Monoclonal Anti-GFAP Antibodies: Extraction, Characteristics, and Immunoenzyme Assay

V. P. Chekhonin, O. I. Gurina, T. B. Dmitrieva, I. I. Shepeleva, I. A. Ryabukhin, and G. N. Babich

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 132, No. 8, pp. 188-191, August, 2001
Original article submitted April 12, 2001

Methods of GFAP purification and obtaining of hybridoma cells producing monoclonal anti-GFAP antibodies and properties of GFAP preparation were described. The immunobloting data on specificity of obtained monoclonal antibodies are presented. A new method of GFAP immunoenzyme assay based on GFAP preparation and anti-GFAP antibodies was elaborated. Standardization of the immunoenzyme system was shown in tests for specificity, accuracy, and reproducibility.

Key Words: *glial fibrillary acidic protein; monoclonal antibodies; immunoenzyme assay; astrocytes; blood-brain barrier*

Glial fibrillary acidic protein (GFAP) is the main component of astrocyte intermediate filaments forming cytoskeleton of differentiated astrocytes. GFAP was identified and described during morphological examination of specific plaques in the brain of patients with multiple sclerosis [5]. Highly purified GFAP preparations were recently isolated and analyzed by physicochemical methods, monospecific polyclonal anti-GFAP antisera were obtained, and immunoenzyme test systems for evaluation of GFAP content in the liquor and serum were elaborated [1,7]. Different immunochemical methods of GFAP determination are used for diagnostics and control of therapy of some neuropsychic disorders and brain tumors [1,2,4,6,11,14].

Immunoenzyme test systems based on polyclonal antibodies (AB) were used in practically all pilot studies of GFAP. Despite their high specificity, immunoenzyme assay systems based on polyclonal antibodies can reveal different antigen epitopes, which makes impossible the comparison of the results and elaboration of standard diagnostic test systems.

Elaboration of monoclonal AB isolation technique in 1975 [9] opened a prospect for standardization of immunochemical test systems and, consequently, for adequate evaluation and comparison of clinical laboratory data.

The purpose of the present study was to obtain hybridoma cells producing monoclonal anti-GFAP AB, to isolate and characterize these monoclonal AB, and to elaborate immunoenzyme test system for detection of GFAP in biological fluids.

MATERIALS AND METHODS

GFAP was isolated as described elsewhere [1], the purity of the preparation was evaluated by disk electrophoresis in polyacrylamide gel (PAAG) with subsequent immunodevelopment [1], and in PAAG with sodium dodecyl sulfate (SDS) [12].

Monoclonal anti-GFAP AB were obtained by a standard method [9] modified in our laboratory.

Sp2/0-Ag14 myeloma cells (10^5-10^6 cells/ml) were cultured in RPMI-1640 (Sigma) supplemented with 10% fetal calf serum (FCS, Gibco). One day before fusion the culture medium was replaced by RPMI-1640 with 15% FCS and high glucose content (4.5 g/liter).

Adult BALB/c mice were immunized with purified GFAP according to the previously described

Laboratory of Immunochemistry, V. P. Serbskii State Research Center of Social and Forensis Psychiatry, Moscow

0007-4888/01/1322-0772$25.00

scheme [1]. The mice were decapitated and the blood was collected for subsequent testing. Aseptically isolated spleen was placed in a 60-mm Petri dish with cold (4°C) serum-free RPMI-1640 and perfused with the same medium until complete isolation of all cells. Isolated cells were centrifuged at 1000*g* and the supernatant was removed. The cells were resuspended in 5 ml medium, counted, tested for viability, and washed 3 times by the same medium.

Sp2/0-Ag14 myeloma cells were washed 3 times with warm (36°C) serum-free RPMI-1640 medium.

After the last washing, spleen cells and Sp2/0-Ag14 myeloma cells were placed in one tube. The optimal ratio between spleen B-lymphocytes from immunized mouse and myeloma cells was 1:5.

After centrifugation, the cells were resuspended and fusion was performed in the presence polyethylene glycol/DMSO (Sigma). After fusion, the cells were centrifuged (4 min at 1000*g*), incubated for 2 h in DMEM supplemented with 20% FCS in a CO_2-zincubator, and transferred into 96-well plates with 100 μl HAT medium (Sigma) [9].

After fusion, the cells were maintained in HAT medium for 10 days and then transferred to DMEM with 20% FCS. Clones appeared within 2-4 weeks. When necessary, the medium was partially replaced with a fresh portion. The cells were cultured in DMEM containing 20% FCS for 2-3 weeks.

The cultural medium was first tested, when the clone contained 150-200 cells. A total of 3 tests were performed. If the titer of anti-GFAP AB increased, the cells were transferred to a 96-well plate coated with a feeder (1 cell per well). After recloning, the hybrid cell were grown and, then injected (3×10^6) intraperitoneally to BALB/c mice 3 weeks after intraperitoneal injection of 0.5 ml pristane (2,6,10,14-tetramethylpentadecane, Sigma). Other hybrid cells were frozen and stored in liquid nitrogen (aliquots 5×10^6 cells/ml).

Monoclonal anti-GFAP AB produced by clones were isolated from ascitic fluid using the corresponding CNBr-Sepharose immunosorbents and extrapure GFAP preparation according to a routine technique [15].

The class of the obtained monoclonal AB was determined by immunoenzyme assay with anti-IgG and anti-IgM AB.

Subclasses of monoclonal AB were identified by double diffusion of hybridoma-secreted immunoglobulins against rabbit anti-mouse AB (Dako).

Specificity of monoclonal AB was confirmed by immunoblotting [13].

Anti-GFAP AB were conjugated with horseradish peroxidase as described elsewhere [10]: 200 μl 0.015% anti-GFAP AB from clone D4 in 0.05 M Na-carbonate buffer (pH 9.6) was added to wells of immunoenzyme assay plates and incubated for 12 h at 4°C. Then the wells were washed with 0.05 M phosphate buffer (pH 7.4, washout buffer). After washout 200 μl tested biological fluid of standard solutions containing known GFAP concentrations were added to the experimental and control wells, respectively. After 2-h incubation at room temperature, the wells were washed with the buffer to remove unbound proteins and horseradish peroxidase-conjugated anti-GFAP AB was added. After 3-h incubation at room temperature the wells were thoroughly washed free from unbound conjugate.

AB-conjugated peroxidase was developed with a substrate mixture (phenylene diamine and 0.1% H_2O_2) for 1 h and optical density was measured at 490 nm on a Dynatech single-channel spectrophotometer.

RESULTS

Homogenous highly purified GFAP was isolated from human brain. Analytical disk electrophoresis in PAAG revealed a single major band corresponding to α_1-globulin zone, which attests to high purity of GFAP preparation.

Molecular weights of GFAP determined by gel-filtration on Sephadex G-150 (Pharmacia) and disk electrophoresis in SDS-PAAG were similar: 55.0±4.3 and 51.0±2.1 kDa, respectively (Fig. 1, *b*) [12].

NH_2-terminal amino acid analysis identified glycine residue only, which together with disk electrophoresis data points to high homogeneity of the preparation.

General chemical analysis showed that 100 mg GFAP preparation contains 96.12 mg protein; the preparation contains also sulfate (total protein content was determined by the method of Lowry, carbohydrates by phenylsulfate and orcein methods, sialic acids according to L. Warren, sulfate by infrared spectroscopy). Amino acid analysis of GFAP (Table 1) confirmed published data on the prevalence of monoaminodicarboxylic and aliphatic acids [5] and the absence of prosthetic groups in the molecule. High content of monoaminodicarboxylic acids ensures acidic properties and negative charge of GFAP molecule.

Highly purified GFAP preparation was used for immunization of BALB/c mice. B-lymphocytes isolated from immune mice were fused with myeloma cells and hybrids producing monoclonal antibodies interacting with human GFAP were obtained. The procedure of selection included immunoenzyme assay of conditioned media from hybridoma cells with subsequent cloning of hybrids producing monoclonal anti-GFAP AB.

The next stage of the selection procedure consisted of the analysis of conditioned media for determination of the class and subclass of monoclonal AB using immunoenzyme and immunodiffusion assays, respectively. Two hybridoma clones (D4 and D8) pro-

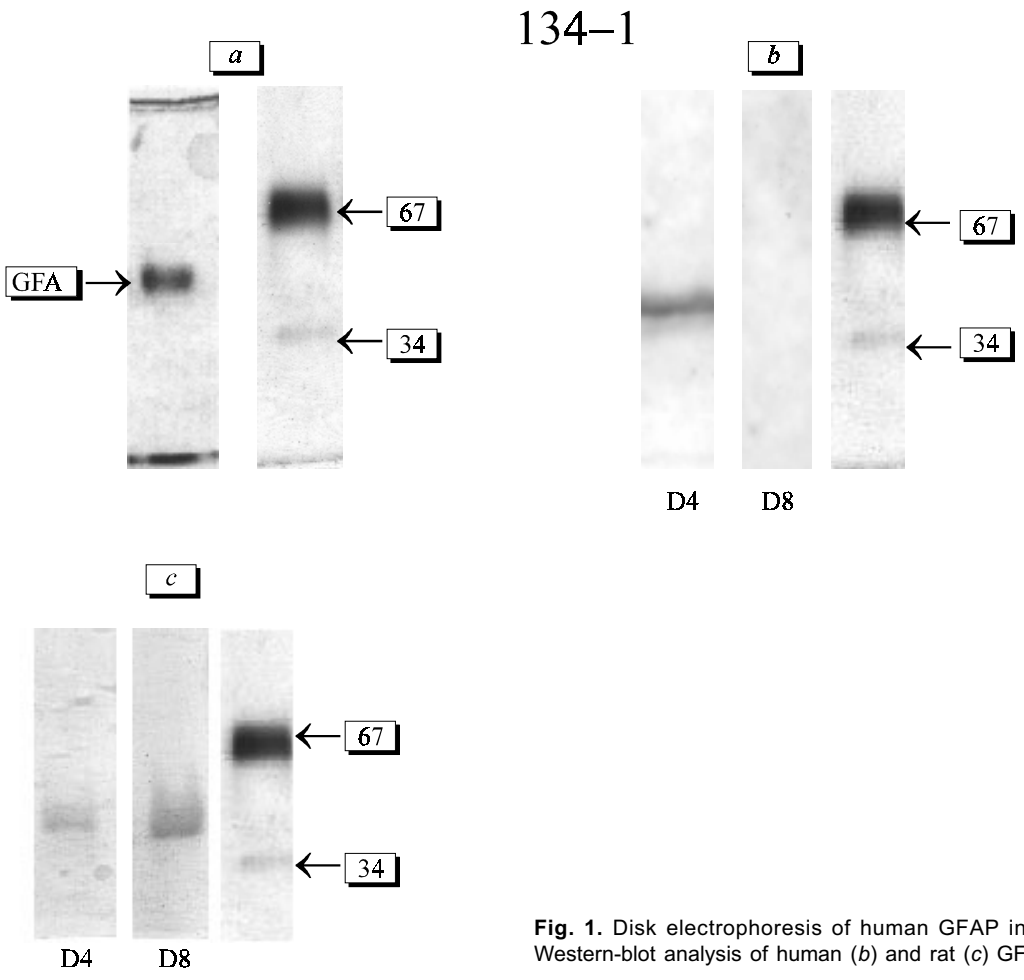

Fig. 1. Disk electrophoresis of human GFAP in PAAG (*a*) and Western-blot analysis of human (*b*) and rat (*c*) GFAP.

ducing IgG_1 monoclonal AB were selected for further experiments.

Immunoblotting with monoclonal AB from D4 and D8 hybridoma clones (Fig. 1, *a, b*) yielded bands corresponding to 51.0±2.1 kDa, *i.e.* electrophoretic mobility of GFAP isolated from human brain.

We also studied binding of mouse monoclonal AB against human GFAP produced by D4 and D8 hybrid clones with rat brain GFAP. Specific staining of 51.0±2.1 kDa protein (the zone of GFAP localization) during immunoblot analysis was revealed only with monoclonal AB from D4, but not from D8 hybridoma (Fig. 1, *c*). Thus, monoclonal AB produced by D4 hybridoma reveal a species-nonspecific antigen epitope present at least in human and rat GFAP, while anti-GFAP AB produced by D8 hybridoma are specific only for human GFAP.

TABLE 1. Amino Acid Composition of GFAP

Amino acid	Content, %	Amino acid	Content, %
Cys	0.82	Asp	10.44
Tre	4.21	Ser	5.12
Glu	17.91	Pro	2.74
Gly	4.16	Ala	12.23
Val	4.54	Met	1.42
Ile	3.77	Tyr	2.50
Phe	2.38	His	2.10
Lys	5.25	Arg	9.51
Leu	10.56	Trp*	N. d.

Note. *Destroyed during acid hydrolysis. N. d. — not detected.

On the basis of highly purified GFAP and monoclonal anti-GFAP AB we elaborated an immunoenzyme test system for GFAP detection in human and animal biological fluids in a concentration range of 0.4-128 ng/ml and constructed a calibration curve (Fig. 2).

Tests for specificity of immunoenzyme GFAP analysis revealed the absence of immunochemical cross-reactions with human, rat, canine, cattle, and porcine serum proteins, neurospecific proteins S-100, NSE, antigens 14-3-3, 10-40-4, α_1-BG, α_2-BG, and α_2-GP, clathrin, vimentin, tubulin, and calmodulin.

Testing of the elaborated systems for parallelism by the method of serial dilutions of human brain extract allowed to obtain curves parallel to the standard ones. Tests for accuracy, reproducibility, and

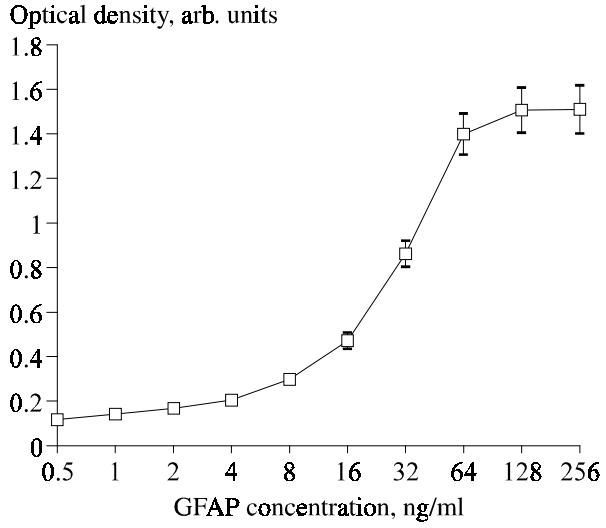

Fig. 2. Calibration curve of GFAP immunoenzyme assay.

reliability of the GFAP analysis systems showed the possibility of introduction of these immunoenzyme tests into clinical and laboratory practice.

Thus, immunocompetent cells from BALB/c mice immunized with purified GFAP preparation after fusion with Sp2/0-Ag14 myeloma cells formed hybridoma clones producing monoclonal anti-GFAP AB.

Immunoenzyme test systems for GFAP detection in biological fluids based on mouse monoclonal anti-GFAP AB are characterized by high specificity, accuracy, and reliability and can be recommended for evaluation of the blood-brain barrier permeability in various diseases and extreme influences associated with the impairment of its functions.

REFERENCES

1. V. P. Chekhonin, T. B. Dmitrieva, Yu. A. Zhirkov, *Immunoenzyme Assay of Neurospecific Antigens*, Moscow (2000).
2. M. S. Abaza, F. Shaban, and R. K. Narayan, *Anticancer Res.*, **18**, No. 2B, 1333-1340 (1998).
3. H. Brisby, K. Olmarker, and L. Rosengren, *Spine*, **15-24**, No. 8, 742-746 (1999).
4. L. Dotevall, L. E. Rosengren, and L. Hagberg, *Infection*, **24**, 125-129 (1996).
5. L. F. Eng, *Scand. J. Immunol.*, **15**, Suppl. 9, 41-51 (1982).
6. L. F. Eng and R. S. Ghirnikar, *Brain Pathol.*, **4**, No. 3, 229-237 (1994).
7. L. F. Eng, R. S. Ghirnikar, and Y. L. Lee, *Neurochem. Res.*, **25**, No. 9-10, 1439-1451 (2000).
8. M. Herrmann, P. Vos, and M. T. Wunderlich, *Stroke*, **31**, No.11, 2670-2677 (2000).
9. G. Kohler and C. Milstein, *Nature*, **256**, 495-497 (1975).
10. P. K. Nakane and A. Kawaoi, *J. Histochem. Cytochem.*, **22**, 1084-1091 (1974).
11. K. Nishigaya, S. Yagi, and T. Sato, *Acta Neuropathol. (Berl.)*, **99**, No. 3, 231-237 (2000).
12. A. L. Shapiro, E. Vinuela, J. V. Maizel, *Biochem. Biophys. Res. Comm.*, **28**, 815-820 (1967).