Development of Enzyme Immunoassay of Glial Fibrillary Acidic Protein on the Basis of Recombinant Antigen

K. A. Pavlov, O. I. Gurina, T. B. Dmitrieva, A. V. Makarov, and V. P. Chekhonin

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Quantitative enzyme immunoassay of glial fibrillary acidic protein of astrocyte intermediate filaments opened new prospects for highly selective diagnosis and monitoring of pathological processes in the CNS. Immunochemical screening of glial fibrillary acidic protein in biological fluids helps to adequately evaluate the permeability of the blood-brain barrier in CNS diseases associated with violation of its functions, such as hypoxic and ischemic disorders, neuroinfections, glial tumors, brain injuries, *etc.* Wide-scale introduction of enzyme immunoassays into clinical laboratory practice implies the development of biotechnological approaches to the creation of methods for obtaining EIA components. This paper presents a method for creation of a test system for EIA of glial fibrillary acidic protein (GFAP) on the basis of recombinant GFAP and antibodies obtained by immunization with recombinant GFAP. Due to this approach, a highly standardized test system for the analysis of GFAP in human biological fluids was created.

Key Words: *glial fibrillary acidic protein; recombinant protein; enzyme immunoassay; central nervous system; blood-brain barrier*

Glial fibrillary acidic protein (GFAP) as an objective marker of the astroglia status and blood-brain barrier resistance in health and disease attracts great recent attention [7,11]. Measurements of GFAP in human biological fluids over the course of disease development helps to objectively monitor the disease course and treatment efficiency and even predict the disease outcome [9].

Enzyme immunoassay test systems are widely used for GFAP analysis [3,5,8]. These systems have some flaws, the main of which is the use of native antigen preparations for plotting the calibration curve. Since the native antigen is usually obtained by methods of preparative protein chemistry from heterogeneous biological material, the preparations in different lots can vary quantitatively and qualitatively. This creates certain problems for standardization of the results of GFAP measurements and sometimes creates insurmountable difficulties in interpretation of the results.

We developed an EIA method on the basis of a standard recombinant GFAP produced by a strain of transformed bacteria.

MATERIALS AND METHODS

The main procedures for cloning, expression, and purification of recombinant GFAP preparation were described previously [2]. The resultant producer strain was used for isolation and purification of recombinant GFAP. The protein was obtained and purified by metal chelation chromatography. Suspension (2 ml) of the producer strain fresh stock was added to 100 ml broth medium (LB, 30 μg/ml kanamycin) and incubated in a rotation shaker incubator (37°C, 200 rpm) until optical density OD_{600}=

Laboratory of Immunochemistry, Department of Biological Psychiatry, V. P. Serbsky Center of Social and Forensic Psychiatry, Moscow, Russia. ***Address for correspondence:*** pkanew@mail.ru. K. A. Pavlov

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0.7 was attained. Isopropyl-β-D-thiogalactoside (IPTG) was then added to the suspension to a final concentration of 1 mM and incubation was continued under the same conditions for 4 h. After incubation, the suspension was centrifuged (3500*g* at 4°C, 30 min) for cell precipitation. The precipitate was 3 times frozen in liquid nitrogen and desfrosted in a water bath (37°C), lyzed in buffer A (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris Cl, pH 8.0) in proportion 5 ml per 1 g wet precipitate for 45 min on a rotation shaker (200 rpm at 20°C). The lysate was twice passed through a syringe with a needle (d=0.2 mm) in order to reduce its viscosity and centrifuged (13,000*g*, 30 min, 4°C). Ni-NTA agarose equilibrated with buffer A was added to collected supernatant and incubated at moderate shaking for 30 min at 20°C. The suspension was then transferred to the column. The carrier was washed with 10-fold volume of buffer B (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris×Cl, pH 6.3). GFAP preparation was eluted with 2-fold volume of the column with buffer C (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris Cl, pH 4.5) and the eluate aliquots (0.5 ml) were collected. The samples were stored at -20°C. The purity of the resultant preparation was evaluated by SDS-PAAG electrophoresis. Immunochemical characterization was performed by immunodiffusion and immunoblotting methods. Immunochemical identification of GFAP was carried out using commercial antibody preparations (Signet, 471-01).

Polyclonal antibodies were obtained by immunization of outbred female rabbits (1-1.5 years). Recombinant human GFAP dialyzed against PBS was used for immunization. The preparation (200 g in 0.5 ml PBS) was emulsified with 0.5 ml complete (immunization 1) or incomplete (subsequent immunizations) Freund's adjuvant and injected subcutaneously into the cervical lymph node area. Immunization was carried out once a week, its efficiency was evaluated by immunodiffusion and direct EIA. The blood was collected at the peak of antibody production from the rabbit ear marginal vein. Antisera containing anti-GFAP were stored at 4°C no longer than 1 year.

Monoclonal antibodies were obtained by immunization of female BALB/c mice (3-6 months) with 20 μg human recombinant GFAP in 50 μl PBS, emulsified in complete/incomplete Freund's adjuvant. Immunization was carried out every other week. Its efficiency was evaluated by immunodiffusion and direct EIA. The preparation was injected subcutaneously to the tail base, in the zone of cervical lymph nodes, along the abdominal lymph node chain, and into the paw pads. The spleens were collected at the peak of antibody production, fusion was carried out directly after spleen collection.

Hybrydomas secreting monoclonal antibodies were obtained as described previously [2]. The clones were screened by direct EIA.

Antibodies were purified by immunoadsorption chromatography using RAP Affinity Purification Kit (Invitrogen). The specificity of the resultant antibodies was evaluated by immunochemical methods and by immunoblotting analysis in comparison with commercial anti-GFAP antisera (Signet lab.).

A sandwich EIA variant was developed for measurement of GFAP concentration in human biological fluids. Monoclonal and polyclonal anti-GFAP were diluted with bicarbonate buffer (0.1 M $NaHCO_3$, pH 9.2) to a final concentration of 10 mg/liter and used directly after dilution. Our preparation of monoclonal anti-GFAP in a concentration of 10 mg/liter (0.1 M $NaHCO_3$, pH 9.2) was pipetted (50 μl per well) into wells of a 96-well plate (Sarsdedt, 82.1581) and incubated in a humid chamber for at least 12 h at 4°C. The wells were then washed 3 times with a washing buffer (100 mM PBS, 150 mM NaCl, 0.2% BSA, 0.05% Twin-20). Washing at this stage and further on was carried out on an ELX50 automated washer (Bio-Tek). In order to block free binding sites on the plate, blocking buffer (100 μl/well; 100 mM PBS (pH 7.2), 1% BSA, 0.5% Twin-20, 0.02% thymerosal) was added into the wells and incubated for 30 min at 20°C. The wells were then washed 3 times in washing buffer; the fluid was aspirated if necessary, and the plates were stored at -80°C.

Buffer for antigen (1 ml; 100 mM PBS, 150 mM NaCl) was added to 100 ng lyophilized GFAP and incubated for 30 min at ambient temperature. At the next stage, serial dilutions of GFAP (from 100 to 0.75 ng/ml in buffer for antigen) were prepared, put into wells (150 μl), and the mixture was incubated for 60 min at 20°C and slight shaking. Two wells with buffer without protein served as controls. The studied samples (plasma, liquor; 150 μl) diluted 1:2 with buffer for antigen were put into the rest wells. After incubation, the wells were washed 3 times with washing buffer.

At the next stage of the study, 100 μl rabbit polyclonal anti-GFAP (10 μg/ml) were put into all wells and incubated for 30 min at 4°C with gentle shaking. After incubation, the wells were washed 3 times in washing buffer and biotinylated goat antibodies to rabbit IgG (0.3 μg/ml; Vector Labs, BA2000) were added, the mixture was incubated for 30 min, and the plates were washed 3 times. At the next stage, 150 μl horseradish peroxidase conjugate with streptavidine (Vector Labs, BA2000)

prepared from standard solutions according to the manufacturer's recommendations was added into wells and the mixture was incubated for 30 min. Peroxidase activity was developed for 15 min with substrate solution containing 0.3% H_2O_2 in substrate buffer (0.04 M $C_6H_8O_7$, 0.27 M $NaHPO_4 \times 2H_2O$; pH 4.5) and 0.04% *o*-phenylene diamine. Peroxidase activity in the wells was detected on an ELx-800 Reader photometer (Bio-Tek) at λ=450 nm.

A standard antigen was calibrated by measuring optical density 6 times for every concentration in order to evaluate the accuracy and reproducibility of the calibration curve of GFAP enzyme immunoassay. Confidence interval and standard deviation were analyzed using Excel software. Reference calibration ("return test") was carried out using serum samples with a known concentration of GFAP. Then a known volume of GFAP (1 ng/µl) was added to 2 samples. The result was evaluated as a proportion of expected to actual result. In order to test the linearity of straight lines for different dilutions of samples, a sample of 0.3% H_2O_2 in substrate buffer (0.04 M $C_6H_8O_7$, 0.27 M $NaHPO_4 \times 2H_2O$; pH 4.5) was titered from 1:1 to 1:8. The result was evaluated as a proportion of expected to actual result.

RESULTS

Electrophoretic analysis of the eluate obtained after metal chelation chromatography on Ni-NTA agarose detected the only band with a molecular weight of 49 kDa immunochemically visualized by commercial anti-GFAP antibodies (Fig. 1). The purity of the protein preparation in densitometric analysis was at least 98±2%. This preparation was used for immunization of animals to obtain mono- and polyclonal antibodies. Immunization of mice provided high antibody titers as soon as after 3-4 cycles; for rabbits the mean period was 5-6 cycles of immunization. No animal mortality was observed in the course of repeated immunizations.

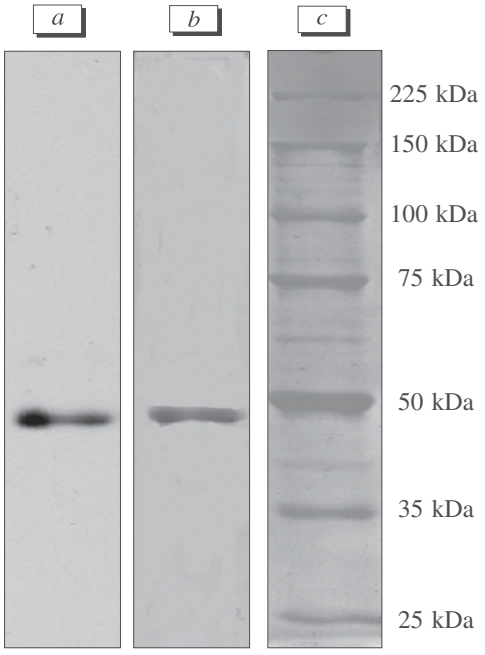

Fig. 1. Results of disk electrophoresis (7.5% acrylamide gel; AA) and immunoblotting of human recombinant GFAP. *a*) rec_hGFAP (ECL, immunoblotting); *b*) rec_hGFAP (49 kDa, GelAnalysis, v. 1.0; SDS-PAAG, 7.5% AA); *c*) protein preparations with calibrated molecular weights.

A total of 5 and 10 mg poly- and monoclonal antibody preparations, respectively, were obtained for EIA development. The data of immunocytochemical and immunohistochemical staining of GFAP-positive astrocytes with antibodies obtained by immunization with recombinant antigen (Fig. 2) and the results of comparative immunodiffusion analysis of recombinant and native antigen and antibodies with commercial anti-GFAP sera (Fig. 3) are presented. Continuous precipitation line (no cross-reactions and "spurs") indicates complete immunochemical identity of the native and recombinant antigens and complete immunochemical identity of the respective antibody preparations.

The results characterizing the test system developed in our study are presented in Tables 1-3. The accuracy and reproducibility of calibration are

TABLE 1. Accuracy and Reproducibility of Calibration Curve of GFAP Enzyme Immunoassay

GFAP concentration, ng/ml	Number of measurements	Mean E_{425}	Confidence interval	Standard deviation	Coefficient of variations, %
1.0	6	0.095	0.091-0.099	0.0014	4.78
2.0	6	0.103	0.100-0.106	0.0232	3.83
4.0	6	0.133	0.129-0.138	0.0130	3.90
8.0	6	0.157	0.151-0.163	0.0440	4.80
16.0	6	0.213	0.207-0.220	0.0548	3.88
32.0	6	0.289	0.275-0.304	0.0632	6.29

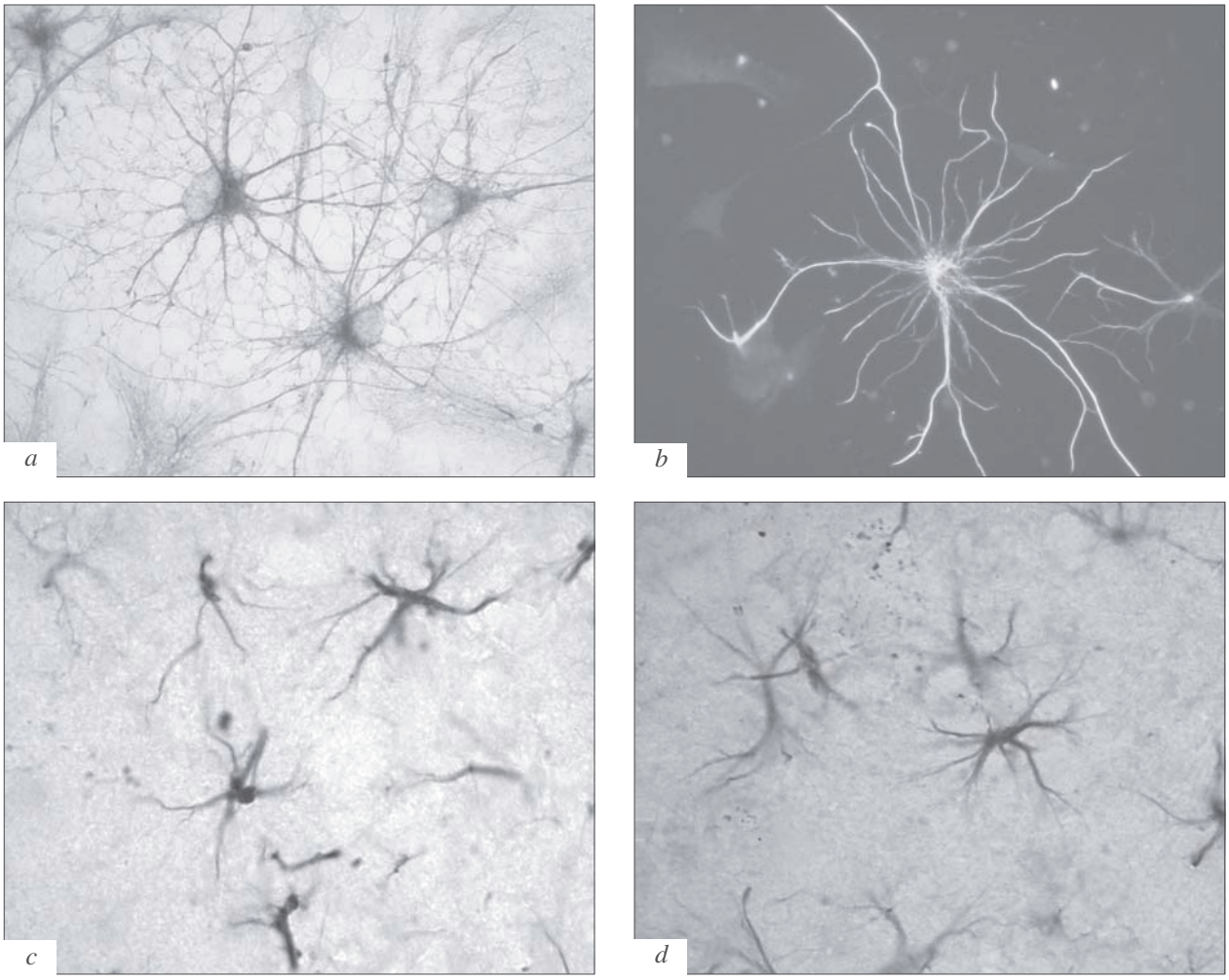

Fig. 2. Immunocytochemical development of GFAP in primary culture of astrocytes (*a*, *b*), the rat brain section (*c*, *d*) with antibodies obtained by immunization with recombinant GFAP. *a*) peroxidase immunocytochemical staining; *b*) immunofluorescent staining; *c*, *d*) peroxidase immunohistochemical staining. *a*, *c*) first antibodies: mouse monoclonal antibodies obtained by immunization with recombinant GFAP; second Ab: BA2000 (Vector Labs), ABC kit (*a*, *c*). *b*, *d*) first antibodies: polyclonal rabbit antibodies, obtained by immunization with recombinant GFAP; second Ab: R6393 (Invitrogen), Rhodamine (*b*), BA2005 (Vector Labs), ABC kit (*d*).

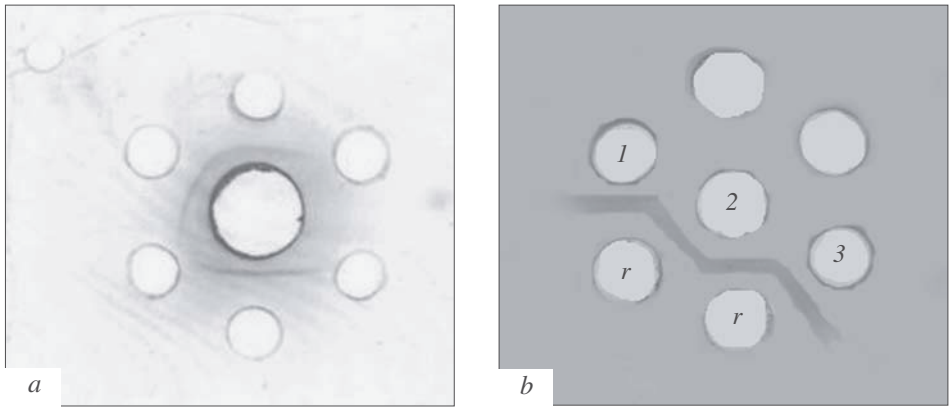

Fig. 3. Immunodiffusion analysis of recombinant and native antigens (*a*) and antibodies with commercial anti-GFAP antisera (*b*). *a*) central well: polyclonal rabbit serum; other wells: recombinant GFAP in different titers. *b*) *r*: recombinant GFAP; *1*) rabbit serum after immunization with recombinant GFAP; *2*) commercial anti-GFAP antiserum (Signet); *3*) serum from immunized mice.

TABLE 2. Reference Calibration ("Return Test") in GFAP Enzyme Immunoassay

Sample No. 1: 2.7 ng/ml			Sample No. 2: 0 ng/ml		
volume (ng) added into GFAP sample per ml	expected/actual result, ng/ml	proportion, %	volume (ng) added into GFAP sample per ml	expected/actual result, ng/ml	proportion, %
2	4.7/5.0	107	2	2/1.74	87
4	6.7/7.7	115	4	4/3.72	93
6	8.7/10.7	123	6	6/5.82	97

TABLE 3. Calibration Curve Parallelism Test in Antigen Dilution

Sample No. 1: 5.80 ng/ml			Sample No. 2: 17.80 ng/ml		
dilution	expected/actual result, ng/ml	proportion, %	dilution	expected/actual result, ng/ml	proportion, %
1	5.80	—	1	17.80	—
1:2	2.90/2.48	85	1:2	8.90/10.10	113
1:4	1.95/1.84	94	1:4	4.45/5.17	116
1:8	0.98/1.10	91	1:8	2.23/2.86	128

confirmed by the confidence interval and coefficient of variations, surpassing 5% only at a concentration of 32 ng/ml. The "return test" results demonstrate a significant (more than 20%) deviation from the expected result only for 3-fold increased concentration of GFAP. The test for curve parallelism in sample dilutions led to significant distortions of the result only at dilution 1:8.

The use of recombinant preparations in EIA test systems for measurements of bioactive substances is now becoming the most prospective trend in the creation of test systems [10]. This can be explained primarily by impossibility to obtain high-quality standard antigens for plotting the calibration curves and by serious differences in the characteristics of mono- and polyclonal antibodies obtained by immunization of animals with native antigens from different lots. The procedure for obtaining native preparations of this or that antigen from biological material virtually cannot be standardized because of differences in individual characteristics of the material [11].

Immunization with recombinant protein preparations leads to the development of immune response and production of antibodies specifically recognizing recombinant and native antigen. No differences in antibody preparations obtained using these two approaches are detected by methods of classical immunochemistry.

As for GFAP, our studies do not confirm the presence of antigenic determinants resulting from posttranslation modification in its structure. That is why the prokaryote system of protein expression is fairly adequate for obtaining recombinant GFAP for the development of enzyme immunoassay system.

Our variant of solid-phase sandwich EIA provides reliable and accurate measurements of GFAP concentrations in human serum and liquor. The working range for optimal measurement of antigen concentrations is 1-32 ng/ml. Parallelism testing of the system carried out by using multiple dilutions of recombinant GFAP, showed curves virtually parallel to the standard curve. Analysis of the results presented in Tables 1-3 indicates that this test system is highly specific, accurate, reliable, and reproducible.

Introduction of these test systems into clinical practice will appreciably supplement the armory of clinical laboratory methods for the diagnosis and monitoring of the course and efficiency of therapy of patients with diseases of the nervous system. On the other hand, the use of these systems for the analysis of blood-brain barrier resistance opens new prospects for studies of autoimmune aspects in the pathogenesis of nervous and mental diseases.

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