# MONOCLONAL ANTIBODY TO SOLUBLE GUANYLATE CYCLASE OF RAT BRAIN

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## 1. Introduction

Guanylate cyclase (GTP pyrophosphate-lyase, cyclizing, EC 4.6.1.2), the enzyme responsible for the synthesis of cyclic GMP, is detected mostly in soluble fraction and partly associated with particulate fraction of mammalian tissues [1–6]. In rat brain, guanylate cyclase is found mainly in synaptosomal soluble fraction suggesting its involvement in the neural transmission [6]. We have purified the soluble guanylate cyclase from bovine brain to an apparent homogeneity and produced the antibody to the enzyme by immunizing a rabbit [7]. The antibody effectively inhibited the soluble guanylate cyclase activity from various mammalian tissues, but not the Triton-dispersed particulate guanylate cyclase from tissues.

A method for preparing a monoclonal antibody has been established [8]. Because of its high specificity, a monoclonal antibody would offer a method for studying the different properties of enzyme molecules and for histochemically demonstrating the cellular as well as subcellular localization of guanylate cyclase. We report here production and properties of the monoclonal antibody to the soluble guanylate cyclase of rat brain.

## 2. Materials and methods

### 2.1. *Purification of guanylate cyclase*

Guanylate cyclase was purified from supernatant fraction (300 × *g*) of rat brain by pH precipitation, $(NH_4)_2SO_4$ fractionation, phenyl-Sepharose, DEAE-Sepharose and Sephacryl S-300 columns as described in [7]. The specific activity of the enzyme preparation was 97 nmol cyclic GMP formed . $min^{-1}$. mg $protein^{-1}$. The purified guanylate cyclase was stored at −80°C until use.

### 2.2. *Preparation of monoclonal hybridoma cell lines*

The purified guanylate cyclase (640 μg) was injected into BALB/c mouse intraperitoneally with $2 \times 10^9$ heat-inactivated pertussis organisms. The booster injections were carried out twice at a 3-week interval without pertussis organisms. Spleen cells were taken from immunized mouse 3 days after the last booster injection and fused with mouse myeloma cells P3-NS I/1-Ag4-1 (NS I) as in [9]. Spleen cells ($1.6 \times 10^8$) were fused with $4 \times 10^7$ myeloma cells by the addition of 1 ml 50% polyethylene glycol 4000. The cells were suspended in growth medium (RPMI 1640 supplemented with 15% fetal bovine serum, 2 mM L-glutamine and 1 mM pyruvate) and inoculated in two 96-well Nunc Microtest Plates. Two days later, 0.1 ml of HAT selective medium (growth medium supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine) was added and half of the medium was replaced 3 times during the subsequent 10 days. Fourteen days after cell fusion, culture fluid (60 μl) was assayed for anti-guanylate cyclase antibody by measuring enzyme inhibition as described below. Monoclonal hybridoma cell lines were obtained by a limiting dilution method repeated twice.

### 2.3. *Inhibition assay of guanylate cyclase activity*

Enzyme preparation was preincubated with anti-guanylate cyclase IgG at 4°C for 12 h in a total volume of 0.1 ml. Goat antimouse IgG (15 μg) was then added and incubated at 4°C for 1 h. The resulting precipitate was removed by centrifugation at 105 000 × *g* for 30 min, and the supernatant fluid was assayed for guanylate cyclase activity. Guanylate cyclase activity was assayed as in [10].

### 2.4. *Immunochemical titration*

Immunochemical titration was performed by a

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modification of the method in [11]. Rat tissues were homogenized with 5 volumes of 25 mM Tris–HCl (pH 7.6) and centrifuged at 105 000 × *g* for 1 h. Varying amounts of the supernatant were incubated with 1.92 μg IgG in a total volume of 80 μl at 4°C for 12 h. Then, 10 μl of 10% (v/v) suspension of heat-inactivated *Staphylococcus aureus* [12] was added and incubated at 4°C for 1 h. The resulting precipitate was removed by centrifugation, and the supernatant fluid was assayed for guanylate cyclase activity.

## 3. Results and discussion

Seven out of 190 proliferating hybridomas secreted anti-guanylate cyclase antibody as determined by the double antibody precipitation method. Four cell lines with a high titer of antibody were chosen for cloning by limiting dilution method. After repeated subcloning, seven stable hybridoma clones producing antibody to soluble guanylate cyclase were obtained. The clone (7-B7) of the highest titer of antibody was used in the following experiments. When the supernatant of rat brain was preincubated with the monoclonal antibody and the enzyme–antibody complex was removed by the addition of goat antimouse IgG followed by centrifugation, guanylate cyclase activity in the supernatant markedly decreased (fig.1A). Approximately 50% inhibition occurred with 3 μg IgG, which was 50-fold more active than rabbit polyclonal antibody prepared in [7]. However, when the supernatant of rat brain was incubated with the antibody alone, guanylate cyclase activity was inhibited by only 30%, indicating that the antibody reacted with the enzyme without blocking the catalytic site. The antibody did not affect the activity of guanylate cyclase solubilized from the synaptic membrane fraction of rat brain.

The antibody inhibited guanylate cyclase activity in the supernatants of brain, liver, lung and heart of rat (fig.1B and table 1), while the soluble enzyme from the brains of mouse, rabbit, chick and monkey, and from neuroblastoma N1E 115 cells was not inhibited by the antibody (fig.1B and table 2). A 70% inhibition was observed with the soluble guanylate cyclase from bovine brain.

The guanylate cyclase activity per unit enzyme protein was compared in various tissues of rat by an immunochemical titration method using the monoclonal antibody. A fixed amount of the antibody (1.92 μg) was incubated with various amounts of the

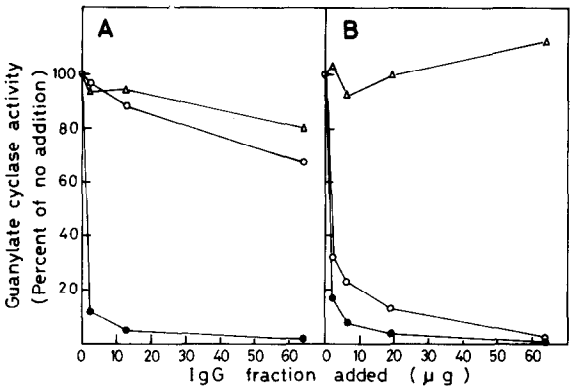


Fig.1. Titration curves of monoclonal antibody *vs* various enzyme preparations. (A) Supernatant (• and ○) or Triton-dispersed synaptic membrane fraction (△) of rat brain was preincubated with various quantities of monoclonal antibody at 4°C for 12 h. Goat antimouse IgG (1.3 μg/μg of monoclonal antibody) was then added and incubation was continued at 4°C for 1 h. The enzyme–antibody complex was then removed by centrifugation, and the supernatant was assayed for guanylate cyclase activity (• and △). Another series of brain supernatant was assayed without addition of goat antimouse IgG and centrifugation (○). (B) Rat brain supernatant (•), rat lung supernatant (○) or mouse brain supernatant (△) was used as an enzyme preparation. Assays were performed as described above. The values are the means of duplicate determinations.

Table 1
Effect of monoclonal antibody on soluble guanylate cyclase from various tissues of rat

| Tissues | Guanylate cyclase activity (pmol/assay) | | % activity of control IgG |
|---|---|---|---|
| | +Control IgG | +Monoclonal IgG | |
| Brain | 360 | 12.2 | 3.4 |
| Liver | 368 | 24.7 | 6.7 |
| Lung | 338 | 34.4 | 10.1 |
| Heart | 315 | 11.0 | 3.5 |

Rat tissues were homogenized with 5 volumes of 25 mM Tris–HCl (pH 7.6) and centrifuged at 105 000 × *g* for 1 h. The supernatant was adjusted to pH 5.2 and resulting precipitate was collected by centrifugation and dissolved in 25 mM Tris–HCl (pH 7.6). Enzyme activity/volume was adjusted to the same level among various tissues. The enzyme preparations were preincubated with 10 μg of monoclonal antibody at 4°C for 12 h. Goat antimouse IgG (13 μg) was then added and incubation was continued at 4°C for 1 h. The enzyme–antibody complex was removed by centrifugation, and the supernatant was assayed for guanylate cyclase activity. The values are the means of duplicate determinations

Table 2
Effect of monoclonal antibody on soluble guanylate cyclase from brains of various vertebrates

| Species | Guanylate cyclase activity (pmol/assay) | | % activity of control IgG |
|---|---|---|---|
| | +Control IgG | +Monoclonal IgG | |
| Rat | 976 | 25 | 3 |
| Mouse | 1034 | 905 | 88 |
| Rabbit | 1709 | 1519 | 89 |
| Chick | 1133 | 961 | 85 |
| Bovine | 1122 | 365 | 32 |
| Monkey | 1162 | 1126 | 97 |
| Neuroblastoma N1E 115 | 1072 | 1021 | 95 |

Preparation and assay of soluble guanylate cyclase from the brains of various animals were performed as described in table 1. The values are the means of duplicate determinations

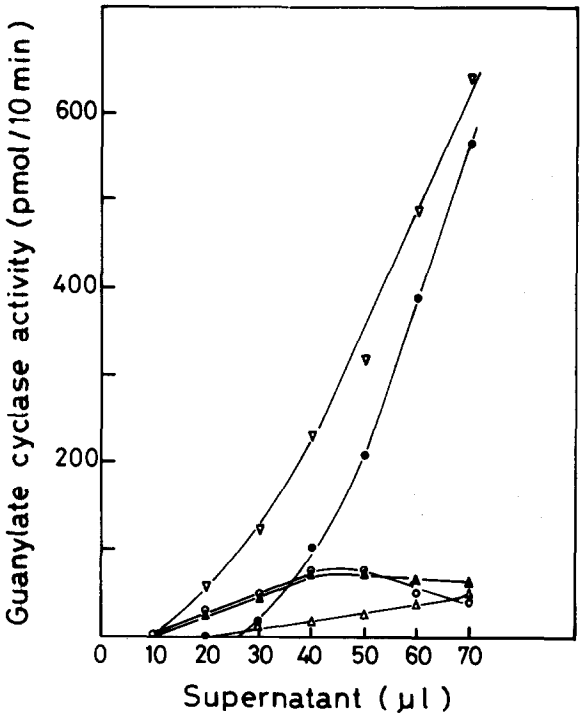


Fig.2. Immunochemical titration of soluble guanylate cyclase of rat tissues. Synaptosomal soluble fraction (●) and the supernatants of brain (○), liver (▲), heart (△) and lung (▽) of rat were used as enzyme preparations. The experiment was performed as in section 2. The values are the means of triplicate determinations.

enzyme preparations of tissues followed by removal of the enzyme–antibody complex and the remaining enzyme activity in the supernatant was determined (fig.2). From the intercepts of abscissa and the slopes of the activity, the guanylate cyclase activity bound to 1 μg of IgG was calculated (table 3). The activities of synaptosomal soluble fraction and lung supernatant were 29- and 4-fold higher than those of the supernatants of brain, liver and heart, respectively. However, when guanylate cyclase was precipitated from the synaptosomal soluble fraction at pH 5.2 and dialyzed, the enzyme activity bound to 1 μg of IgG decreased to 7.8 pmol/10 min, which was comparable to those of the supernatants of brain, liver and heart. The observation indicates that the guanylate cyclase in the synaptosomal soluble fraction of rat brain is in a highly activated state, and is consistent with our previous

Table 3
Immunochemical titration of soluble guanylate cyclase of rat tissues

| Tissues | Guanylate cyclase activity bound to 1 μg antibody (pmol/10 min) |
|---|---|
| Brain | 9.9 |
| Lung | 36.5 |
| Liver | 9.9 |
| Heart | 7.3 |
| Synaptosomal soluble fraction | 289 |

The guanylate cyclase activities bound to 1 μg IgG were calculated from the intercepts of abscissa and the slopes of activity in fig.2

finding that synaptosomal soluble fraction contained an endogenous activator for guanylate cyclase [6,13]. The observation also coincided with the reports that activating factors were present in the supernatant of rat lung [14,15].

We have previously prepared the antibody to soluble guanylate cyclase purified from bovine brain by immunizing a rabbit [7]. The antibody cross-reacted with the soluble enzyme from various tissues of rat, mouse, bull and neuroblastoma cells, but did not react with the particulate enzyme of various tissues. The monoclonal antibody to the soluble guanylate cyclase from rat brain also reacted with the soluble enzyme from rat tissues, but not with the particulate guanylate cyclase of synaptic membrane, indicating that particulate enzyme is immunologically distinct from soluble enzyme. We have obtained 7 monoclonal antibodies, all of which exhibited the same properties as the clone (7-B7) reported here. Garbers has shown that the antibody to the particulate guanylate cyclase from sea urchin inhibited the particulate enzyme from rat tissues, but not the soluble enzyme from rat tissues [16]. The differing properties between soluble and particulate guanylate cyclase have been indicated by the difference in kinetics [5,17], metal requirement [5,17–19], apparent $M_r$ [17,20] and the sensitivity to proteolysis [21,22]. On the other hand, Zwiller et al. reported that the antibody to soluble guanylate cyclase of rat brain they prepared cross-reacted with particulate enzyme, indicating a partial similarity between soluble and particulate guanylate cyclase [23].

Lewicki et al. have recently reported the production of monoclonal antibodies to soluble guanylate cyclase from rat lung [24,25]. Their antibodies recognized the soluble lung enzymes from rat, beef and pig, but did not, or only slightly cross-reacted with the soluble enzymes from rabbit and mouse lung [25]. The monoclonal antibody prepared here also discriminates between soluble enzymes of rat and other species of vertebrates, indicating that soluble guanylate cyclases from different species of animals contain a different structure in the enzyme proteins. A specific monoclonal antibody with a very high titer would enable us to study the protein structure of the enzyme and the localization of soluble guanylate cyclase in tissues immunohistochemically.

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