Novel Type of Monoclonal Antibodies against Cyclic GMP and Application to Immunocytochemistry of the Rat Brain

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Abstract—A novel type of monoclonal antibodies against cyclic GMP were produced to study the immunocytochemical distribution of cyclic GMP in the rat brain. Cyclic GMP conjugated to bovine serum albumin with glutaraldehyde was used as an immunogen, and monoclonal antibodies were produced. The one monoclonal antibody which did not crossreact against other nucleotides was applied to the immunocytochemistry of the rat brain. Cyclic GMP immunoreactivities were distributed unevenly in the rat brain. The cerebellar cortex, hippocampus and cerebral cortex contained a high degree of cyclic GMP immunoreactivity, while most of the white matter was not stained. In the cerebellar cortex, stellate cells and Golgi cells showed intense immunoreactivities, but granule cells showed weak immunoreactivities. Approximately 60–80% of the Purkinje cells showed intense immunoreactivities, while the remaining ones showed only weak staining. The pyramidal cells in the cerebral cortex and hippocampus also showed intense immunostaining. Some glial cells adjacent to the Purkinje cells also stained. The nuclei of cyclic GMP-immunoreactive cells were not stained. These immunocytochemical distributions of cyclic GMP are in fairly good agreement with reported the biochemical data and the immunocytochemical distribution of guanylate cyclase. These monoclonal antibodies should be helpful for elucidating the physiological role of cyclic GMP in the brain.

Cyclic GMP is widely distributed in the central nervous system and peripheral organs (1) and may be involved in intracellular signal transduction in vasodilatation (2, 3) and in photoreceptors (4). On the other hand the role of cyclic GMP in the central nervous system is obscure, so the immunocytochemical localization of cyclic in the brain had to be given attention. Steiner et al. (5) prepared antibodies using as an immunogen the succinyl derivative of cyclic GMP conjugated to protein. These antibodies have been used in radioimmunoassays (1) and immunocytochemistry (6, 7). De Vente et al. (8) reported a modified immunocytochemical approach for visualization of cyclic GMP using formaldehyde as a cross-linker to couple cyclic GMP to protein and as a tissue fixative. However, immunocytochemical localizations of cyclic GMP in rat brain obtained with these antibodies are not consistent with the biochemical data (9). In the cerebellum, an area containing the largest amount of cyclic GMP in the brain (10), no neuronal cells are stained with these antibodies (6), while guanylate cyclase has been shown to be present in the neurons (11).

We chose as the immunogen cyclic GMP conjugated to bovine serum albumin (BSA) by glutaraldehyde. Cyclic GMP-specific monoclonal antibodies were then produced and used to immunocytochemically analyze the rat brain.

Materials and Methods

Preparation of conjugates and immunization procedure: BSA was chosen as a carrier protein for the cyclic GMP-conjugate used as the immunogen. The coupling procedures were similar to those used for the γ -amino-butyrate-BSA conjugate (12). In brief, cyclic GMP (0.1 mmol, Sigma) was mixed with...
BSA (60 mg) in 10 ml of 0.1 M phosphate buffer (PB, pH 7.4). After the addition of glutaraldehyde (1 mmol) as a linking agent, the reaction mixture was stirred for 2 hr at room temperature, in the dark, and then 1 mmol of sodium borohydride was added, and the preparation was dialyzed against a large volume of PB. Dialyzed solution was stored frozen at -20°C until use. An analogous procedure was used to couple BSA or bovine thyroglobulin (BTG, 60 mg; Sigma) to the nucleotides: cyclic GMP, guanosine 5'-monophosphate (GMP), guanosine 5'-diphosphate (GDP), guanosine 5'-triphosphate (GTP), cyclic AMP, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), 3',5'-cyclic inosine monophosphate (cyclic IMP), 3',5'-cyclic cytosine monophosphate (cyclic CMP) and 3',5'-cyclic uridine monophosphate (cyclic UMP). The mol/mol ratio of coupled cyclic GMP/BSA or BTG was determined using a trace amount of 3H-cyclic GMP (NEN Research Products, specific activity of 10.2 Ci/mmol) and calculated from the fraction of radioactivity remaining in the dialyzed solution and the concentration of BSA or BTG.

Cyclic GMP-BSA conjugate (0.2 mg protein in 0.25 ml) was emulsified with an equal volume of Freund's complete adjuvant (Difco) and intraperitoneally injected into four week old female BALB/c mice (Charles-River Japan). After 4, 7 day interval booster injections of the immunogen mixed with Freund's incomplete adjuvant (Difco), the splenocytes were taken and used for hybridization.

Hybridization, cloning and selection of hybridoma cell lines: Four days after the last immunization, the mice were killed by cervical dislocation and the splenocytes removed. A total of 10⁸ splenocytes were mixed with 2×10⁷ mouse myeloma cells (P3U1) and intraperitoneally injected into four week old female BALB/c mice (Charles-River Japan). After 4, 7 day interval booster injections of the immunogen mixed with Freund's incomplete adjuvant (Difco), the splenocytes were taken and used for hybridization.

Production of ascites tumors and purification of monoclonal antibody: Ascites was raised in BALB/c mice of both sexes after pretreatment of 0.5 ml of pristane (2,6,10,14-tetramethyl-pentadecane, Aldrich), intraperitoneally, one week before the intraperitoneal injection of 10⁶ hybridoma cells. When ascites was evident, the fluid was collected and tested for the presence of monoclonal antibody by ELISA, as described below. Monoclonal antibody was purified from ascites fluids by ammonium sulphate precipitation and affinity chromatography, as follows: Cyclic GMP conjugated to BTG was chosen as a specific ligand for the antibody. The ligand (20 mg protein) was immobilized to 3.5 ml of cyanogen bromide-activated Sepharose 4B (Pharmacia) in 0.1 M NaHCO₃ (pH 8.3). The gel was packed into a column and 1 ml of monoclonal antibody partially purified by ammonium sulphate precipitation and affinity chromatography was applied to the column. After washing the gel with 5–10 volumes of 0.1 M PB containing 0.15 M NaCl (PBS), the antibody was eluted by 0.2 M glycine-HCl buffer (pH 2.8). The eluted antibody was neutralized by 1 M Tris-HCl buffer (pH 8.0). Monoclonal antibodies had a purity exceeding 95% as determined by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (data not shown), after the affinity purification step.

Detection of antibody activity by ELISA: In screening the clones for production of antibody against cyclic GMP, Immunoplate II (Nunc) 96-well polystyrene microtiter plates were coated with 0.1 ml of cyclic GMP-BTG plates (Falcon) containing 10⁵/well thymus cells as feeders, at a density of 10⁶ cells per well.

At 10–14 days post-fusion, the wells were screened for reactivity using an enzyme-linked immunosorbent assay (ELISA) technique, as described below. Seven strongly reactive wells were identified, and cell lines from these wells were expanded and tested for tissue staining. Cells from positive wells were cloned by repeating limiting dilution in 96-well culture plates containing 10⁶ thymus cells/well, as feeders. Clonal hybridoma cell lines were expanded, kept in liquid nitrogen and used for production of large quantities of monoclonal antibodies in ascites fluids.
conjugate (2 μg protein/ml) in 50 mM bicarbonate buffer (pH 9.6). The plates were incubated overnight at 4°C and then washed three times with PBS containing 0.01% Triton X-100. Plates were incubated with 1% skim milk solution (Difco) in 0.1 M PBS to prevent the non-specific binding of antibody. Again the plates were washed and then 0.1 ml of culture supernatant was added per well and allowed to stand for 1 hr at room temperature. Then the plates were washed three times and 0.1 ml of the monoclonal antibodies were examined by the ELISA method as follows: (i) Serially diluted monoclonal antibodies (10^{-11}-10^{-6} g/ml) were applied to the microtiter plates coated with nucleotides conjugated to BTG (2 μg protein/ml). (ii) Microtiter plates were coated with 0.1 ml of serially diluted nucleotides conjugated to BTG (2 × 10^{-16}-2 × 10^{-3} g protein/ml), and then 0.1 ml of diluted purified monoclonal antibodies (1 ng/ml) was added. (iii) Purified monoclonal antibodies (0.1 ng/ml, 0.1 ml) were preincubated with each of the serially diluted conjugated or unconjugated nucleotides (0.1 ml) for 60 min at 37°C and then applied to cyclic GMP-BTG coated (2 μg protein/ml) microtiter plates. These plates were treated as described above and then the values were corrected by subtracting the blank ones.

Tissue preparations and immunocytochemical staining: Under ether anesthesia, male Wistar rats were perfused transcardially with isotonic saline, followed with 0.5% glutaraldehyde, 4% formaldehyde, and 0.2% picric acid in 0.1 M PB. The tissues were removed, immersed in the same fixative but omitting the glutaraldehyde, for 12 hr at 4°C, and then stored in 30% sucrose in 0.1 M PBS. The tissues were cut on a cryostat (Leitz) at 10 μm and used for immunocytochemical staining. The sections were pretreated with 0.5% H$_2$O$_2$ to block the endogenous peroxidase and then with 5% normal goat serum to prevent the non-specific binding of the antiserum. The preparations were incubated with affinity-purified cyclic GMP monoclonal antibody (2 ng/ml) for 18 hr at 4°C. The sections were washed with PBS containing 0.01% Triton X-100 and incubated for 6 hr in goat anti-mouse IgG diluted 1:1000. After rinsing, the sections were incubated with the PAP complex (Miles Scientific) diluted 1:1000. The paper was washed well and reacted with 0.2 mg/ml 3,3′-diaminobenzidine (DAB, Sigma), and 0.005% H$_2$O$_2$ in 50 mM Tris-HCl (pH 7.4) for 5 min at room temperature.
(Fuji Film) was used for photography.

The specificity of the method was tested by replacing the monoclonal antibodies with normal mouse immunoglobulin purified from mouse serum, using Protein A Sepharose CL-4B (Pharmacia), at the same concentration of protein (2 ng/ml). The specificity of the monoclonal antibodies was also examined by adsorption of the diluted antibodies. The monoclonal antibodies were preadsorbed with free (10^{-6}–10^{-3} mM) and glutaraldehyde-conjugated nucleotides (10^{-5}–10^{-2} g protein BTG/ml). Preadsorbed monoclonal antibodies were applied to rat brain preparations, and immunohistochemical examinations were then made.

**Results**

**Characterization of the antibodies:** ELISA screening using cyclic GMP-BTG conjugate yielded 7 positive clones out of 960 wells containing growing hybridomas.

The specificity and titer of these monoclonal antibodies were examined by ELISA. The affinity-purified cyclic GMP monoclonal antibody was diluted with PBS containing 0.01% Triton X-100, at a dilution of 10^{-12}–10^{-6} g protein/ml, and the preparation was then applied to microtiter plates coated with cyclic GMP-BTG conjugate. As shown in Fig. 1A, the monoclonal antibody that showed the highest titer and specificity for cyclic GMP (designated as cG-1) recognized the cyclic GMP-BTG conjugate at a concentration of 10 pg protein/ml, and the maximal reaction was at 10 ng/ml. Other nucleotide conjugates or BTG were not recognized by the monoclonal antibody at a concentration of over 10 ng protein/ml.

The results obtained by ELISA in serial dilution experiments of nucleotide conjugates on microtiter plates showed that cG-1 could recognize the cyclic GMP conjugate to BTG at a concentration of 2 fg protein of BTG/ml (corresponding to 0.1 fM of conjugated cyclic GMP) (Fig. 1B). The 50% effective concentration against cyclic GMP conjugate was 20 pg protein of BTG/ml (1 pM of conjugated cyclic GMP). This monoclonal antibody did not react with other nucleotide conjugates coated on microtiter plates.

![Fig. 1.](image)

**Fig. 1.** Immunoreactivity of the monoclonal antibody (cG-1) in ELISA plates. **A.** Serially diluted purified monoclonal antibody (10^{-12}–10^{-6} g/ml) was applied to the microtiter plates coated with nucleotides conjugated to BTG (2 μg protein/ml). **B.** The purified monoclonal antibody (1 ng/ml) was applied to the microtiter plates coated with serially diluted nucleotide conjugates to BTG (2×10^{-12}–2×10^{-3} g protein/ml). (1) cyclic GMP-BTG, (2) GMP-BTG, (3) cyclic AMP-BTG, (4) AMP-BTG, (5) cyclic IMP-BTG, (6) BTG.

From displacement curves (Fig. 2A) established in competition experiments, the most immunoreactive conjugate was cyclic GMP-BTG. Self-displacement occurred at a concentration of 20 pg protein of BTG/ml (1 pM of conjugated cyclic GMP) and the 50%
displacement concentration was 20 ng protein of BTG/ml (1 nM of conjugated cyclic GMP). Other nucleotide conjugates showed little or no competition. This monoclonal antibody recognized the unconjugated form of cyclic GMP. The 50% displacement concentration of unconjugated cyclic GMP was 10 nM, that is, approximately 10 fold greater than that of the conjugated cyclic GMP (Fig. 2B). Other unconjugated nucleotides were without effect.

The specificity of the monoclonal antibody was also estimated by the model system, using nitrocellulose paper. The affinity purified monoclonal antibodies were used at a concentration of 2 ng protein/ml. Although all monoclonal antibodies recognized cyclic GMP, there were differences in the titer, and some monoclonal antibodies recognized other nucleotides (data not shown). As shown in Fig. 3, cG-1 recognized 100 pmol of cyclic GMP dotted on the nitrocellulose paper and reacted in a dose-dependent manner. Other nucleotides could not be recognized by this monoclonal antibody even when 1000 times greater amounts were dotted on the paper.

Fig. 2. Displacement curves from competition experiments using the ELISA method between coated cyclic GMP-BTG (2 μg protein/ml) and (A) conjugated or (B) unconjugated nucleotides previously incubated with monoclonal antibody (cG-1) (0.1 ng/ml). A, Conjugated nucleotides: (1) cyclic GMP-BTG, (2) GMP-BTG, (3) cyclic AMP-BTG, (4) AMP-BTG, (5) cyclic IMP-BTG and (6) BTG were used at a concentration of 2×10^-13-2×10^-15 g protein/ml. B, Unconjugated nucleotides: (1) cyclic GMP, (2) GMP, (3) cyclic AMP, (4) AMP, (5) cyclic IMP and (6) cyclic GMP-BTG were used at a concentration of 10^-15-10^-14 M. The ratio between the absorbance (490 nm) with (B) and without (Bo) competition is plotted.

Fig. 3. Testing for antibody specificity using a nitrocellulose paper model system. The nitrocellulose paper, pretreated with BSA, was spotted with 1 μl of serially diluted nucleotide solution (10^-11-10^-7 mol/μl) and fixed by 4% for maldehyde and 0.5% glutaraldehyde and 0.2% picric acid. This paper was incubated with cyclic GMP monoclonal antibody (cG-1) at a concentration of 2 ng/ml and then stained according to the PAP method.
Fig. 4. Microphotographs of cyclic GMP-immunoreactive structures in the rat brain. A, Cyclic GMP-immunoreactive cells in cerebellar cortex. Cyclic GMP-immunoreactive cells were found in the molecular layer (M), Purkinje cell layer (P) and granular layer (G), but white matter (W) was free of immunoreactivity. Bar: 100 μm. B, High magnification of A. Purkinje cells (arrows) and basket cells (arrowheads) showed cyclic GMP immunoreactivities. Bar: 25 μm. C, Some Purkinje cells had only weak cyclic GMP immunoreactivity compared with adjacent Purkinje cells. Arrows indicate two differentially stained Purkinje cells. Small cells adjacent to Purkinje cells (arrowheads) were also stained by cyclic GMP monoclonal antibody. Bar: 25 μm. D, Granule cells showed a weak cytoplasmic immunoreactivity, and Golgi cells (arrows) showed a relatively stronger immunoreactivity in the same layer. Bar: 25 μm. E, Cyclic GMP-immunoreactive pyramidal cells in the hippocampus. The cytoplasm of pyramidal cells were stained positively, but in the nuclei, cyclic GMP immunoreactivity was nil. Bar: 25 μm. F, The pyramidal cells in the cerebral cortex also showed cyclic GMP immunoreactivity. Bar: 25 μm.
**Fixation condition:** To study the distribution of cyclic GMP-immunoreactivity in the rat brain, we tested three fixatives: glutaraldehyde alone, formaldehyde alone, and the combination of glutaraldehyde and formaldehyde. Cyclic GMP was fixed with all these compounds on the nitrocellulose, and it was recognized by the monoclonal antibody. No cyclic GMP immunoreactivity was observed when the brain preparation was not or weakly fixed, and the addition of formaldehyde increased the intensity of cyclic GMP immunoreactivity in the nitrocellulose paper model system and the brain preparation (data not shown). An intense staining pattern and good preservation of the tissue were constantly obtained; therefore, we used the fixative containing 0.5% glutaraldehyde, 4% formaldehyde, and 0.2% picric acid in 0.1 M PB.

**Immunocytochemical staining:** Cyclic GMP immunoreactivities were widely but unevenly distributed throughout the rat brain. Cerebral cortex, hippocampus and cerebellar cortex were rich in these immunoreactivities, while most of the white matter was stain-free.

In the cerebellar cortex, many cyclic GMP immunoreactive structures were observed (Figs. 4A, B, C and 5A, D). Stellate cells and basket cells in the molecular layer were intensely stained (Figs. 4A, B, C and 5A, D). Approximately 60–80% of the Purkinje cells

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**Fig. 5.** Microphotographs of the rat cerebellar cortex demonstrated by PAP immunohistochemistry. A, Cerebellar cortex stained with cyclic GMP monoclonal antibody (cG-1). Cyclic GMP immunoreactive structures were observed. Bar: 25 μm. B, Cerebellar cortex stained with normal mouse immunoglobulin. No specific immunoreactivity was shown. Bar: 25 μm. C, After adsorption of cyclic GMP monoclonal antibody with cyclic GMP-BTG conjugate (1 mg protein BTG/ml), cyclic GMP immunoreactivity in the cerebellar cortex was completely abolished. Bar: 25 μm. D, Cyclic GMP immunoreactivity in the cerebellar cortex was not reduced by adsorption of monoclonal antibody with cyclic AMP-BTG conjugate (1 mg protein of BTG/ml). Bar: 25 μm.
showed intense immunoreactivities (Figs. 4A, B, C and 5A, D), while the remaining showed only weak staining (Fig. 4C). Most reactive Purkinje cells stained exclusively in the cytoplasm. Small cells adjacent to Purkinje cells which were presumably Bergman glial cells, also stained intensely (Figs. 4B, C and 5A, D). Granule cells showed a weak cytoplasmic immunoreactivity and sparse, larger Golgi cells showed a relatively stronger immunoreactivity, in the same layer, and no immunoreactivity in the white matter (Fig. 4D).

In the hippocampus, intense cyclic GMP-immunoreactivities were observed in pyramidal cells (Fig. 4E). The cytoplasms of these cells were stained positively, but in the nuclei, cyclic GMP immunoreactivity was nil.

Cyclic GMP-immunoreactive cells were also found in the cerebral cortex. As shown in Fig. 4F, cyclic GMP immunoreactivities were present in the cytoplasm of the pyramidal cells, but absent in the nuclei.

Glia cells in the caudate-putamen complex and corpus callosum also showed intense cyclic GMP immunoreactivities (data not shown).

When higher concentrations (>1%) of glutaraldehyde were used in the fixatives, neurophils were stained in several brain regions such as the molecular layer in the cerebellar cortex and hippocampus (data not shown).

Staining in these areas was specific for cyclic GMP: (a) no specific staining was observed when sections were incubated with immunoglobulin from non-immunized mice (Fig. 5B); (b) cyclic GMP monoclonal antibody preadsorbed with cyclic GMP or cyclic GMP-BTG led to no immunostaining (Fig. 5C). (c) cyclic GMP immunoreactivity was not reduced by adsorption of this monoclonal antibody with other nucleotides (Fig. 5D).

Discussion

We developed a novel type of monoclonal antibodies against cyclic GMP and used them to immunocytochemically study the rat brain. Earlier studies (10, 14) of cyclic GMP in the central nervous system demonstrated that this compound is widely and unevenly distributed in the brain. The concentration of cyclic GMP in the cerebellum is higher than in other areas of the brain, perhaps because of the low activity of cyclic GMP phosphodiesterase in the cerebellum (15). Guanylate cyclase that catalyzes the formation of cyclic GMP is found in tissue homogenates in all regions, and it is widely distributed in various neuronal cell types throughout the brain, as ascertained by immunohistochemistry (16). Thus, a neuronal localization of cyclic GMP is concluded, whereas immunohistochemical studies using the Steiner-type antibody showed cyclic GMP to be present only in glial cells, in the cerebellum (6) or in other brain areas (17). This discrepancy has been explained by loss of cyclic GMP in the sections present in a soluble form during the staining procedures (18). Chan-Palay and Palay (19) reported that both glial and neuronal cells in the cerebellum showed cyclic GMP immunoreactivity in the thoroughly fixed tissue sections using Steiner-type antibody. However, De Vente et al. (8) mentioned in their report that they could not reproduce these results, and we also failed to observe any specific cyclic GMP immunoreactivity using Steiner-type antibody under the conditions used by Chan-Palay and Palay (19). De Vente et al. (8) reported an approach for visualization of the soluble pool of cyclic GMP using formaldehyde as a cross-linker to couple cyclic GMP to protein and as a tissue fixative. In their report, although widely distributed cyclic GMP immunoreactivities were demonstrated throughout the rat brain, including the hippocampus and cerebral cortex, cyclic GMP immunoreactivity was absent in the cerebellar cortex, an area containing the largest amount of cyclic GMP. In contrast, our immunostaining results are in good agreement with the reported biochemical data and the distribution of guanylate cyclase and cyclic GMP-dependent protein kinase (10, 11, 20). A neuronal localization of cyclic GMP in the cerebellum was thus demonstrated for the first time in the present work. Purkinje cells which have been shown to contain guanylate cyclase and cyclic GMP-dependent protein kinase were immunocytochemically shown to contain cyclic GMP. Variation in the staining intensity in the cytoplasm may reflect different states of neuronal
activity.

De Vent et al. used formaldehyde as a cross-linker, because the coupling ratio was higher than that of glutaraldehyde (8). However, as described in their report, the cyclic GMP-formaldehyde-protein conjugate was unstable; that is, the dissociation of the conjugate occurred rapidly, and the half was less than 6 hr at 37°C. Although they claimed that the dissociation rate is slow enough to isolate the conjugate and the conjugate is stable enough to give rise to specific antibodies in rabbits, this instability of the immunogen may result a degraded product which would give rise to antibodies against compounds other than cyclic GMP. Purification of specific antigen-affinity chromatography may circumvent these problems; however, this seems unlikely due to the instability of the antigen. These factors may explain the inconsistency between their immunostaining results and the biochemical data, as described above.

We found that despite the low coupling ratio (approximately 5%) the cyclic GMP-glutaraldehyde-protein conjugate is stable when treated with sodium borohydride (data not shown). This cyclic GMP-glutaraldehyde-protein conjugate was immobilized on an agarose matrix and utilized for affinity purification of the monoclonal antibodies, as described above. Since the conjugate is stable, the affinity resin can be used for more than ten purifications and remains active even after more than four months.

The stability of the cyclic GMP-glutaraldehyde-protein conjugate also enabled simple screening and characterization of the monoclonal antibodies using ELISA. In contrast, the specificity of the antisera was evaluated only by the “gelatin model system” in the case of polyclonal antiserum against the formaldehyde-conjugated cyclic GMP (8). Characterization of the monoclonal antibodies with ELISA and the nitrocellulose paper model system showed that the monoclonal antibody cG-1 was the most specific for cyclic GMP. Immuno-inhibition experiments showed that the monoclonal antibody (cG-1) reacted with both the unconjugated and conjugated cyclic GMP.

Since our novel type anti-cyclic GMP monoclonal antibodies are highly specific and applicable to thoroughly fixed tissue preparations, these antibodies should be pertinent laboratory tools which can be used to elucidate the physiological role of cyclic GMP in the brain.

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