Monoclonal antibodies to choline acetyltransferase of rat brain

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Received 28 March 1983

Monoclonal antibodies to rat brain choline acetyltransferase were produced by the hybridoma technique. Two stable cell lines, Ab-57 and Ab-60, secreted immunoglobulin of subclass IgG1. The monoclonal antibodies bound to choline acetyltransferase without blocking catalytic activity. Affinity of Ab-57 was 100-times higher than that of Ab-60. Both antibodies bound to the rat enzyme in a mutually exclusive fashion. The antibodies showed cross-species reactivity with choline acetyltransferase from several mammalian brains.

Choline acetyltransferase *Monoclonal antibody* *Cross-species reactivity* *Cholinergic neuron*

1. INTRODUCTION

Choline acetyltransferase (ChAT, acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) is the enzyme responsible for the biosynthesis of the neurotransmitter acetylcholine (ACh). This enzyme is currently the most reliable marker for cholinergic neurons. The antibody to the enzyme would offer a useful method to demonstrate cholinergic neurons immunohistochemically and to study the biochemical regulation of cholinergic activity. Although a number of attempts have been made to purify ChAT from mammalian brains and to generate a specific antiserum to the enzyme, the specificity of the antiserum has been criticized due to the impurity of the enzyme preparations used as antigens [1,2]. Two laboratories have purified ChAT from bovine and porcine brains to a high degree and produced the antisera against the enzymes [3–5]. However, the cross-reactivity of the antibodies to ChAT from other mammalian brains and the usefulness for immunohistochemical and biochemical studies have not been evaluated yet.

An alternative method for producing the monospecific antibodies is the newly developed monoclonal antibody technology [6], which allows the use of a partially purified enzyme as an antigen. The monoclonal antibody would be also useful for studying the different properties of enzyme molecules among various species. We report here the production and characterization of the monoclonal antibodies to ChAT purified from rat brain.

2. MATERIALS AND METHODS

2.1. *Purification of ChAT*

ChAT was purified from rat brain (300–350 g) as in [7] with the modifications that acetone powder of the tissue was used as a starting material [8] and that blue dextran–Sepharose was used instead of CoA–Sepharose [4]. The procedure included homogenization and solubilization of acetone powder, acid precipitation at pH 4.5, $(NH_4)_2SO_4$ fractionation and CM–Sephadex, blue dextran–Sepharose and hydroxylapatite column chromatographies sequentially. The specific activities of the enzyme preparations from different batches of rat brain were ~10 μmol ACh formed.min^{-1}.mg $protein^{-1}$.

2.2. *Production of monoclonal antibodies*

A BALB/c mouse was injected intraperitoneally with 243 μg purified ChAT with 1.5×10^8 heat-inactivated pertussis vaccine. The animal received a booster (96 μg ChAT) without pertussis vaccine

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00145793/83/$3.00

intraperitoneally 21 days after the first immunization. The animal was sacrificed 3 days after the booster and spleen cells (4×10^8) were fused with mouse myeloma cells P3-NS I/1-Ag4-1 (1.5×10^8) by the addition of 2 ml 50% polyethylene glycol 4000. The cells were suspended in HAT selective medium (RPMI 1640 medium supplemented with 15% fetal calf serum, 2 mM L-glutamine, 1 mM pyruvate, 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine), and inoculated into eight 96-well microtest plates (5×10^5 spleen cells/well); 4 and 6 days after the cell fusion, 50 μl of HAT medium was added. Cultures showing hybrid growth were screened for production of anti-ChAT antibodies as described below. Positive cells were cloned by a limiting dilution method 4 times.

2.3. *Purification of antibodies*

Ascites tumors were induced by injecting 1×10^7 hybridoma cells into Pristane-primed BALB/c mice intraperitoneally. Antibody was purified by a pH stepwise gradient elution after adsorption to a protein A–Sepharose column (1 cm × 6 cm) [9].

2.4. *Detection of anti-ChAT antibodies*

For the detection of antibody, ChAT was partially purified from acetone powder of rat brain up to pH 4.5 precipitation. ChAT preparation (~0.3 nmol/min) was incubated with 50 μl culture fluid for 1 h at 20°C in 100 μl total vol. Then, 20 μl rabbit anti-mouse IgG serum coupled to formalin-fixed *Staphylococcus aureus* (10%, v/v) was added and incubated for an additional 1 h at 20°C. The enzyme–antibody complex was removed by centrifugation, and the supernatant was assayed for ChAT activity as in [10]. Formation of the enzyme–antibody complex did not differ between the incubation at 20°C for 2 h and the incubation at 4°C overnight.

2.5. *Scatchard plot analysis of antibody titration curve*

An estimated activity of 100 μmol.min^{-1}.mg protein^{-1} [11] and M_r 68000 [12] for rat ChAT were used to calculate the quantities of ChAT bound to various amounts of antibody. Antibody titration curves were analyzed by the method of Scatchard [13] using a linear least squares curve-fitting routine.

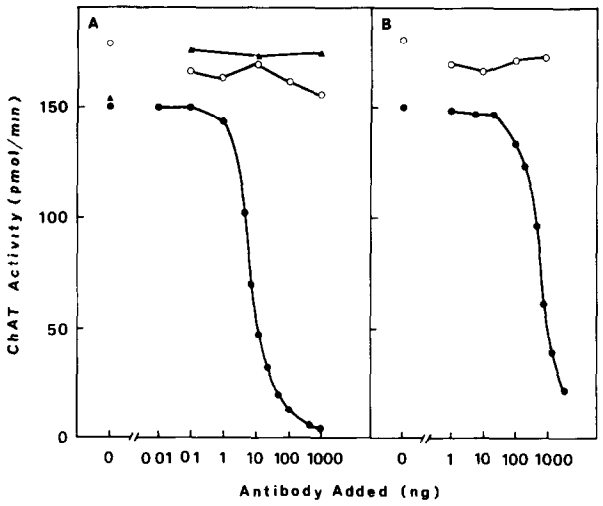

Fig.1. Titration of rat ChAT with monoclonal antibodies. Rat ChAT preparation was incubated with Ab-57 (A) or Ab-60 (B) as indicated and ChAT activity remaining in the supernatant (●) was assayed as in section 2. As a negative control (▲), monoclonal antibody to hemagglutinin of influenza virus (PR-8 strain) was used. In another series of experiments, ChAT activity was assayed without addition of anti-mouse IgG coupled to *S. aureus* and centrifugation (○). The values were the means of duplicate determinations.

2.6. *Analysis of immune complexes*

Interaction of the antibodies with ChAT was analyzed with velocity sedimentation of immune complexes. Rat ChAT preparations (~0.8 nmol/min) were incubated with Ab-57 (2.4 μg) or Ab-60 (22 μg), or both in 235 μl total vol. In a control tube, the antibodies were omitted. Incubation was carried out for 1 h at 20°C and for 4 h at 4°C. Samples were layered over 5–20% sucrose density gradients in phosphate buffered saline (pH 7.4), and centrifuged at 86000 × g for 14 h at 4°C. The samples were fractionated into 19 aliquots which were analyzed for ChAT activity. Standard proteins were treated in an identical manner and detected with absorbance at 280 nm.

3. RESULTS

After 2 weeks of selection in HAT medium, 66% of the wells contained growing hybridomas. Out of 4 wells initially positive for anti-ChAT activity only two wells survived repetitive cloning. Two cell

lines, Ab-57 and Ab-60, were established after 4 cloning operations. Both cell lines secreted immunoglobulin of subclass IgG1, because both antibodies were eluted at pH 6.0 from a protein A–Sepharose column. Ab-57 and Ab-60 produced ascites fluids containing 1.6 mg and 100 μg antibody/ml, respectively. Purified antibodies from ascites fluids were used in the following experiments.

Titration of rat ChAT by the antibodies was shown in fig.1: 50% inhibition was observed with 5 ng Ab-57 and 500 ng Ab-60, respectively. When ChAT preparation was incubated with antibody alone, all the enzyme activity was recovered in the supernatant. ChAT activity in the supernatant decreased only when the enzyme–antibody complex was removed by precipitation with anti-mouse IgG coupled to *S. aureus*. ChAT activity was recovered in the precipitate, although the formation of the enzyme–antibody–anti-mouse IgG–*S. aureus* complex decreased the enzyme activity to the half of the expected value. Control incubation using anti-hemagglutinin of influenza virus did not inhibit ChAT activity. These results indicated that

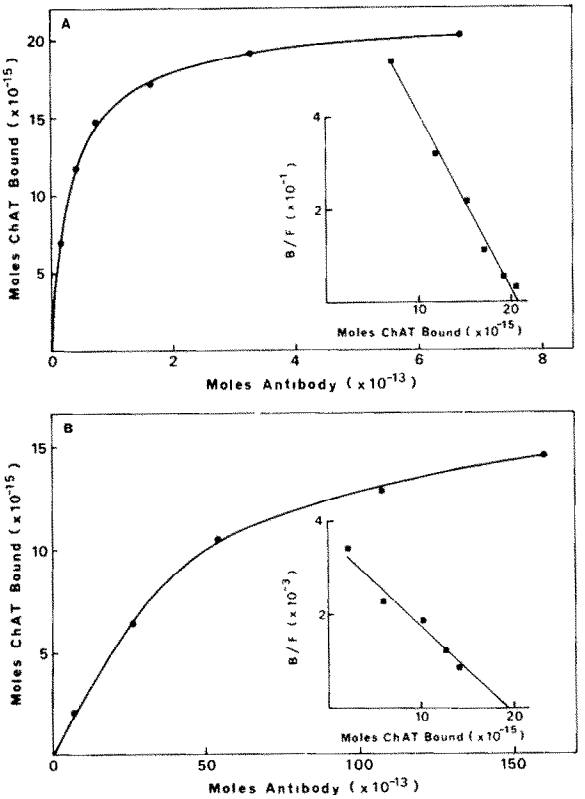

Fig.2. Titration curves and Scatchard plots of rat ChAT with Ab-57 (A) and Ab-60 (B). Procedure and assays were performed as in section 2. The values were the means of duplicate determinations.

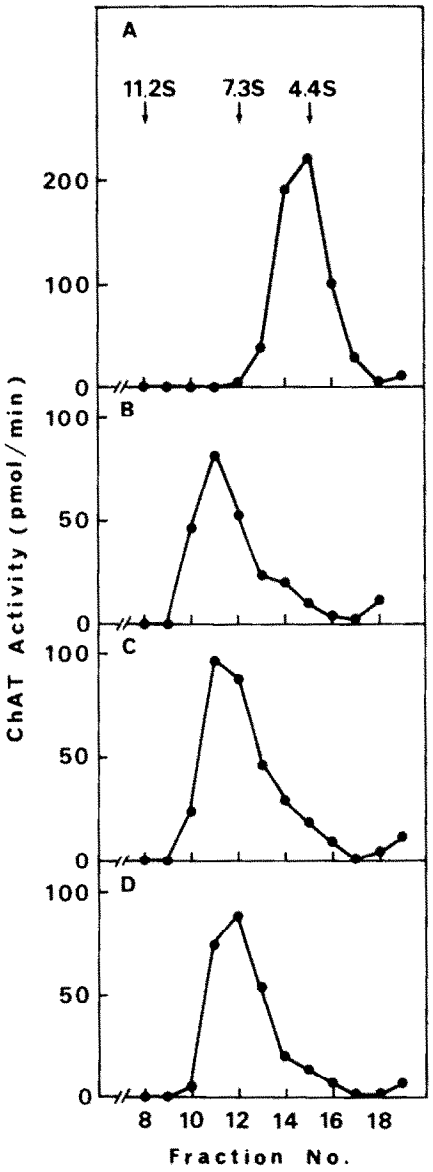

Fig.3. Effects of monoclonal antibodies on velocity sedimentation of rat ChAT. Rat ChAT preparations were incubated without (A) or with Ab-57 (B), Ab-60 (C) and Ab-57 plus Ab-60 (D). *S* values indicated were obtained by the sedimentation of bovine serum albumin (4.4 S), bovine γ-globulin (7.3 S) and catalase (11.2 S).

Table 1

Cross-reactivity of monoclonal antibodies with ChAT from brains of various vertebrates

Species	No addition	Ab-57 (10 ng)		Ab-57 (1 μg)		Ab-60 (1 μg)	
	ChAT activity (pmol/min)	ChAT activity (pmol/min)	% activity of no addition	ChAT activity (pmol/min)	% activity of no addition	ChAT activity (pmol/min)	% activity of no addition
Rat	151	22	15	10	6	22	14
Mouse	133	37	28	12	9	42	32
Guinea pig	103	100	97	55	53	114	111
Cat	81	27	34	2	3	39	48
Monkey	122	114	94	21	18	112	91
Chicken	144	116	81	118	82	117	81
Turtle	103	100	97	98	95	101	99
Frog	33	32	98	23	70	34	102
Carp	128	116	91	119	93	120	93

ChAT preparations of various species were prepared in a similar manner to rat ChAT preparation and incubated with monoclonal antibodies as indicated for 1 h at 20°C. ChAT activity remaining in the supernatant was assayed as in section 2. The values were the means of duplicate determinations

both antibodies reacted with the enzyme without blocking the catalytic site.

Affinity of each antibody for rat ChAT was calculated from titration curves and Scatchard plots (fig.2) assuming spec. act. 100 μmol.min^{-1}.mg protein^{-1} and M_r 68000 for rat ChAT. Scatchard plots were linear indicating that each antibody recognized a single binding site. Affinity (K_a) was calculated to be 3.81×10^9 M^{-1} for Ab-57 and 1.91×10^7 M^{-1} for Ab-60, respectively.

The effect of antibodies on the sedimentation profile of rat ChAT activity was shown in fig.3. An identical shift in sedimentation pattern was observed when ChAT was incubated with either Ab-57 or Ab-60, or both. This indicated that both antibodies bound to rat ChAT in a mutually exclusive fashion. ChAT activity of the enzyme–antibody complex decreased to the half of that of the enzyme alone.

Cross-reactivity of both antibodies with various mammalian brain ChAT was shown in table 1. Both Ab-57 and Ab-60 antibodies cross-reacted with ChAT from mouse and cat with affinities similar to rat ChAT. A high dose of Ab-57 (1 μg) showed partial reactivity with ChAT from guinea pig and monkey. Both antibodies showed no or marginal cross-reactivity with ChAT from non-mammalian species.

4. DISCUSSION

This paper reports the establishment and characterization of the monoclonal antibodies to ChAT from rat brain. During this investigation, several reports have appeared dealing with the production of monoclonal antibodies to ChAT from rat [14,15], bovine [16], porcine [5] and *Drosophila* [17]. Two antibodies established here seem to bind to neighboring regions of the enzyme surface and have a similar cross-species reactivity with mammalian enzymes. However, both antibodies have different affinities. A remarkably high affinity of Ab-57 with an order of 10^9 M^{-1} would be a useful feature for the quantification of ChAT molecule to study the kinetics and regulatory mechanisms of ACh synthesis. However, a moderate affinity of Ab-60 with an order of 10^7 M^{-1} may offer a suitable ligand to prepare immunoaffinity chromatography. Cross-species reactivity of both antibodies with mammalian ChAT offers a tool for biochemical and immunohistochemical studies of cholinergic neurons in various mammalian brains. Several antibodies to bovine ChAT have cross-species reactivity with mammalian ChAT [16]. Using the antibodies obtained here, we are trying to immunohistochemically demonstrate the cholinergic neurons in mammalian brains.

ACKNOWLEDGEMENTS

We thank Dr Tamie Ando for providing monoclonal antibody to hemagglutinin of influenza virus (PR-8 strain) and helpful advice in preparing monoclonal antibodies, and Dr Masaki Nakane for useful discussion. This work was supported in part by Grant-in-Aids for Encouragement of Young Scientists to I.I. and for Special Project Research on 'Metabolic Responses and their Disorders' to T.D. from the Ministry of Education, Science and Culture of Japan.

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