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Production and characterization of monoclonal antibodies to soluble rat lung guanylate cyclase

(cyclic GMP/hybridoma/myeloma/immunoprecipitation/redox)

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ABSTRACT Four monoclonal antibodies to rat lung soluble guanylate cyclase [GTP pyrophosphate-lyase (cyclizing) EC 4.6.1.2] have been produced by fusing spleen cells from immunized BALB/c mice with SP-2/0 myeloma cells. The antibodies were detected by their ability to bind immobilized guanylate cyclase and by immunoprecipitation of purified enzyme in the presence of second (rabbit anti-mouse) antibody. After subcloning by limiting dilution, hybridomas were injected intraperitoneally into mice to produce ascitic fluid containing 2–5 mg of antibody per ml. The four antibodies obtained had titers of between 1:1580 and 1:3160 but were detectable at dilutions greater than 1:20,000. Soluble guanylate cyclase from several rat tissues were crossreactive with the four monoclonal antibodies, suggesting that the soluble enzyme from different rat tissues is antigenically similar. The antibodies also recognized soluble lung enzyme from rat, beef, and pig, while enzyme from rabbit was not crossreactive and mouse enzyme was recognized by only one of the antibodies. Particulate guanylate cyclase from a number of tissues had only minimal crossreactivity with the antibodies. Immunoprecipitated guanylate cyclase retained catalytic activity, could be activated with sodium nitroprusside, and was inhibited by cystamine. None of the antibodies were inhibitory under the conditions examined. These antibodies will be useful probes for the study of guanylate cyclase regulation and function under a variety of physiological conditions.

Guanylate cyclase [GTP pyrophosphate-lyase (cyclizing) EC 4.6.1.2] catalyzes the formation of cyclic GMP from GTP. In all tissues examined, the enzyme exists in two forms, a membrane-bound form and a soluble form that has different properties. Work in many laboratories has also led to the finding that hydroxyl radical, hydrogen peroxide, nitric oxide, nitrosamines, and a variety of other nitro and nitroso compounds can activate both forms of the enzyme (for review, see ref. 1). Studies on the mechanisms of these activations have been hindered by the lack of highly purified preparations of the enzyme. However, the recent purification of the soluble enzyme from liver, lung, and brain (2–6) should permit detailed studies on protein structure and the mechanism of redox regulation of guanylate cyclase.

Other observations have suggested that the steady-state level of guanylate cyclase, as well as its intracellular distribution, can be altered. For example, in regenerating liver, fetal liver, and transplantable tumors, particulate guanylate cyclase activity increases while soluble activity decreases (7–10). These and other studies suggest that an understanding of the regulation of guanylate cyclase activity and its intracellular abundance could shed new light on the function of the enzyme and cyclic GMP. Such studies would be greatly aided by antibodies to the membrane-bound and soluble forms of the enzyme. Questions about redistribution and abundance of the enzyme could be examined by measuring the actual quantities of guanylate cyclase protein under various conditions and with immunohistochemical techniques. The development of highly specific antibodies to the enzyme could also be used for affinity purification procedures, guanylate cyclase turnover studies, and other experiments to examine the regulation and function of the enzyme and cyclic GMP. We have succeeded in obtaining four monoclonal antibodies to soluble guanylate cyclase; in this report, the production and characterization of these antibodies are described. Some of these observations have been reported as abstracts (11, 12).

METHODS

Preparation of Tissue Homogenates. Tissues were removed from animals and 10% homogenates were prepared with 50 mM Tris·HCl, pH 8/1 mM EDTA/1 mM dithiothreitol/250 mM sucrose in a Teflon homogenizer. The homogenates were centrifuged at 105,000 × *g* for 60 min at 2–4°C, and the supernatant and particulate fractions were stored at −70°C.

Purification of Rat Lung Soluble Guanylate Cyclase. The details of the purification procedure have been published (6). Briefly, the procedure involved isoelectric and ammonium sulfate precipitation, affinity chromatography on GTP-sepharose, and preparative polyacrylamide gel electrophoresis. This protocol led to preparations having specific activities of 40–60 nmol mg^{-1} min^{-1} with Mg^{2+}-GTP as substrate, which represents a purification of ≈3000-fold. The purified enzyme showed a single protein band on analytical isoelectric focusing and polyacrylamide gels that migrated coincident with enzyme activity and, under denaturing conditions, a single band of 72,000 daltons was obtained (6).

Guanylate Cyclase Assay. Enzyme samples were incubated with 50 mM Tris·HCl, pH 7.6/0.02% bovine serum albumin/1 mM GTP/4 mM $MnCl_2$ or $MgCl_2$ at 37°C for 10 min. With crude enzyme preparations, 10 mM theophylline/7.5 mM creatine phosphate/14 μg of creatine phosphokinase (135 units/mg) were included as described (7, 13). Incubations were terminated with cold 50 mM NaOAc, pH 4, followed by boiling. Cyclic GMP formed was determined by radioimmunoassay (14). Protein was measured by the method of Lowry *et al.* (15) using bovine serum albumin as standard.

Cell Culture Conditions. SP-2/0 myeloma cells (provided by D. Benjamin, University of Virginia) were grown in RPMI 1640 media (GIBCO)/2 mM glutamine/50 μM 2-mercaptoethanol/12% horse serum/penicillin/streptomycin (complete RPMI 1640). Cells were grown at 37°C in humidified 10% CO_2/90% air. Stock cultures were routinely maintained at 1–50 × 10^5 cells/ml and, for 4 days before fusion, were kept at 1–2 × 10^5 cells/ml. Prior to fusion, cells were grown on 6-thioguanine (6 μg/ml) for 6 days, followed by 1 day with no drug present.

Immunization and Spleen Cell Preparations. Male BALB/c mice (Flow Laboratories, McLean, VA) were injected subcutaneously with 3–10 μg of purified rat lung soluble guanylate

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cyclase in 50 mM Tris·HCl, pH 7.6/Freund's complete adjuvant (1:1). Subsequent immunizations were performed 4 times at monthly intervals with 3–10 μg of purified guanylate cyclase in Freund's incomplete adjuvant. Four days after the final immunization, mice were sacrificed by cervical dislocation, and each spleen was placed in 10 ml of Ca^{2+}-free, serum-free media as described by Schneiderman *et al.* (16). The spleens were teased apart, filtered through Nitex monofilament screens, and centrifuged at 800 × *g* for 10 min at 25°C. Two additional washes with Ca^{2+}-free, serum-free media were performed, and the spleen cells were then fused with SP-2/0 myeloma cells.

Cell Fusion and Hybridoma Production. Spleen cells from one immunized mouse (1–2 × 10^8 cells) were fused with 1.5 × 10^7 myeloma cells as described by Kennet (17), using 37% polyethylene glycol (M_r 1000, Koch–Light). After fusion, the washed cells were suspended in complete RPMI 1640 media, mixed with freshly prepared thymocytes from one mouse, and distributed (100 μl) into each of 960 wells of $CoStar^{96}$ microtiter plates (Costar, Cambridge, MA). The following day, hypoxanthine (8 μg/ml), aminopterin (0.17 μg/ml), and thymidine (27 μg/ml) (18) were added; growth was then continued for 2 weeks with fresh media every 3 or 4 days. Clones found to be positive for antiguanylate cyclase activity were subcloned by limiting dilution (i.e., one cell per well). The resultant monoclonal populations were used to produce ascitic fluid in mice that had received 0.5 ml of pristane (Aldrich) 2 weeks prior to intraperitoneal injection of 5 × 10^6 hybridoma cells.

Plate Cost Assay for Anti-Guanylate Cyclase. Highly purified guanylate cyclase (40 ng) in 20 mM Tris·HCl, pH 7.6, was added to each well of a set of polyvinyl chloride microtiter plates (Cooke Industries, Alexandria, VA). To another set of wells, an equal volume of Tris buffer without enzyme was added. The plates were incubated at 25°C for 3 hr, after which the fluid was removed and the wells were washed twice with 1% gamma globulin-free horse serum in phosphate-buffered saline (buffer A). Two hundred μl of 20% gamma globulin-free horse serum in phosphate-buffered saline was added to all wells, and the mixtures were incubated for 1 hr at 25°C. The wells were washed three times with buffer A. Fifty-microliter aliquots of hybridoma media were added in duplicate to wells that had been incubated with guanylate cyclase and also to a well incubated with Tris buffer, which served as a minus-antigen control. After the plates had been incubated for 3 hr at 25°C, samples were removed, and wells were washed twice with buffer A. To all wells, 100 μl of purified ^{125}I-labeled rabbit anti-mouse IgG (10–15 ng, 35,000–45,000 cpm) was added, and samples were incubated for 15 hr at 25°C. Samples were removed, and the wells were washed twice with buffer A and eight times with water. The plates were air dried, and the individual wells were assayed for ^{125}I.

Preparation of ^{125}I-Labeled Rabbit Anti-Mouse IgG. Purified rabbit anti-mouse IgG (200 μg) (Cappel Laboratories, Cochranville, PA) was iodinated with 1 mCi of $Na^{125}I$ (1 Ci = 3.7 × 10^{10} becquerels), using lactoperoxidase enzymobeads (Bio-Rad). After iodination, the sample was applied to a Sephadex G-25 column in buffer A. The peak fractions of protein had a specific activity of 2.5 mCi/mg of IgG; these samples were stored at −70°C. Fresh preparations of ^{125}I-labeled rabbit anti-mouse IgG were made every 2 or 3 weeks.

Immunoprecipitation of Guanylate Cyclase. Twenty microliters of either hybridoma media or ascitic fluid (diluted 1:100 to 1:32,000) was incubated with 20 μl of purified guanylate cyclase (20 ng of protein) for 2 hr at 2–4°C. Twenty microliters of carrier mouse IgG (130 μg) was added, followed by 20 μl of rabbit anti-mouse IgG antisera (Cappel Laboratories). The incubations were continued for 60 min and then the mixtures were centrifuged for 30 min at 2000 × g. The supernatant fractions were assayed for guanylate cyclase activity. The immunoprecipitates were washed with two 500-μl portions of 20 mM Tris·HCl, pH 7.6, and resuspended in 100 μl of guanylate cyclase reaction mixture containing 1 mM dithiothreitol. Samples were assayed for guanylate cyclase activity as described above. In some cases, cystamine or sodium nitroprusside was added to the incubation mixtures.

RESULTS

After three injections of purified rat lung guanylate cyclase (10 μg per injection) over a period of 3 months, a BALB/c mouse was found to contain antiguanylate cyclase activity in serum, as shown by immunoprecipitation of enzyme activity. The mouse was then immunized for a fourth time and, 4 days later, the spleen cells were fused with SP-2/0 mouse myeloma cells. Within 12–22 days, 233 hybridomas were identified and screened for antiguanylate cyclase activity. The initial screen of all hybridomas was the ability of hybridoma media to bind guanylate cyclase on polyvinyl chloride microtiter plates, followed by detection with ^{125}I-labeled rabbit anti-mouse IgG. The data from the screening of 33 hybridomas by this "plate coat" method are summarized in Fig. 1. Four hybridomas, designated B_1, B_2, B_3, and B_4, were found to produce anti-guanylate cyclase activity. A clone was scored positive if the amount of ^{125}I-labeled rabbit anti-mouse IgG bound to immobilized guanylate cyclase was at least 2 or 3 times the amount binding to wells that had not been pretreated with purified guanylate cyclase. Such a minus-antigen control was necessary, as we obtained some hybridoma media that gave increased and similar amounts of ^{125}I binding to wells with or without guanylate cyclase pretreatment. When these media were further tested by immunoprecipitation of guanylate cyclase, they were found to be devoid of antiguanylate cyclase activity. Such "plate coat artifacts" are probably the result of nonspecific interaction of antibody with the polyvinyl chloride plates or with the horse serum, rather than with the specific antigen. Moreover, the testing of each media with wells plus and minus antigen clearly separated such artifacts from authentic antiguanylate cyclase,

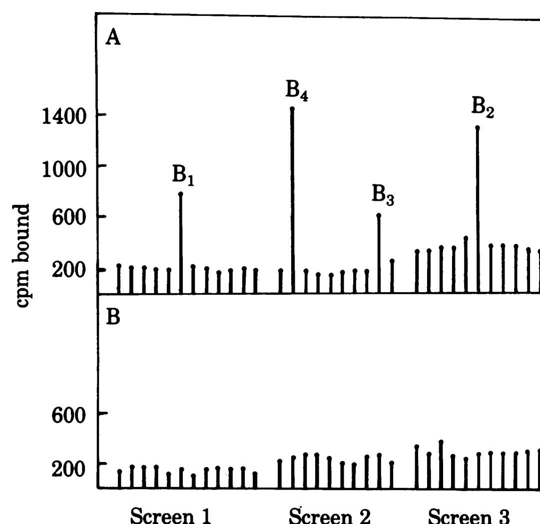

FIG. 1. Plate-coat assay for anti-guanylate cyclase. (*A*) Purified guanylate cyclase was immobilized on polyvinyl chloride plates and incubated with 50 μl of hybridoma media. The wells were aspirated, washed, and ^{125}I-labeled rabbit anti-mouse IgG was added. After overnight incubation, the wells were aspirated, washed, and assayed for radioactivity. (*B*) Minus-antigen control.

and subsequent examination with immunoprecipitation confirmed these findings.

The four hybridomas detected by using the plate-coat assay were grown for 7 additional days, rescreened to detect stable clones, and then subcloned by limiting dilution. Clones that grew from single cells were rescreened. With all four of these hybrids, all clones were found to be positive, demonstrating the monoclonal nature of the hybridomas. The hybridomas were injected intraperitoneally into pristane-treated BALB/c mice and, 12–15 days later, ascitic fluid was obtained for each hybridoma. Control ascitic fluid was similarly derived from a hybridoma that produced no antiguanylate cyclase activity. Elution profiles of the antibodies from protein A-Sepharose columns (19) indicate that antibodies B_1, B_2, and B_4 are of the IgG_1 type, while B_3 is of the IgG_{2A} type.

An alternative method for detecting antiguanylate cyclase was the immunoprecipitation of enzyme activity by using saturating amounts of a second (rabbit anti-mouse IgG) antibody to facilitate precipitation. Under these conditions, hybridomas B_1–B_4 showed a disappearance of guanylate cyclase activity from the supernatant fraction of incubation mixtures that contained enzyme, buffer, and hybridoma media (or ascites fluid) and a corresponding appearance of enzyme activity in the washed immune precipitate. It should be noted that, in the absence of second antibody, the four antibodies did not precipitate or inhibit guanylate cyclase activity. Furthermore, the complex of enzyme with first and second antibody possessed full catalytic activity. Thus, these antibodies do not appear to be directed against the active site of the enzyme. Titration curves for the precipitation of purified guanylate cyclase by the ascitic fluid from the four hybridomas are shown in Fig. 2. The titers of the antibodies (defined as the dilution giving 50% precipitation of enzyme activity) were 1:2190, 1:1580, 1:1300, and 1:5160 for B_1, B_2, B_3, and B_4, respectively. However, we were able to detect the antibodies, especially B_4, at dilutions greater than 1:20,000. Control ascites at all dilutions produced less than 0.5 pmol of cyclic GMP. Cell culture media from these positive hybridomas had titers of 1:20–1:50 when measured in a similar manner.

The ability of the four antibodies to crossreact with soluble guanylate cyclase from other rat tissues was investigated by immunoprecipitation of enzyme activity from crude supernatant fractions by using a second antibody as described above (Table 1). All four antibodies precipitated soluble guanylate cyclase from all rat tissues examined. Antibodies B_1, B_2, and B_4 gave

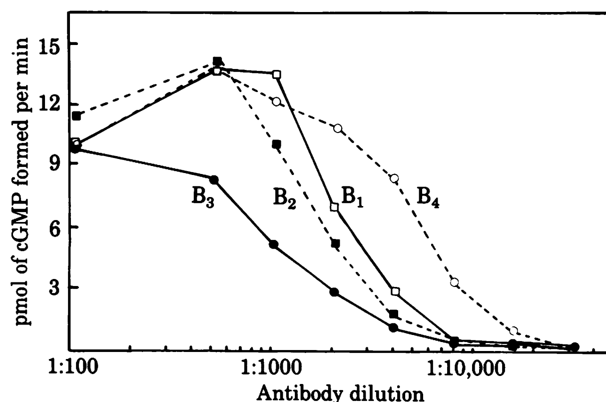

FIG. 2. Immunoprecipitation of guanylate cyclase by monoclonal antibodies. Various dilutions of ascites fluid were incubated with 50 ng of purified guanylate cyclase. After addition of the second antibody, immunoprecipitates were collected, washed, and assayed for guanylate cyclase activity. cGMP, cyclic GMP.

Table 1. Precipitation of soluble guanylate cyclase from rat tissues by using monoclonal antibodies

Rat tissue	Guanylate cyclase precipitated, % of total activity				
	Control	B_1	B_2	B_3	B_4
Cortex	3.0	135	123	98	118
Lung	1.3	86	85	57	75
Liver	1.6	63	63	38	55
Heart	0.2	45	46	29	44
Kidney	0.8	74	73	37	46
Testes	0.3	45	43	24	36

Ascites fluid was diluted 1:500 and incubated with 20 μl of 20 mM Tris, pH 7.6 and 20 μl of rat tissue supernatant fraction. After addition of a second antibody, immunoprecipitates were washed and assayed for guanylate cyclase activity as described.

similar results, while antibody B_3 gave less precipitation with all tissues tested. Antibodies B_1, B_2, and B_4 resulted in a small activation of the enzyme from cerebral cortex, and somewhat less soluble enzyme from heart and testes preparations was precipitated compared with other tissues. These studies suggest, however, that the soluble enzymes from these rat tissues are antigenically quite similar.

Crossreactivity of the antibodies toward soluble lung guanylate cyclase from other species was also examined by immunoprecipitation. All four antibodies crossreacted similarly with soluble lung enzyme from rat, beef, and pig (Table 2). There was a small activation of the beef and pig enzyme. None of the antibodies reacted with the rabbit enzyme. Antibodies B_1, B_2, and B_3 showed some weak crossreactivity with mouse enzyme, while antibody B_4 reacted more strongly, precipitating 60% of the activity. These studies suggest that the soluble lung enzyme from rat, pig, and beef are antigenically similar, while mouse and rabbit enzyme are different from each other and the other species examined. In addition, we have tested detergent-solubilized particulate guanylate cyclase from a number of tissues and found little or no crossreactivity with these antibodies.

The antibodies were also used to immunoprecipitate purified guanylate cyclase and examine the effects of sodium nitroprusside and cystamine on the precipitated antibody-bound enzyme (Table 3). We found that, when guanylate cyclase was precipitated, the enzyme could be activated 6- to 9-fold with 100 μM sodium nitroprusside and inhibited almost completely by 2 mM cystamine. Thus, these observations are similar to our earlier reports with purified soluble enzyme (4, 6, 20). These data suggest that, although all four antibodies are capable of binding to guanylate cyclase, the sites on the enzyme required for nitroprusside and nitric oxide activation and mixed disulfide inhibition are still accessible and presumably different from the antibody binding site.

Table 2. Immunoprecipitation of lung soluble guanylate cyclase from different species

Species	Guanylate cyclase precipitated, % of total activity				
	Control	B_1	B_2	B_3	B_4
Rat	0.9	119	117	87	100
Beef	0.4	146	124	124	146
Pig	0.1	106	80	95	143
Mouse	0.3	25	17	8	59
Rabbit	0	0.1	0.3	0	0

Ascites fluid was diluted 1:500 and incubated with supernatant fractions from tissue homogenates. Immunoprecipitates were collected, washed, and assayed for guanylate cyclase activity.

Table 3. Effect of sodium nitroprusside and cystamine on immunoprecipitated guanylate cyclase

Addition	Guanylate cyclase activity, pmol/10 min				
	Control	B_1	B_2	B_3	B_4
None	0.1	2.8	1.9	2.0	2.8
Sodium nitroprusside (100 μM)	0.4	22.5	15.9	11.7	19.9
Cystamine (2 mM)	0.1	0.3	0.1	0.7	0.1

Ascites fluid was diluted 1:500 and incubated with 20 ng of purified guanylate cyclase from rat lung. After the addition of a second antibody, immune precipitates were collected, washed, and assayed for guanylate cyclase activity in the presence of sodium nitroprusside or cystamine using Mg^{2+}-GTP as substrates.

DISCUSSION

Guanylate cyclase exists in two forms, a soluble and a particulate form, that differ in their kinetic characteristics, apparent size, and their relative abundance under some conditions (1). It has been observed that, under conditions such as tumorigenesis or organ regeneration, there is an alteration in the intracellular distribution of the two enzyme forms such that the particulate becomes predominant (7–10). These studies have been compromised by the measurement of enzyme activity rather than of enzyme protein and would clearly be aided by specific antibodies capable of detecting and quantitating small amounts of the enzymes. Although the soluble form of the enzyme has been purified (2–6), the particulate form has only recently been solubilized from membranes and partially purified (21, 22). At present, the physicochemical relationship between the two enzyme forms is unknown.

Another area in the study of guanylate cyclase has been the regulation of enzyme activity on an acute short-term basis. Considerable effort has failed to demonstrate any reproducible hormonal effects on the enzyme(s), although several naturally occurring agents have been shown to activate it. Fatty acids, the hydroxyl radical, and a wide variety of nitro and nitroso compounds are all capable of activating the enzyme, and the general theory that has emerged is one of regulation of guanylate cyclase by free radicals (1, 23). The known involvement of free radicals in a number of biological, as well as pathological, events (24) makes this an interesting mechanism whereby cyclic GMP production may be coupled to cellular events. However, one major unanswered question has been the molecular changes that accompany such activations. Physicochemical studies of the enzyme to answer these and other related questions require larger quantities of purified enzyme than have been obtained with conventional purification methods.

In preparing to study some of the questions raised, we have sought to develop antibodies to soluble guanylate cyclase from rat lung. Although one group has recently reported low-titer antibodies to soluble brain guanylate cyclase in rabbits (5), the purified liver and lung enzyme in our hands have not proved to be very antigenic in rabbits or sheep for reasons that are currently unknown. This prompted us to consider production of antibodies in mice, with the eventual generation of mouse hybridomas secreting monoclonal antibodies. Such monoclonal antibodies have the marked advantage of being homogeneous and directed against a single antigenic determinant on the enzyme, be it a primary sequence or a three-dimensional conformation. It is obvious that a library of monoclonal antibodies, each to a different site, would be extremely useful in studying the types of problems described above. We have reported here on the successful fusion of mouse SP-2/0 myeloma cells with spleen cells from an immunized BALB/c mouse and on the characterization of four monoclonal antibodies to soluble guanylate cyclase from rat lung. After cell fusions and growth of hybridomas, the antibodies were detected by their ability to bind immobilized guanylate cyclase, followed by detection with ^{125}I-labeled rabbit anti-mouse IgG. This method of screening was relatively fast, could accommodate large numbers of test hybridoma media, and positive clones were quite obvious. Once detected, the positive clones were selected for stability and subcloned by limiting dilution. Injection of the subcloned hybridomas into BALB/c mice led to the production of ascitic fluid containing 2–5 mg antibody per ml of ascitic fluid. Antibodies B_1, B_2, and B_4 are of the IgG_1 type, while B_3 is an IgG_{2A}. Monoclonal antibodies alone are nonprecipitating; however, when a second rabbit anti-mouse IgG antibody was included in incubation mixtures, the four antibodies were able to precipitate guanylate cyclase over a wide range of ascitic antibody dilution (1:100 to 1:10,000). Soluble guanylate cyclase from supernatant fractions of cerebral cortex, liver, heart, kidney, and testes homogenates also crossreacted with all four monoclonal antibodies with some small differences. Soluble lung enzyme from rat, pig, and beef reacted similarly with the antibodies, while lung enzyme from rabbit was nonreactive and mouse lung enzyme was partially reactive. All antibodies had only minimal reactivity with the particulate enzyme from several rat tissues. Although these studies suggest that soluble rat enzyme from several tissues is antigenically similar and different from rat particulate enzyme or the soluble enzyme of certain other species, additional studies are needed.

Although previous work has shown that guanylate cyclase can be activated by nitroso compounds, including azide, sodium nitroprusside, and nitric oxide (1, 4, 13), the mechanism for this activation is unknown. In addition, we have recently reported on the reversible inhibition of guanylate cyclase on mixed disulfide formation with cystamine (20). This work demonstrated the importance of free sulfhydryl groups for both basal and nitric oxide-activated enzyme activity (20). In the present work, we have observed that, when guanylate cyclase is immunoprecipitated with these four monoclonal antibodies, the precipitated antibody-bound enzyme can be activated by nitroprusside and inhibited by cystamine. These data indicate that the site(s) responsible for these modulations of enzyme activity are not blocked by immunoprecipitation and must be different from the antibody-binding site.

The development of monoclonal antibodies to soluble guanylate cyclase should allow us to develop a number of techniques to study questions concerning this enzyme and may help to elucidate the role(s) of cyclic GMP in biological systems.

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