2 at 37°C in DMEM medium with the same additions.

Gel filtration of lysates of infected insect cells. Sf21 cells infected with the recombinant baculovirus BVi67 expressing VARV-CrmB [17] were resuspended in growth medium, and the resulting suspension was subjected to freezing-thawing cycling three times. After removing debris by low speed centrifugation, the supernatant was applied on a 2.5 × 84-cm column with Biogel A-0.5m

(100-200 mesh; BioRad, USA) equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The column was eluted at 25 ml/h, and fractions of 4-ml volume were collected.

The resin, according to the specification of the producer, allows separation of proteins in the range 10-500 kDa. To calibrate the column, the following protein standards were used: ovalbumin (45 kDa), BSA (66 kDa), catalase (232 kDa), ferritin (440 kDa) (all these proteins were from Serva, Germany), and blue dextran (2000 kDa; Sigma), the latter marking the free volume of the column. The protein concentration in fractions was determined using an Ultraspec 3000 spectrophotometer (Amersham Pharmacia Biotech). TNF-neutralizing activity in the fractions was determined as described in [18].

Analysis of recombinant protein glycosylation. The recombinant protein VARV-CrmB was isolated from the infected insect cells Sf21 by affinity chromatography as described in [19]. The protein concentration was determined by Bradford's method [23]. Electrophoretic fractionation of proteins in polyacrylamide gel was done by Laemmli's method [24] under reducing and non-reducing conditions achieved by the presence or absence of 2-mercaptoethanol (0.2 M) in the sample application buffer. Polypeptides were stained by Coomassie Blue R250 and GelCode Glycoprotein Staining according to the producer's recommendations. The extent of glycosylation of recombinant protein VARV-CrmB was determined using Glycoprotein Carbohydrate Estimation kit in the format of 96-well plates, comparing the absorption of experimental samples and standard proteins measured with an EL$_X$808 Microplate Reader (Bio-Tek Instruments, USA) at 550 nm wavelength. We modified the producer's protocol so that the results are represented by the ratio of A_{550} to the protein amount used for the analysis (A_{550}/mg).

Determination of biological activity of protein VARV-CrmB *in vitro*. The biological activity of VARV-CrmB protein and commercial TNF antagonists was determined by their ability to inhibit cytotoxic activity of hTNF (or hLTα) in mouse fibroblast cell culture L929 as described in [25]. The results are presented as a ratio (%) of surviving cells to the number of cells in controls not treated with cytokines. Each point was measured thrice, and the mean of survival (%) was calculated using the formula:

$$(A_{TNF + CrmB} - A_{TNF})/(A_{cells} - A_{TNF}) \times 100.$$

Absorbance (A) was measured at 550 nm.

RESULTS

Characteristics of recombinant protein VARV-CrmB. The recombinant protein was isolated by affinity chromatography from the culture medium of destroyed insect cells of Sf21 line infected with recombinant baculovirus
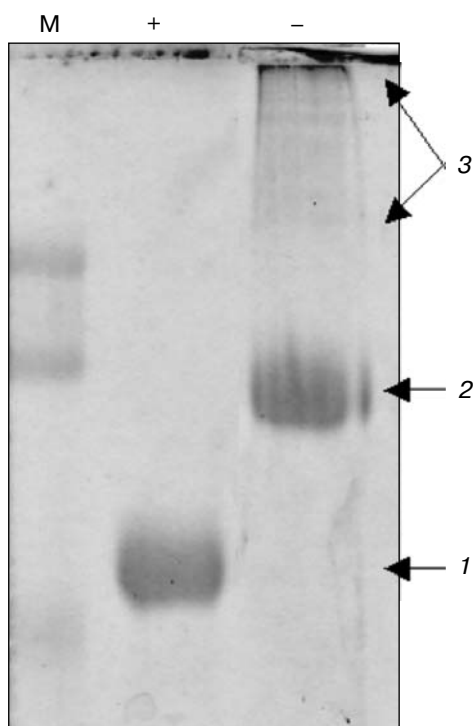
Fig. 1. Electrophoretic fractionation of recombinant protein VARV-CrmB in 10% polyacrylamide gel. In samples **(+)** the application buffer contained 1% 2-mercaptoethanol, in samples (−) 2-mercaptoethanol was absent. The gel was loaded with 8 μg protein per lane. Arrows indicate monomeric (*1*), dimeric (*2*), and oligomeric (*3*) VARV-CrmB forms. M, proteins with molecular weight 114, 88, and 50 kDa (β-galactosidase, BSA, and ovalbumin, respectively) (Prestained SDS-PAGE Standards, low range; BioRad).

[19]. The preparation was fractioned in polyacrylamide gel reducing and non-reducing conditions. As we have shown before [20], under non-reducing conditions VARV-CrmB is detected by staining the gel with Coomassie Blue R250 as a polypeptide with molecular weight 90 kDa, and under reducing conditions as a 47-kDa protein. High molecular weight, supposedly oligomeric forms of VARV-CrmB are revealed by staining with Coomassie Blue R250 during fractionation of the protein (8 μg) (Fig. 1). The monomer molecular weight (47 kDa) is higher than expected from the amino acid composition of VARV-CrmB (38.2 kDa) [20]. The difference between experimental and theoretically assumed molecular weight of the polypeptide is probably explained by glycosylation of VARV-CrmB in insect cells. Differential staining of VARV-CrmB and protein standards glycosylated to different extents after fractionation in polyacrylamide gel (Fig. 2, a and b) confirm the glycosylation of VARV-CrmB. To estimate the extent of glycosylation of the desired product, we used the Glycoprotein Carbohydrate Estimation kit. The oxidation of *cis*-glycol glycoprotein groups to aldehydes and subse-
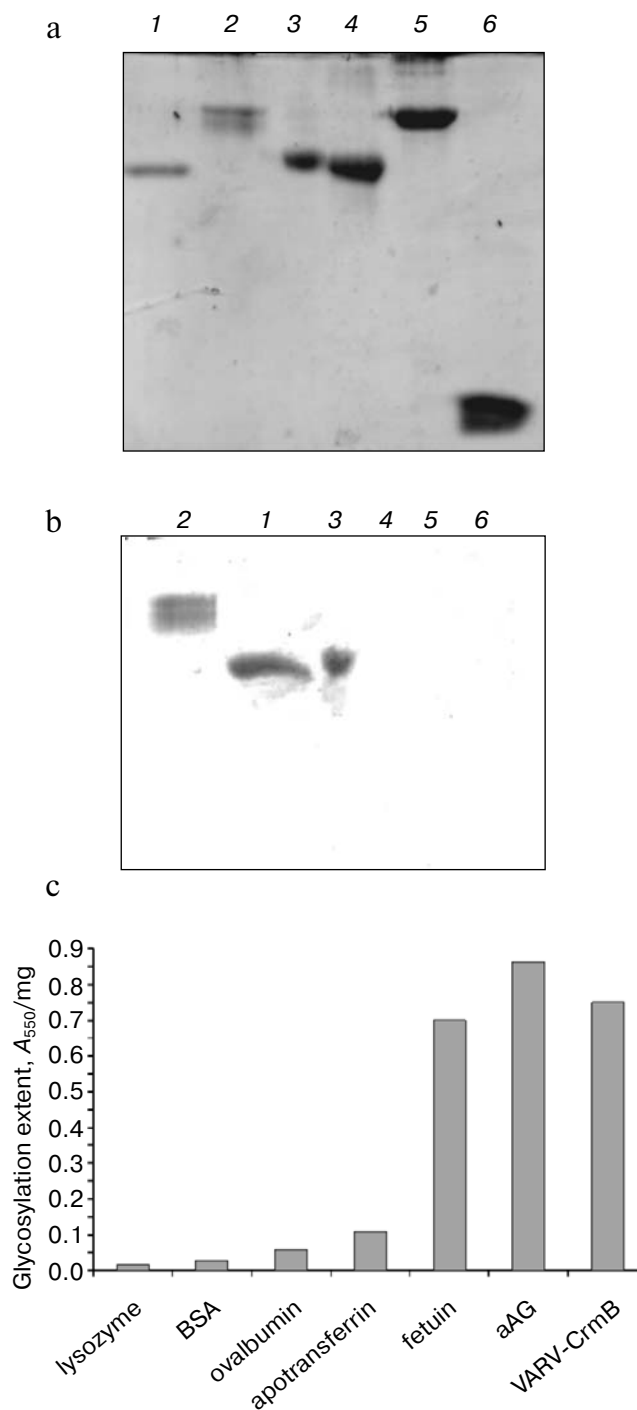
Fig. 2. a, b) Glycosylation of VARV-CrmB protein. Recombinant protein VARV-CrmB (lane *3*) and marker proteins aAG, fetuin, ovalbumin, BSA, and lysozyme in two repeats (lanes *1*, *2*, *4-6*, correspondingly) were fractionated by electrophoresis in 12% polyacrylamide gel. One half of the gel was stained with Coomassie Blue R250 (a), and the second by reagents from the GelCode Glycoprotein Staining kit (b) (samples *1* and *2* are applied in reverse). c) Number of carbohydrate residues estimated with the Glycoprotein Carbohydrate Estimation kit. Ordinate axis, the ratio of A_{550} to the protein amount taken for the analysis is presented (A_{550}/mg).

quent staining of the reaction products allows us to estimate the percentage of carbohydrate residues in samples by measuring their absorption at 550 nm. The results of such determination are shown in Fig. 2c. The extent of VARV-CrmB glycosylation can be estimated as intermediate between fetuin (22.9% glycosylated) and α_1-acid glycoprotein (aAG) (41.4%). However, considering the difference of glycosylation systems in mammalian and insect cells [26], the result should be consider as qualitative rather than quantitative.

Gel filtration of lysates of Sf21 insect cells infected with recombinant virus BVi67. The appearance of oligomeric forms of the recombinant protein in preparations of affinity purified VARV-CrmB necessitates determining its conformation under native conditions, i.e. during the expression in insect cells. For that purpose, clarified lysate of infected insect cells was fractioned on a column with Biogel A-0.5m. The fractions were studied in the test of inhibition of the cytolytic action of hTNF on the cells of L929 line. The results of this experiment are shown in Fig. 3. TNF-neutralizing activity is mostly found in the fractions corresponding to the free volume of the column, and therefore it should correspond to oligomeric forms of VARV-CrmB whose molecular weight exceeds 500 kDa. Minor TNF-neutralizing activi-

ty is determined in protein fractions with molecular weights of 270 and 90 kDa; these could correspond to hexameric and dimeric forms of VARV-CrmB.

Effectiveness of recombinant VARV-CrmB *in vitro*. To investigate the ability of recombinant protein VARV-CrmB to prevent interaction of hTNF or hLTα with receptors of eukaryotic cells, we performed an experiment similar to that we have described before [25] in which we used cells of mouse fibroblasts L929 line as a target, hTNF or hLTα as cytotoxic agents, and recombinant protein VARV-CrmB as an inhibitor. In the mentioned study [25], we showed that the addition of VARV-CrmB (in concentrations up to 0.2 mg/ml) to the growth medium of L929 cells did not influence their viability. In the present study, to the growth medium of L929 cells we added serial twofold dilutions (starting from 0.2 μg/ml concentration) of hTNF and hLTα and the same dilutions of cytokines in the presence of VARV-CrmB (50 μg/ml). In all plate wells containing hTNF or hLTα, we observed 100% cell death (Fig. 4, curves *a* and *b*), and the addition of VARV-CrmB to the growth medium effectively inhibited cell death mediated by the cytokines (Fig. 4, curves *c* and *d*).

To estimate therapeutic potential of the recombinant viral protein, we compared in a similar test on hTNF-
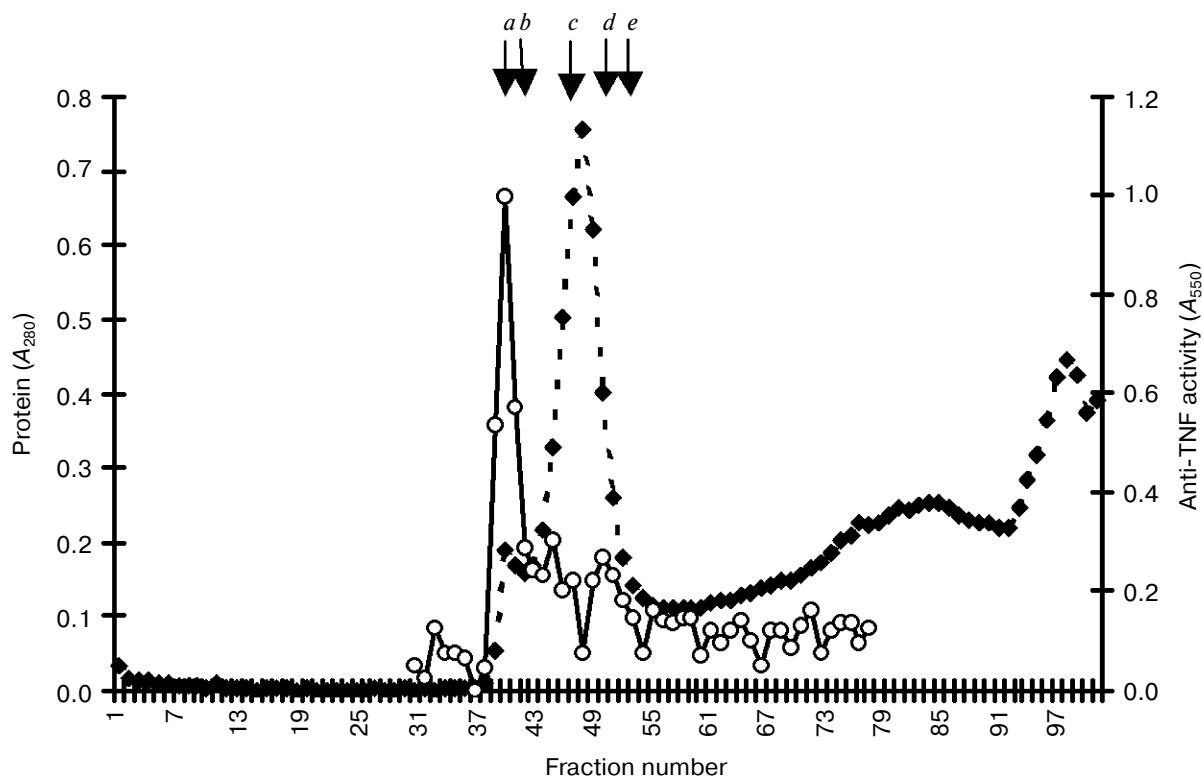
Fig. 3. Profiles of protein concentrations and TNF-neutralizing activity of the lysates of Sf21 insect cells infected with recombinant baculovirus BVi67 after gel filtration on Bio-Gel A-0.5m. The column was calibrated using markers with molecular weight whose elution is indicated with arrows (*a* −2000 kDa blue dextran; *b* − 440 kDa ferritin; *c* − 232 kDa catalase; *d* − 67 kDa BSA; *e* − 45 kDa ovalbumin). Dashed line, protein concentrations, A_{280}; solid line, TNF-neutralizing activity, A_{550}.
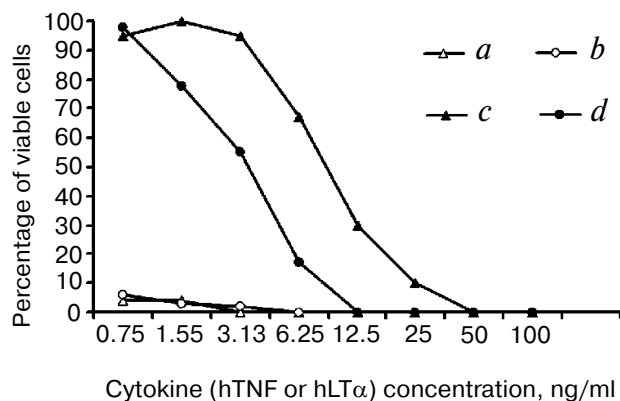
Fig. 4. VARV-CrmB protein inhibiting TNF- and LTα-mediated cytolysis of L929 mouse fibroblasts. Cell viability was estimated by staining with neutral red [25] after 18-h incubation with twofold dilutions of TNF (curve a) or LTα (curve b) and also with the presence of 50 μg/ml VARV-CrmB in samples (curves c and d, respectively). The mean values from three independent experiments are presented.

neutralizing activity known TNF antagonists (soluble cell TNF receptors type I and II, neutralizing monoclonal antibody MAK195, commercial drug Remicade). For that purpose, we modified the experimental scheme so that the L929 cells were exposed to hTNF (2 ng/ml) and serial twofold dilutions of VARV-CrmB or each of the abovementioned TNF antagonists. Protein concentrations at which 50% inhibition of cytotoxic hTNF action was observed (IC_{50}) are presented in the table. The IC_{50} values for soluble cell and chimeric (containing Fc-immunoglobulin domain [9]) receptors (hTNFsRI,II or

Efficiency of *in vitro* neutralization of cytotoxic hTNF action by different TNF-antagonists (aTNF)

Preparations of TNF-antagonists (aTNF)	hTNF/aTNF concentration ratio at IC_{50}
VARV-CrmB	2 ng/ml : 4-8 ng/ml
mAb MAK 195	2 ng/ml : 12-15 ng/ml
Ab$_{TNF}$	2 ng/ml : 40-80 ng/ml [25]
Remicade	2 ng/ml : 0.5-1 ng/ml*
hTNFsRI	2 ng/ml : 0.2-0.3 μg/ml
hTNFsRII	2 ng/ml : 2-3.5 μg/ml
*hTNFsRI***	0.25 ng/ml : 30-60 ng/ml
*hTNFsRII***	0.25 ng/ml : 0.2-0.6 ng/ml
*hTNFsRI/Fc Chimera***	0.25 ng/ml : 0.4-1 ng/ml
*hTNFsRII/Fc Chimera***	0.25 ng/ml : 4-16 ng/ml

 * Infliximab concentration in Remicade.
** Data from R&D Systems catalog.

hTNFsRI,II/Fc Chimera, correspondingly), taken from the catalog of R&D Systems, are also presented in the table. Good correspondence of our experimentally determined IC_{50} for TNFsRI and TNFsRII serves as an inner control and indicates that the effectiveness of inhibition of cytotoxic action of hTNF by the recombinant protein VARV-CrmB *in vitro* is comparable with the activity of the chimeric TNF receptors and TNF-neutralizing monoclonal antibody mAb MAK195, but it is somewhat less effective than the commercial drug Remicade.

DISCUSSION

A range of human diseases (rheumatoid arthritis, Crohn's disease, sepsis) is etiologically connected with disbalance of the production of proinflammatory cytokines, especially TNF. Good results in treating rheumatoid arthritis and Crohn's disease are achieved by anti-TNF-therapy using drugs Enbrel and Remicade. The active ingredient of the first drug is a dimeric chimeric protein combining the extracellular ligand binding domain of cellular TNF receptor type I and the Fc-fragment of human IgG1 [9], and the second drug is a hybrid monoclonal antibody cA2 containing the constant human domain and the variable mouse domain [5]. Effective domestically produced drugs similar to those cited above are absent, so the development of original drugs for TNF-system blockade is topical.

Though it is proven that the cascade of reactions initiated by bacterial endotoxins in the organism (septic shock syndrome) is provoked by a high level of TNF, the therapy of septic shock by known TNF antagonists is rather inefficient [14], and the present situation with sepsis is poor: the fatality rate is still high, about 30% [27].

The rate of viral pathogenicity for in humans corresponds with their ability to effectively neutralize the action of antiviral response mediators. Viruses from the *Orthopoxvirus* genus of Poxviridae family determine the synthesis of secreted protein viroceptors that function as cytokine-binding proteins, and one of those viroceptors from each of the viruses VARV, CPXV, and MPXV is a product of the *crmB* gene − TNF-binding protein [15].

In our earlier experiments on comparing orthopoxvirus proteins CrmB we discovered that only VARV-CrmB is able to display large therapeutic effect on a mouse model of septic shock [20]. So, in this work we studied the basic characteristics of VARV-CrmB produced in insect cells.

We found that VARV-CrmB during synthesis in insect cells results in oligomeric forms (Figs. 1 and 3). During fractionation of affinity-isolated VARV-CrmB in polyacrylamide gel under non-reducing conditions it is detected by Coomassie Blue R250 staining essentially as a polypeptide with molecular weight ~90 kDa, and under reducing conditions as a 47-kDa polypeptide (Fig. 1).

The detection of TNF-neutralizing activity of the culture medium after destruction of insect cells of Sf21 line infected with recombinant baculovirus BVi67 by freeze-thaw cycling (Fig. 3) showed that this activity is connected with fractions containing high molecular weight proteins. Supposedly, oligomeric forms of VARV-CrmB are rather labile, and the method we used for separation of the recombinant protein [19] produced mostly the more stable dimeric form (Fig. 1). We have also shown that the synthesized viral protein is effectively glycosylated in insect cells (Fig. 2); this might positively influence the stability of the protein during potential therapeutic application. The results of this study indicate that the recombinant viral protein VARV-CrmB neutralizes *in vitro* cytotoxic action of hTNF and hLTα (Fig. 4). The efficiency of hTNF neutralization is comparable with the activity of chimeric cell receptors and monoclonal antibody mAb MAK195 (the table). TNF-neutralizing activity of Remicade somewhat exceeds the analogous activity of VARV-CrmB (the table). We have also shown earlier that VARV-CrmB is 50-100 times more effective in neutralizing hTNF cytotoxicity compared to polyclonal antibodies against TNF [25].

In work [28], TNF-binding protein VARV (strain Bangladesh-1975) in a baculovirus expression system was produced by incorporating the corresponding nucleotide changes in the sequence of the camel variola gene using site-directed mutagenesis. The authors estimated the interaction affinity of the recombinant protein with hTNF by surface plasmon resonance method (SPR), defining the dissociation constant $K_d = 278$ pM. So, the affinity of VARV-CrmB to hTNF is comparable with the affinity of cell receptors that is reported by different authors as being between 300-500 pM for TNFRII (p75) and 0.7-1.5 nM for TNFRI (p55) [30-32]. On this basis, we can assume that the difference in the efficiency of neutralization of hTNF and hLTα toxicity by the viral protein and cell receptors is not due to a difference in affinity. The critical factor might be the ability of the viral protein to form dimers and oligomers increasing the avidity of the complex.

In addition to effective TNF-neutralizing activity *in vitro*, we have also shown earlier [20] that VARV-CrmB has strong therapeutic action in an experimental model of LPS (lipopolysaccharide)-induced endotoxic shock in SPF Balb/C mice, significantly increasing survival. Histological study of inner organs and brain of experimental animals showed [33] that VARV-CrmB mitigates against myocardial infarctions, decreases the rate of blood circulation failure in the vessels of inner organs, brain, and mesentery, and prevents the development of acute kidney failure observed in animals of the control group.

Recently, it was found that CrmB, along with the N-terminal TNF-binding domain, contains a C-terminal SECRET-domain that binds the viral protein with chemokines [28]. Possibly, not only TNF-binding activity but also chemokine-binding VARV-CrmB properties are important for the therapeutic effect of this protein against septic shock.

Our data suggests the recombinant protein VARV-CrmB as a novel potential TNF antagonist. It is too early to discuss its application for TNF-mediated pathologies and septic shock in particular. It is necessary to study the chemokine-binding activity of VARV-CrmB in more detail and to continue experiments *in vivo* on the model of LPS-induced septic shock [20], applying not only a preventive but also a therapeutic scheme for use of the drug.

Also, the possibility of gene therapy should be considered − the delivery of the *crmB* VARV gene into mammalian cells in an appropriate vector in case of corresponding diseases for decreasing TNF production rate. This possibility is demonstrated in [34]. The insertion of recombinant adenovirus encoding the rat chimeric protein TNFsRI/Fc to rats with experimentally induced arthritis led to a decrease in proinflammatory cytokines and a decrease in inflammation and destruction of bone tissue.

REFERENCES

1. Mc Devitt, H., Munson S., Ettingen, R., and Wu, A. (2002) *Arthritis Res.*, **4** (Suppl. 13), 141-152.
2. Idriss, H. T., and Naismith, J. H. (2000) *Microsc. Res. Tech.*, **50**, 184-195.
3. Bazzoni, F., and Beutler, B. (1996) *N. Engl. J. Med.*, **334**, 1717-1725.
4. Nepomnyashchikh, T. S., Antonets, D. V., Gileva, I. P., and Shchelkunov, S. N. (2007) *Uspekhi Sovrem. Biol.*, **127**, 576-587.
5. Knight, D. M., Trinh, H., Le, J., Siegel, S., Shealy, D., McDonough, M., Scallon, B., Moore, M. A., Vilcek, J., Daddona, P., and Ghrayeb, J. (1993) *Mol. Immunol.*, **30**, 1443-1453.
6. Siegel, S. A., Shealy, D. J., Nakada, M. T., Le, J., Woulfe, D. S., Probert, L., Kollias, G., Ghrayeb, J., Vilcek, J., and Daddona, P. E. (1995) *Cytokine*, **7**, 15-25.
7. Weinblatt, M. E., Keystone, E. C., Furst, D. E., Moreland, L. W., Weisman, M. H., Bizbara, C. A., Teoh, L. A., Fischoff, S. A., and Chartash, E. K. (2003) *Arthrit. Rheumat.*, **48**, 35-45.
8. Mohler, K. M., Torrance, D. C., Smith, C. A., Goodwin, R. G., Stremler, K. E., Fung, V. P., Madani, H., and Widmer, M. B. (1993) *J. Immunol.*, **151**, 1548-1561.
9. Bathon, J. M., Martin, R. W., Fleischmann, R. M., Tesser, J. R., Schiff, M. H., Keystone, E. C., Genovese, M. C., Wasko, M. C., Moreland, L. W., Weaver, A. L., Harkenson, J., and Fink, B. K. (2000) *N. Engl. J. Med.*, **343**, 1586-1593.

10. Lo, E., Rezaik, K., Evans, A. T., Madariaga, M. G., Phillips, M., Brobbey, W., Schwartz, D. N., Wang, Y., Weinstein, R. A., and Trenholm, G. M. (2004) *Clin. Infect. Dis.*, **40**, 636-637.

11. Arend, S. M., Breedveld, F. C., and van Dissel, J. T. (2003) *Neth. J. Med.*, **61**, 111-119.

12. Calabrese, L. H., Zein, N., and Vassilipoulos, D. (2004) *Ann. Rheum. Dis.*, **63** (Suppl. 2), 18-24.

13. Scallon, B. J., Trinh, T., Nedelman, M., Brennan, F. M., Feldmann, M., and Ghrayeb, J. (1995) *Cytokine*, **7**, 759-770.

14. Moreland, L. W., Baumgartner, S. W., Schiff, M. H., Tindall, E. A., Fleischmann, R. M., Weaver, A. I., Ettinger, R. E., Cohen, S., Koopman, W. J., Mohler, K., Widmer, M. B., and Blosch, C. M. (1997) *N. Engl. J. Med.*, **337**, 141-147.

15. Shchelkunov, S. N. (2003) *Mol. Biol. (Moscow)*, **37**, 41-53.

16. McFadden, G., and Murphy, P. M. (2000) *Curr. Opin. Microbiol.*, **3**, 371-373.

17. Gileva, I. P., Ryazankin, I. A., Maksutov, Z. A., Totmenin, A. V., Ageenko, V. A., Nesterov, A. E., and Shchelkunov, S. N. (2003) *Russian Federation Patent No. 2241754.*

18. Gileva, I. P., Ryazankin, I. A., Maksutov, Z. A., Totmenin, A. V., Lebedev, L. R., Nesterov, A. E., Ageenko, V. A., Shchelkunov, S. N., and Sandakhchiev, L. S. (2003) *Doklady RAN*, **390**, 160-164.

19. Lebedev, L. R., Ryazankin, I. A., Sizov, A. A., Ageenko, V. A., Odegov, V. N., Afinogenova, G. N., and Shchelkunov, S. N. (2001) *Biotekhnologiya*, **6**, 14-18.

20. Gileva, I. P., Nepomnyashchich, T. S., Antonets, D. V., Lebedev, L. R., Kochneva, G. V., Grazhdantseva, A. A., and Shchelkunov, S. N. (2006) *Biochim. Biophys. Acta*, **1764**, 1710-1718.

21. Reinhart, K., Wiegand-Lohnert, C., Grimminger, F., Kaul, M., Withington, S., Trecher, D., Eckart, J., Willatts, S., Bouza, C., Krausch, D., Stochenhuber, F., Eiselstein, J., Daum, L., and Kempeni, J. (1996) *Crit. Core Med.*, **24**, 733-742.

22. Scallon, B., Cai, A., Solowski, N., Rosenberg, A., Song, X., Shealy, D., and Wagner, C. (2002) *Pharmacology*, **301**, 418-426.

23. Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248-254.

24. Laemmli, U. (1970) *Nature*, **227**, 680-685.

25. Gileva, I. P., Ryazankin, I. A., Nepomnyashchich, T. S., Totmenin, A. V., Maksyutov, Z. A., Lebedev, L. R., Afinogenova, G. N., Pustoshilova, N. M., and Shchelkunov, S. N. (2005) *Mol. Biol. (Moscow)*, **39**, 218-225.

26. Aumiller, J. J., Hollister, J. R., and Jarvis, D. L. (2003) *Glycobiology*, **13**, 497-507.

27. Slade, E., Tamber, P. S., and Vincent, J.-L. (2003) *Crit. Care*, **7**, 1-2.

28. Alejo, A., Ruiz-Arguello, M. B., Ho, Y., Smith, V. P., Saraeva, M., and Alcami, A. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 5995-6000.

29. Tartaglia, A., Pennica, D., and Goeddel, D. V. (1993) *J. Biol. Chem.*, **268**, 18542-18548.

30. Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and Scheurich, P. (1995) *Cell*, **38**, 793-802.

31. Hohmann, H. P., Brockhaus, M., Baeuerle, P. A., Remy, R., Kolbeck, R., and van Loon, A. P. (1990) *J. Biol. Chem.*, **265**, 22409-22417.

32. Medvedev, A. E., Espevik, T., Ranges, G., and Sundan, A. (1996) *J. Biol. Chem.*, **271**, 9778-9784.

33. Gileva, I. P., Malkova, E. M., Nepomnyashchikh, T. S., Vinogradov, I. V., Lebedev, L. R., Kochneva, G. V., Grazhdantseva, A. A., Ryabchikova, E. I., and Shchelkunov, S. N. (2006) *Cytokines Inflamm. (Russ.)*, **1**, 44-49.

34. Sandalan, Z., Bruckheimer, E. M., Lustig, K. H., and Burstein, H. (2007) *Mol. Ther.*, **15**, 264-269.