ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 4, pp. 424-429. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © V. A. Spiridonova, A. S. Lygina, M. M. Anohina, N. N. Tupitsyn, 2007, published in Biokhimiya, 2007, Vol. 72, No. 4, pp. 525-532.

Preparation of Functionally Active Recombinant Human Interleukin-6

V. A. Spiridonova[1]*, A. S. Lygina[2], M. M. Anohina[3], and N. N. Tupitsyn[3]

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181; E-mail: spiridon@belozersky.msu.ru*
[2]*Chemical Faculty, Lomonosov Moscow State University, 119992 Moscow, Russia; E-mail: lygina@mail.ru*
[3]*Blokhin Cancer Research Center, Russian Academy of Medical Sciences, Kashirskoe Shosse 24, 115478 Moscow, Russia; fax: (495) 324-1430; E-mail: cannt@aha.ru*

Received October 10, 2006
Revision received December 8, 2006

Abstract—The gene of human interleukin-6 (hIL-6) with an additional 20 amino acids on the N-end, including six histidine residues, was cloned into the expression plasmid pET28b(+). The conditions were elaborated for preparing highly active protein both using denaturing agents and without them. Application of a dialysis cascade allowed us to prepare a functionally active hIL-6 of 90-95% purity with the yield of 3 mg from liter of the cell culture. The highest activity was detected by ELISA in the preparation obtained without denaturing agents. The functional activity of hIL-6 was studied by flow cytofluorimetry. Addition of hIL-6 to the cells of immortal lines of human multiple myeloma resulted in dimerization of the gp130 receptor molecule.

DOI: 10.1134/S0006297907040098

Key words: interleukin-6, cloning, highly active protein, ELISA, gp130 receptor dimerization

Human interleukin-6 (hIL-6) is involved in various cell processes: proliferation, differentiation, inhibition of apoptosis, etc. The clinical application of hIL-6 for cancer therapy seems very promising; also, the search for inhibitors suppressing its numerous functions, on both the level of individual proteins and cell complexes, is important [1]. The SELEX (Systematic Evolution of Ligands by Exponential enrichment) method is now widely used for this purpose. This method can be used to prepare aptamers, small DNA or RNA molecules that specifically and with high affinity bind with their protein targets and can either regulate or completely inhibit the activity of cell proteins. The method and its application have been described elsewhere [2-6]. Aptamers are properly functional analogs of monoclonal antibodies. To prepare aptamers to hIL-6, a large amount of the recombinant protein with high biological activity is needed.

Interleukin-6 is produced by various cell types. It is a glycoprotein with molecular weight of 21-26 kD depending on the isolation source. The extraction of proteins from human and mammalian tissues and blood serum is a rather difficult and not productive process; therefore, methods for obtaining superproducers of useful proteins in bacterial cells have been intensively developed during the last decade. This approach for production of a protein material is characterized by availability and the possibility to control the amount and quantity of the desired product. However, when *E. coli* are used for cloning genes of eukaryotic proteins, it is necessary to have in mind the lack of glycosylation apparatus in bacteria; thus, the functions under study of the protein of interest must be independent of the presence of glycoside moiety.

Analysis of data on its structure (PDB 1ALU) has shown that hIL-6 fits the requirements for bacterial superproducers [7]. In fact, the glycosylation site is located in the region of the hIL-6 N-terminal residues, which are remote from epitopes involved in the binding with receptors. The main difficulty in the hIL-6 preparation is the low level of its expression and also the formation of inclusion bodies; therefore, the protein is usually isolated and purified under denaturing conditions. The renaturation stage is often the most serious problem in the preparation of the useful product. To enhance the protein yield, some authors

Abbreviations: FAB-fragments) fluorescently labeled secondary antibodies; hIL-6) human interleukin-6; IPTG) isopropyl-β-D-thiogalactoside; MCA) monoclonal antibodies.

* To whom correspondence should be addressed.

used constructions of hIL-6 genes fused with the marker protein genes and introduction of signaling sequences.

To enhance the solubility of hIL-6 in the *E. coli* cytoplasm, hIL-6 was fused with thioredoxin [8]. The yield of the protein increased to 1-5 mg from liter of the culture, but the product had to be cleaved by proteinases, and this additional stage complicated purification of the protein. Expression of the chimeric protein hIL-6 combined with a signaling peptide IL-2 was another attempt to solve the problem [9]. The protein yield was significantly enhanced, but it had a relatively low biological activity and also needed additional stages of detaching the signaling peptide and purification.

An alternative solution was to release hIL-6 into the cell periplasm, and it allowed, first, the proteolysis stage to be bypassed and, second, the released protein to be isolated from cellular peptidases [10, 11]. The signaling sequence of the OMPA protein was used in the work as an export signal [10]. The yield was increased to 8-10 mg hIL-6 from 1 liter of the culture. In the work [11], a leader sequence of chemolysine was used as a secretion signal. The chemolysine signaling peptide ensured the cell secretion of the protein into the extracellular space, and the hIL-6 penetration across the cell membrane promoted its regular folding. However, this recombinant protein was expressed markedly worse (0.18 mg/liter).

The protein preparation for X-ray crystallographic analysis (PCA, PDB 1ALU) was obtained by cloning the *hIL-6* gene into the amp^R-plasmid pT7.7 that resulted in appearance of Met(−2) and Ala(−1) residues on the N-end [12]. The expressed protein aggregated into inclusion bodies. On the gene *hIL-6* expression under the trp-promoter control, the protein also aggregated into inclusion bodies that required further stages to disassemble these aggregations. All this prevented obtaining high yields [13].

The main shortcomings of the above-considered approaches are either the low level of *hIL-6* expression or numerous stages of the protein isolation and purification. Yasueda et al. tried to increase the yield by optimizing the nucleotide composition of the *hIL-6* gene [14]. They [14], as well as Li et al. [11], thought the low expression of *hIL-6* to be associated with an incompatibility of the primary/secondary structure of hIL-6 mRNA and the translation apparatus of *E. coli*. Introduction of a six-histidine peptide into the N-end of the protein was used only for preparing porcine recombinant IL-6 [15]. The protein yield was 0.5 mg/ml.

It should be emphasized that the biological activity of all hIL-6 preparations obtained earlier was tested only for one function of the cytokine, and in each case different tests were used, which prevented their comparison. Thus, none of the known methods for preparation recombinant hIL-6 can be used for production of hIL-6 suitable for choosing aptamers.

The present work was designed to create a hIL-6 superproducer with an additional 20 amino acids on the N-end, and this would allow us rapidly and in one step to separate the cell protein and isolate a pure preparation of hIL-6, while not affecting the biological activity of the cytokine. We have tested the recombinant hIL-6 using ELISA and the binding with cell receptors in cell culture.

MATERIALS AND METHODS

Reagents and cell cultures. Reagents used were as follows: salts of domestic production of special and chemical purity; type II agarose (Sigma, USA); acrylamide, N,N′-methylene-bis-acrylamide, and kanamycin (Serva, Germany); N,N,N′,N′-tetramethylethylenediamine (Temed) (BioRad, USA); β-mercaptoethanol, BSA, and Triton X-100 (ICN, USA); bacto-agar, bacto-tryptone (Difco, USA); Ni-NTA-agarose (QIAgen, Germany); 100 mM dNTP and protein markers (Fermentas, Lithuania).

The *hIL-6* gene was kindly presented by Prof. J. Wijdnes (France).

Preparation of the protein producer. The superproducer strain was obtained using a system described in [16]. The *hIL-6* gene was amplified by polymerase chain reaction (PCR) with primers containing recognition sites for restrictases NdeI and XhoI:

forward primer: 5′-CAGCCATATGCCCTGCCCCAGTACC

and

reversed primer: 5′-AGCACTCGAGCTACATTTGCCGAAGAGC.

The *hIL-6* gene was cloned into the expression vector pET28b(+) by the NdeI and XhoI sites. The plasmid pET28b(+) contains a polylinker, six histidine triplets, and the phage T7 promoter under control of the lac-repressor. The *E. coli* strain JM109 was transformed with a mixture of ligases. Of the resulting 36 clones, eight were tested to detect the *hIL-6* gene insertion. As shown by PCR with corresponding primers, the insertion was detected in four clones (Nos. 1, 9, 10, 14). The gene sequence was determined using universal primers.

The *E. coli* strain BL21(DE3) was transformed with the resulting recombinant plasmid pEThIL-6. Synthesis of the hIL-6 protein was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to the final concentration of 1 mM. The production of the protein was assessed by electrophoresis in 12% polyacrylamide gel by the Laemmli method [17].

Isolation and purification of the protein. For preparative isolation of hIL-6 under denaturing conditions, the recombinant strain cells were planted into 500 ml of nutrient medium LB supplemented with kanamycin

(100 mg/liter). The cells were grown for 3-5 h until the absorption A_{600} = 0.6-0.8, and then induced with 1 mM IPTG for 3 h at 37°C. The biomass was precipitated by centrifugation at 7000*g* for 10 min at 4°C. The cells were washed in 50 mM Tris-HCl buffer (pH 7.4) and lysed by ultrasonication; the debris was separated and treated with solution of 6 M guanidine hydrochloride in the same buffer for 12-14 h at 5°C with constant stirring. The resulting suspension was placed onto a column with 300-600 μl of Ni-NTA-agarose pre-equilibrated with solution of 6 M guanidine hydrochloride in 50 mM Tris-HCl buffer (pH 7.4). The sample was incubated with the resin for 1 h. After the incubation, the resin was washed in solution of 6 M guanidine hydrochloride in the same buffer and then in solution of 20 mM imidazole in buffer containing 6 M guanidine hydrochloride. The hIL-6 was eluted from the resin with 1-2 ml of 1 M imidazole solution in buffer containing 6 M guanidine hydrochloride. The protein was renatured using two dialysis modes each consisting of some stages.

In the first mode (preparation 1) the guanidine hydrochloride concentration was decreased, and the preparation was transferred into a storage buffer: a) 1 M guanidine hydrochloride, 50 mM Tris-HCl (pH 7.4), 2 mM reduced glutathione, 0.2 mM oxidized glutathione (dialysis for 12-14 h); b) 50 mM Tris-HCl (pH 7.4), 10% glycerol (dialysis three times for 45 min).

In the other mode (preparation 2) the dialysis system included four stages with a gradually lowered urea concentration: a) 8 M urea, 50 mM Tris-HCl (pH 7.4) (dialysis for 2 h); b) 2 M urea, 50 mM Tris-HCl (pH 7.4), 2 mM reduced glutathione, 0.2 mM oxidized glutathione (dialysis for 12-14 h); c) 1 M urea, 50 mM Tris-HCl (pH 7.4), 2 mM reduced glutathione, 0.2 mM oxidized glutathione (dialysis for 2 h); d) 50 mM Tris-HCl (pH 7.4), 10% glycerol (dialysis three times for 45 min).

The purity of the product was determined by Laemmli electrophoresis of the protein, and the protein concentration was determined by the Bradford method.

During the preparative isolation of hIL-6 under non-denaturing conditions, the protein synthesis was induced by IPTG at 20-22°C. The synthesized protein partially remained in the cytosol and was isolated without guanidine hydrochloride. After the 3-h induction the cells were precipitated by centrifugation, washed, lysed by ultrasonication, and the debris was separated from the supernatant by centrifugation. The resulting supernatant was placed onto the column with 300-600 μl of Ni-NTA-agarose pre-equilibrated with buffer supplemented with 10 mM Mes-KOH (pH 6.1). The sample was incubated with the resin for 1 h, the supernatant was separated, and the resin was washed in the working buffer and then in the same buffer containing 20 mM imidazole. The hIL-6 was eluted from the resin in 1-2 ml of 1 M imidazole solution in the same buffer. Imidazole was removed by dialysis against the buffer supplemented with 0.5 M NaCl, 10 mM Mes-KOH (pH 6.1). If the protein was isolated without denaturing agents, no renaturation stage was required. At the final stage, hIL-6 was dialyzed against the buffer supplemented with 0.5 M NaCl, 10 mM Mes-KOH, and 10% glycerol (pH 6.1) (preparation 3).

Determination of the active hIL-6 concentration. The concentration of functionally active hIL-6 was determined by enzyme immunoassay (ELISA) using antibodies, reaction mixtures, and protocols from Biosource (Belgium). The solution of hIL-6 was diluted (1-4)·10^6-fold in serum to obtain hIL-6 concentration in the range of 25-500 pg/ml. Then the preparation was placed onto a support with immobilized anti-IL-6 monoclonal antibodies (MCA), the support was washed to remove non-specifically bound proteins, treated with a solution of secondary anti-IL-6-biotinylated MCA, and, after the repeated washing, was covered with streptavidin peroxidase. Upon removal of the unbound peroxidase, a substrate with the chromogenic group was added, incubation was performed for 30 min, the reaction was stopped, and the absorption was measured at 450 nm. The active rhIL-6 concentration was calculated by a calibration curve plotted for standard hIL-6 (Biosource).

Determination of functional activity of recombinant human interleukin-6 by flow cytometry. The functional activity of hIL-6 was studied using cells of immortal lines of the human multiple myeloma RPMI8226, A1 antibodies to the epitope in the gp130 molecule dimerization site (kindly presented by J. Brochier, France), and fluorescently labeled secondary antibodies (ITC-labeled FAB-fragments) to immunogenic lines (MedBioSpektr, Russia).

The hIL-6 activity was determined by the method of N. N. Tupitsyn [18] based on redistribution of the gp130 molecule epitopes (in particular, disappearance of epitopes in the A1 dimerization site) on the membrane of the multiple myeloma cells under the influence of hIL-6.

Solution of hIL-6 was dissolved in RPMI-1640 medium to the final concentration of 100 ng/ml. The RPMI8226 cells were collected by centrifugation (1500 rpm, 3 min), washed in PBS, resuspended in 1 ml of PBS, and the cell number was determined in a Goryaev chamber. The total volume was divided into aliquots containing 500,000 cells and collected by centrifugation (1500 rpm, 3 min). The cells were supplemented with 50 μl of the analyzed or commercial hIL-6 in PBS and incubated on ice for 30 min. After three washings, the cells were incubated on ice with A1 antibodies (25 μl, at the concentration of 25 μg/ml) for 30 min. The antibodies were washed off similarly. Then the cells were supplemented with fluorescently labeled antibodies (FAB-fragments) and incubated on ice for 30 min. FAB-fragments were washed off in PBS, and the cells were resuspended in 500 μl of PBS. The cells treated only with FAB-fragments or with the A1 antibodies and FAB-fragment were used as controls. The fluorescence was detected with a flow cytofluorimeter.

RESULTS AND DISCUSSION

Human interleukin-6 plays a crucial role in various defense mechanisms, such as immune response, hemopoiesis, and acute phase reaction. It is involved in many cell processes: proliferation, differentiation, maturation, etc. To clinically test hIL-6 (cancer therapy) and search for inhibitors of its various functions as an individual protein and complexes in the cell, a sufficiently large amount of the hIL-6 protein is required.

We have used a system described in [16] based on the plasmid pET28b(+) with a strong promoter of the T7 phage under the control of lac-repressor, polylinker, and six histidine triplets. The *hIL-6* gene was cloned into the expression vector pET28b(+) by the NdeI and XhoI sites.

The presence of six histidine residues on the N-end considerably facilitates the purification of the protein by affinity chromatography with a special Ni^{2+}-containing sorbent.

To provide for stable storage and amplification, the insertion-containing recombinant DNA was transformed in the *E. coli* strain JM109 lacking the gene of the phage T7 RNA polymerase and unable to express IL-6. It was necessary to use the recombinant plasmid in non-expressing strains because of possible realignments of the plasmid caused by synthesis of a foreign (possibly, toxic for the cell) recombinant protein produced on the promoter "leakage" even in the absence of inducer.

The recombinant plasmids (Nos. 1, 9, 10, 14) were retransformed into the *E. coli* BL21(DE3) strain expressing T7 RNA polymerase, and synthesis of hIL-6 was induced by addition of IPTG. The expression of hIL-6 was detected by electrophoresis according to Laemmli (Fig. 1). Plasmids of these clones were sequenced, the resulting sequences were compared with the original *hIL-6* gene, and a change in the C429G position was found that did not result in the substitution of the Val115 encoded by the corresponding triplet by another amino acid.

Together with the additional 20 amino acids, the molecular weight of the recombinant protein was 22.55 kD.

Conditions of the protein hIL-6 expression and isolation were optimized for the following parameters.

Induction time. The cells were induced by 2 mM IPTG for 2-10 h after the growth for 12 h in LB medium supplemented with kanamycin (50 mg/liter). The amount of hIL-6 produced was determined by electrophoresis of the total cell protein upon lysis of the cells. The hIL-6 fraction in the total cell protein was maximal 2-3 h after the induction beginning and did not markedly change later.

The inducer concentration. To assess the influence of IPTG concentration on the protein expression after the growth for 12 h in LB medium with kanamycin (50 mg/liter), the cells were induced by 1-4 mM IPTG during 3-4 h. The inducer concentration had no influence on the expression level; therefore, the induction was performed with 1 mM IPTG.

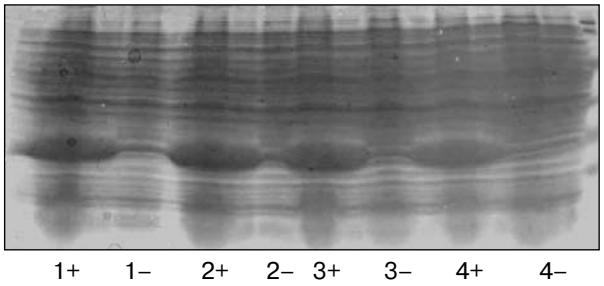

Fig. 1. Laemmli electrophoresis in 12% polyacrylamide gel of the total cell protein of the superproducer *E. coli*. The "+" denotes the IPTG-induced cells, the "–" means the control uninduced cells (Nos. 1, 9, 10, 14).

Concentration of the selective antibiotic. The cells were grown for 12 h, induced by 1 mM IPTG, and after the induction for 3-4 h the expression level was determined by electrophoresis as described earlier. With increase in the kanamycin concentration from 10 to 150 mg/liter, the level of hIL-6 expression significantly increased, which could be associated with an increase in the plasmid copy number. However, high concentrations of kanamycin are undesirable because they are accompanied by an increase in expression of a concomitant 29-kD protein and contamination of the isolated protein preparation.

Induction temperature. Two parallel experiments were performed: the protein synthesis was induced for 3-6 h at 37°C in the first experiment and at 20-22°C in the other. Then the cells were collected by centrifugation, broken by ultrasonication, and the supernatant was separated. The cell debris was treated with 6 M guanidine hydrochloride for 12-14 h. Contents of the recombinant hIL-6 were analyzed by Laemmli electrophoresis in the total cell lysate and debris. In the first experiment, virtually all protein (90%) was present in the cell debris, and in the second case it was partially found in the total cell lysate (Fig. 2).

As a result, the following conditions were chosen for expression of the hIL-6 protein: the cells were grown in LB medium supplemented with kanamycin (100 mg/liter) to absorption of 0.6-0.8 at 600 nm, then induction with 1 mM IPTG was performed for 3 h at 20-22°C. Figure 3 presents the electrophoregram of the rhIL-6 protein preparation isolated using Ni-NTA-agarose.

Because the protein was isolated with denaturing agents, its functional activity depended on the renaturation conditions. The hIL-6 was renatured by stepwise dialyses: in the first case the buffer composition was sharply changed, and in the second case the urea concentration was decreased gradually (see "Materials and Methods").

The renaturation schemes used allowed us to obtain a gradual and, correspondingly, more regular formation of the protein structure. When the protein was isolated

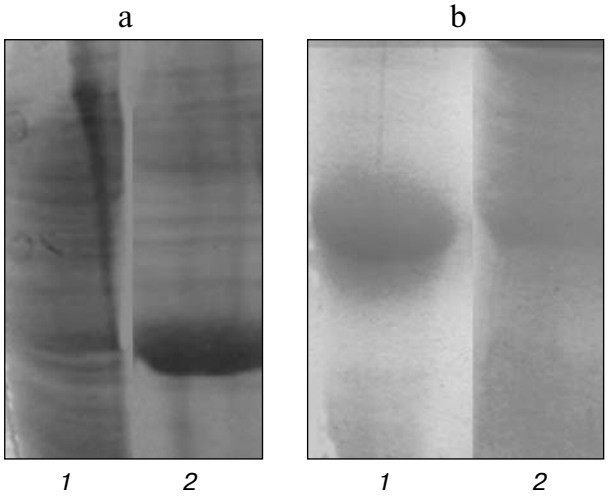

Fig. 2. Laemmli electrophoresis in 12% polyacrylamide gel of the total cell protein expressed upon the induction by IPTG in the *E. coli* BL21(DE3) cells transformed by the pET28IL-6 plasmid. a) The induction was performed at 37°C; b) the induction was performed at 20-22°C. Lanes: *1*) the supernatant after the ultrasonic destruction of the cells; *2*) the cellular debris after separation of the supernatant.

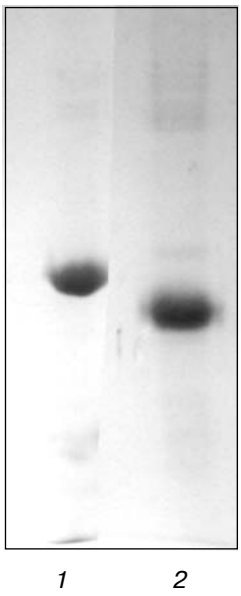

Fig. 3. Analysis by electrophoresis in 12% polyacrylamide gel of the purity of the hIL-6 protein preparation isolated using Ni-NTA-agarose. Lanes: *1*) marker protein, 24 kD; *2*) hIL-6 fraction after purification on the resin.

without denaturing agents, the hIL-6 was dialyzed against buffer supplemented with 10% glycerol to remove imidazole (preparation 3).

The three preparations of the protein were tested for functional activity.

Determination of functional activity of recombinant hIL-6 by ELISA. The concentration of the active form of rhIL-6 in the three preparations was determined by enzyme immunoassay (ELISA). The hIL-6 was sorbed on a support with monoclonal antibodies (MCA) and treated with secondary MCA. The ELISA method allowed us to assess the fraction of the cytokine molecules, and comparison of these data with the protein quantity determined by Bradford's method gave the active protein fraction. The preparations 1-3 of rhIL-6 were analyzed by ELISA. The results are presented in the table.

All three preparations contained a high fraction of active cytokine molecules (table). Preparation 3 isolated without denaturing agents was the most active. The active protein fractions were lower in preparations 1 and 2 isolated with involvement of guanidine hydrochloride. Note that the gradual stepwise removal of the denaturing agent promoted the more regular folding of the protein, as indicated by the higher fraction of the active molecules in preparation 2 than in preparation 1.

Determination of functional activity of recombinant hIL-6 by flow cytofluorimetry. The functional activity of hIL-6 was studied by flow cytometry on cells of human multiple myeloma RPMI8226 immortal lines. Under normal culture conditions, these cells equally express all epitopes of the gp130 molecule. Addition of hIL-6 to these cells leads to dimerization of the molecule and

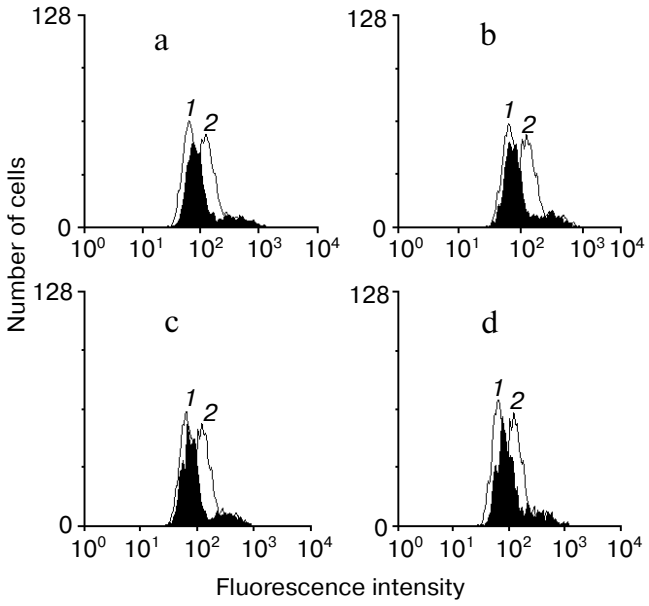

Fig. 4. Functional activity of preparations 1-3 determined by flow cytofluorimetry. The peak *1* maximum position on the abscissa axis corresponds to the fluorescence intensity of cells labeled only with secondary antibodies (FAB-fragments); the peak *2* maximum position corresponds to the fluorescence intensity of cells labeled with both A1 antibodies and FAB-fragments; the dark peak maximum corresponds to the fluorescence intensity of the cells preincubated with the isolated preparations: a) preparation 1; b) preparation 2; c) preparation 3; d) commercial hIL-6.

Determination of active hIL-6 fraction in preparations 1-3

Preparation	Total protein concentration (by Bradford), μg/ml	Final concentration after dilution, pg/ml	Absorption (at 450 nm)	IL-6 concentration by ELISA, pg/ml	Active fraction, %
1	120	30	0.55	24.25	81
		60	0.64	50.36	84
		120	0.76	84.81	71
2	100	25	0.53	21.75	87
		50	0.61	42.86	86
		100	0.78	89.25	89
3	90	22.5	0.54	21.19	97
		45	0.61	43.15	96
		90	0.76	83.14	92

changes in the gp130 immunophenotype, and this underlies the detection of the functional activity of hIL-6. We have considered an epitope (to antibody A1) in the dimerization site of the gp130 molecule.

As controls, we used the cells labeled only with FAB-fragments (presenting the minimal fluorescence intensity, or the background) and cells labeled with A1 antibodies and secondary antibodies, or FAB-fragments (presenting the maximal fluorescence intensity, because A1 epitopes in the gp130 dimerization site were free of A1 antibodies). When the RPMI8226 cells were preincubated with a functionally active preparation of recombinant hIL-6, the gp130 receptors formed dimers, and, as a result, A1 epitopes became unavailable for A1 antibodies, which decreases the fluorescence intensity. Figure 4 presents flow cytometry data on the functional activity of the three preparations of hIL-6 compared with the commercial preparation. All three preparations were functionally active, as indicated by the decreased fluorescence intensity (comparison by the abscissa axis).

The system for superproduction of recombinant hIL-6 developed by us ensures the production of a functionally active 90-95% pure hIL-6 at the yield of 3 mg per liter of cell culture. The system of stepwise dialyses allowed us to obtain active protein preparations isolated with denaturing agents. The preparation isolated without denaturing agents was the most active, as shown by ELISA. By flow cytofluorimetry, the hIL-6 preparation was shown to influence the gp130 receptor dimerization, and this indicated a high activity of the isolated protein.

The authors are grateful to Prof. A. M. Kopylov for his valuable remarks in the discussion of the manuscript and to A. V. Golovin for his help in preparation of the illustrations.

The work was supported by the Russian Foundation for Basic Research (project No. 05-04-49750).

REFERENCES

1. Meager, A. (1996) in *Encyclopedia of Molecular Biology and Molecular Medicine* (Meyers, R. A., ed.) VCH Press, pp. 331-342.
2. Gold, L., Polisky, B., Uhlenbeck, O., and Yarus, M. (1995) *Annu. Rev. Biochem.*, **64**, 763-797.
3. Eaton, B. E., Gold, L., Hicke, B. J., Janjic, N., Jucker, F. M., Sebesta, D. P., Tarasow, T. M., Willis, M. C., and Zichi, D. A. (1997) *Bioorg. Med. Chem.*, **5**, 1087-1096.
4. Osborne, S. E., Matsumura, I., and Ellington, A. D. (1997) *Curr. Opin. Chem. Biol.*, **1**, 5-9.
5. Kopylov, A. M., Spiridonova, V. A., and Park, K. Kh. (1998) *Ros. Khim. Zh.*, **42**, 89-96.
6. Kopylov, A. M., and Spiridonova, V. A. (2000) *Mol. Biol. (Moscow)*, **34**, 1097-1113.
7. Somers, W., Stahl, M., and Seehra, J. S. (1997) *EMBO J.*, **16**, 989-997.
8. Han, B. G., Ma, X. K., Meng, L., Song, X. G., Peng, S. Y., Wang, J. X., and Ling, S. G. (1998) *Biochem. Mol. Biol. Int.*, **46**, 839-846.
9. Tonouchi, N., Oouchi, N., Kashima, N., Kawai, M., Nagase, K., Okano, A., Matsui, H., Yamada, K., Hirano, T., and Kishimoto, T. (1988) *J. Biochem. (Tokyo)*, **104**, 30-34.
10. Barthelemy, I., Gonzalez de Buitrago, G., Carreiro, C., Roncal, F., Perez-Aranda, A., Marquez, G., and Barbero, J. L. (1993) *J. Biotechnol.*, **27**, 307-316.
11. Li, Y., Chen, C. X., von Specht, B. U., and Hahn, H. P. (2002) *Protein Exp. Purif.*, **25**, 437-447.
12. Arcone, R., Pucci, P., Zappacosta, F., Fontaine, V., Malorni, A., Marino, G., and Ciliberto, G. (1991) *Eur. J. Biochem.*, **198**, 541-547.
13. Ejima, D., Watanabe, M., Sato, Y., Date, M., Yamada, N., and Takahara, Y. (1999) *Biotechnol. Bioeng.*, **62**, 301-310.
14. Yasueda, H., Nagase, K., Hosoda, A., Akiyama, Y., and Yamada, K. (1990) *Biotechnology* (N.Y.), **8**, 1036-1040.
15. Lee, D. Y., Cho, Y. W., Kang, S. G., Shin, S. J., and Yoo, H. S. (2004) *J. Vet. Sci.*, **5**, 337-343.
16. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Meth. Enzymol.*, **185**, 60-89.
17. Laemmli, U. (1970) *Nature*, **227**, 680-685.
18. Tupitsyn, N. N., Kadagidze, Z. G., Gaillard, J.-P., Sholokhova, E. N., Andreeva, L. Yu., Liautard, J. P., Duperray, C. J., Klein, B. N., and Brochier, J. D. (1998) *Clin. Lab. Haem.*, **20**, 345-352.