Anti-idiotype and Anti-anti-idiotype Antibodies Generated from Polyclonal Antibodies against Microcystin-LR

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Polyclonal anti-idiotype antibodies (pAb2/m) for microcystin leucine—arginine variant (MCYST-LR) were generated from ascites after immunization with affinity-purified Fab fragment of rabbit polyclonal antibodies (pAb1/r) against MCYST-LR to BALB/c mice. Competitive direct enzyme-linked immunosorbent assay (cd-ELISA) revealed that the binding of free MCYST-LR to solid-phase pAb1/r was inhibited by affinity-purified pAb2/m. pAb2/m can be used as MCYST-LR surrogate in the ELISA. Using the purified pAb2/m as immunogen, polyclonal anti-anti-idiotype antibodies (pAb3/r), which have characteristics similar to those of original pAb1/r, were generated in rabbits. In the pAb3/r-based ELISA, the concentrations causing 50% inhibition (IC_{50}) of binding of MCYST-LR—horseradish peroxidase (MCYST—HRP) to the solid-phase pAb3 by MCYST-LR, MCYST—arginine—arginine variant (MCYST-RR), MCYST—tyrosine—arginine variant (MCYST-YR), and nodularin (NODLN) were 0.7, 24.4, 19.2, and 24.3 ng/mL, respectively. pAb3/r showed a dose-dependent neutralization of the inhibitory effect of MCYST-LR to protein phosphatase 1 (PP1) in an *in vitro* assay system. pAb2/m had no effect on PP1 activities.

Keywords: *Antibody; anti-idiotype; microcystin-LR; PP1; ELISA*

INTRODUCTION

Microcystins (MCYST) are a group of monocyclic hepatotoxins produced by certain freshwater cyanobacteria, including *Anabaena flos-aquae*, *Microcystis aeruginosa*, and *Oscillatoria agardhii* (Carmichael, 1992, 1994). Microcystin leucine—arginine variant (MCYST-LR) has been found to be a potent inhibitor of protein phosphatase 1 and 2A both *in vivo* and *in vitro* (Toshizawa et al., 1990); the inhibitory effect was due to its specific interaction with the enzyme through both covalent and noncovalent binding in liver (Claeyssens et al., 1995; Nishiwaki et al., 1994; Runnegar et al., 1993; Toivola et al., 1994). MCYST-LR has also been shown to be a potent tumor promoter in rats (Falconer, 1991; Nishiwaki-Matsushima et al., 1992). Both polyclonal and monoclonal antibodies (pAb and mAb) against MCYST-LR have been developed for the analysis and biological function of the toxin (An and Carmichael, 1994; Chu et al., 1989; Nagata et al., 1995; Huang and Chu, 1996). Simple and specific enzyme-linked immunosorbent assay (ELISA) has been established for the detection of trace amounts of toxin in water and algae (Chu et al., 1990; Nagata et al., 1995; Chu and Wedepohl, 1994) and in body fluids (Lin and Chu, 1994a). Both pAb and mAb have also been shown to have protective effects on the hepatotoxicity of MCYST-LR *in vitro* and *in vivo* and on MCYST-LR-dependent protein phosphatase inhibition (Lin and Chu, 1994b; Nagata et al., 1995). Such developments have led to a great demand for specific antibodies and related immunochemical reagents for the assay.

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An alternative approach for preparing immunochemical reagents is through generating anti-idiotype antibodies (Ab2), which have gained wide application in diagnostic and therapeutic areas for large molecules (Kennedy et al., 1987; Nisonoff, 1991; Nisonoff and Lamoyi, 1981; Sacks et al., 1982). In the past few years, Ab2 against small molecular weight haptens (Nisonoff, 1991), including insecticides, herbicides (Spinks et al., 1993), hormones (Khole and Hegde, 1992; Wang et al., 1995), and mycotoxins (Chanh et al., 1989, 1990, 1992; Chu et al., 1995; Hsu and Chu, 1994, 1995) and phycotoxins (Chanh et al., 1992; Shestowsky et al., 1992) have been successfully developed. Some Ab2 have been shown to effectively mimic the biological functions of the haptens (Chanh et al., 1992; Nisonoff, 1991; Shestowsky et al., 1993; Wang et al., 1995); they also could be used as antigen surrogates in immunoassays and to generate anti-anti-idiotype antibodies (Ab3) having specificity similar to the original antibody (Ab1) (Chu et al., 1995; Dinca et al., 1993; Shestowsky et al., 1993; Spinks et al., 1993). Using Ab2 and Ab3 as the immunoassay reagents, it is possible to avoid the potentially toxic marker in the assay system and to replace the toxin conjugates as immunogen for antibody production. In the present study, polyclonal antibodies against MCYST-LR that have good cross-reactivities with the MCYST-arginine—arginine variant (MCYST-RR), MCYST—tyrosine—arginine variant (MCYST-YR), and nodularin (NODLN) were chosen as the idiotype antibodies (pAb1/r) for generating pAb2/m in mice. Using the purified pAb2 as immunogen, anti-anti-idiotype antibodies (pAb3/r) were generated in rabbits. Details for the production and characterization of pAb2/m and pAb3/r and their biological effects on protein phosphatase are reported herein.

MATERIALS AND METHODS

Materials. MCYST-LR, MCYST-RR, MCYST-YR, and NODLN were kindly provided by Dr. W. W. Carmichael of Wright State University (Dayton, OH). Virus-free, 6—8-week-

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old, female BALB/c mice were obtained from Harlan Sprague Dawley (Madison, WI). The murine myeloma cell line p3/NS-1/1-AG-4-1 was obtained from American Type Culture Collection (Rockville, MD). Bovine serum albumin (BSA), *o*-phenylenediamine (OPD), Tween 20, and 2,6,10,14-tetramethylpentadecane (pristane, T-7640) were obtained from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) was obtained from United States Biochemicals (USB, Cleveland, OH). Immobilized papain gel (No. 20341), immobilized protein A (No. 20333, immunopure), AminoLink gel, and goat anti-mouse IgG + IgM-peroxidase conjugate (ELISA grade) were obtained from Pierce Chemical Co. (Rockford, IL). Dulbecco modified Eagle's medium (DMEM) was obtained from GIBCO Laboratories (Grand Island, NY). Horseradish peroxidase (HRP; ELISA grade) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). K-B substrate solution (a premixed solution with 1 mM 3,3′,5,5′-tetramethylbenzidine and 3 mM H_2O_2 in potassium citrate buffer, pH 3.9) was supplied by ELISA Technologies (Lexington, KY). Freund's complete adjuvant containing *Mycobacterium tuberculosis* (H37 Ra) and Freund's incomplete adjuvant were obtained from Difco Laboratories (Detroit, MI). *Pasteurella*-negative New Zealand White rabbits were obtained from LSR Industries (Union Grove, WI). Protein phosphatase 1 (PP1) was kindly provided by Dr. E. Y. C. Lee of the University of Miami (Miami, FL). ELISA microwell plates were purchased from Nunc (high binding capacity, Nunc Co. No. 4-69914, Roskilde, Denmark). All other chemicals and organic solvents used were of reagent grade or better.

Purification of pAb (pAb1/r) against MCYST-LR. The original pAb1/r against MCYST-LR were prepared according to the method described before (Chu et al., 1989) and were further purified by affinity chromatography on a column conjugated with MCYST-LR (Lin and Chu, 1994b). In a typical experiment, 1 mL of pAb1/r (21 mg of protein) in 0.01 M phosphate buffer, pH 7.5, containing 0.15 M NaCl (PBS) was applied to the column. After incubation at room temperature for 1 h, the column was washed extensively with 0.01 M PBS until the absorbance of the eluant at 280 nm was near zero. Bound antibodies were eluted from the column with 0.1 M glycine−HCl, pH 2.8. Fractions, 1 mL each, were collected and neutralized immediately by addition of the appropriate amount of 1.0 M Tris-HCl, pH 9.2, to each fraction. The fractions containing specific antibodies were then pooled and dialyzed against 0.01 M PBS, pH 7.5, overnight in a cold room (4−6 °C) and stored at −20 °C.

Preparation of Fab Fragment of pAb1/r. For generating mouse polyclonal anti-Ab1 antibodies (pAb2/m), the Fab fragment of pAb1/r was prepared by digesting the purified antibody with an immobilized papain gel according to the supplier's instructions (Rockford, Pierce, IL). In a typical experiment, 0.5 mL of papain gel was equilibrated and washed twice with 4 mL of digesting buffer [(DB), 20 mM NaH_2PO_4 + 20 mM dithiothreitol (instead of 20 mM cysteine hydrochloride) + 10 mM EDTA-Na_4, pH 7.0]. After centrifugation at 2000 rpm (1700*g*) for 2 min, the gel was suspended in 0.5 mL of DB. The affinity-purified pAb1/r (1.5 mg) obtained above, in 0.5 mL of DB, was incubated with 0.5 mL of papain gel at 37 °C in a shaker for 18 h. The reaction was terminated by addition of 1.5 mL of 0.01 M Tris-HCl, pH 7.5, and centrifuged to remove the papain gel. The supernatant solution was concentrated and then stored at −20 °C. Further purification of the Fab fragment of pAb1/r was achieved by passing the digested solution through a protein A column (2 mL size) according to the manufacturer's instructions (Rockford). The concentrated preparation was applied to the prepacked column and incubated at room temperature for 2 h. The Fab fragment (*ca*. 0.9 mg) was obtained by washing the column with 5 mL of 0.01 M PBS.

Production and Purification of Mouse Polyclonal Anti-idiotype Ab (pAb2). For generating pAb2, 8 female BALB/c mice (6−8 weeks of age) were immunized with the Fab fragment of pAb1 according to the protocols described by Kurpisz et al. (1988). Briefly, each mouse received 40 μg of Fab of pAb1, in 0.3 mL of PBS, via ip injection. Booster injections were made on days 14 and 28 with the same amount of immunogen. The mice were primed with 0.3 mL of pristane on days 3 and 17. On the 31st day after the initial injection, 6×10^6 cells of a nonsecreting myeloma cell NS-1 in 0.3 mL of DMEM were injected ip into each mouse. Two to three weeks after the last booster injection, the ascites fluid was collected, pooled, and centrifuged at 7000 rpm (5900*g*) for 5 min to remove cell debris. The cleared ascites fluid was either frozen at −20 °C or subjected to further purification by ammonium sulfate precipitation (50% saturation for the final solution) and affinity chromatography with a column conjugated with anti-MCYST-LR pAb1 (Chu et al., 1995). Bound antibodies were eluted from the column with 0.1 M glycine−HCl, pH 2.8, and neutralized immediately by addition of the appropriate amount of 1.0 M Tris-HCl, pH 9.2. The fractions containing specific antibodies were pooled, dialyzed against 0.01 M PBS (pH 7.5), and then stored at −20 °C.

Characterization of Anti-idiotype Antibody (pAb2/m) by Competitive Direct ELISA (cd-ELISA). One hundred microliters of idiotype pAb1 (8 μg/mL in PBS, pH 7.5) was coated onto each well of the microtiter plate. After overnight incubation at 4 °C, the wells were washed five times with 350 μL each of washing buffer (PBS-Tween; 0.01 M PBS, pH 7.5, with 0.5% Tween 20) and incubated with 0.1% BSA in PBS (0.01 M, pH 7.5, 170 μL per well) for 30 min. The wells were washed again and incubated with different concentrations of purified pAb2 (50 μL/well) together with 50 μL of MCYST-HRP (0.6 μg/mL) at 37 °C for 1 h. The wells were washed four times with PBS-Tween, and 0.1 mL of substrate OPD solution [10 mg of OPD + 13 μL of 30% hydrogen peroxide in 25 mL of 0.05 M citrate−phosphate buffer (4.8 g of citric acid and 7.1 g of Na_2HPO_4 in 500 mL of distilled water with pH adjusted to 5.0)] was added to each (Chu et al., 1990). After incubation at room temperature in the dark for 10 min, the reaction was terminated by adding 0.1 mL of 1 N HCl. Absorbance at 490 nm was determined in an automatic ELISA reader (THERMO/max microplate reader, Molecular Devices Co., Menlo Park, CA). Triplicate analyses were made.

Production and Purification of Rabbit Polyclonal Anti-anti-idiotype Ab (pAb3/r). The immunization schedule and methods of immunization were essentially the same as described previously (Chu and Ueno, 1977). In the initial immunization, two rabbits were each injected intradermally with 0.25 mg of affinity-purified MCYST-pAb2 in 0.6 mL of PBS (0.01 M, pH 7.5) emulsified with 0.6 mL of complete Freund's adjuvant by a multiple injection method (20−30 sites). Booster injections were made intramuscularly at the fourth week with 0.2 mg of immunogen in 0.6 mL of PBS and 0.6 mL of incomplete Freund's adjuvant at two sites in each thigh. The antisera collected were precipitated twice with $(NH_4)_2SO_4$ to a final saturation of 35%. The precipitates were reconstituted with distilled water to half of the original volume, dialyzed against distilled water for 1 h and then against 0.01 M PB, pH 7.5, overnight at 4 °C, and lyophilized. For pAb3 purification, 1 mL of $(NH_4)_2SO_4$ precipitation-purified and dialyzed antiserum was applied to an affinity column conjugated with MCYST-LR, the same way as in the purification of pAb1.

Characterization of Rabbit Polyclonal Anti-anti-idiotype Ab (pAb3/r). A cd-ELISA similar to the pAb1/r-based regular ELISA was used to characterize pAb3/r (Chu et al., 1990). Instead of coating with pAb1/r, each well was coated with 100 μL of pAb3/r (3.5 μg/mL in PBS, pH 7.5). After incubation at 4 °C overnight and washing, 0.1% BSA in PBS was added to each well for 30 min. Subsequent washing and incubation steps were the same as described above except that in the reaction step, the wells were each incubated with 50 μL of different concentrations of MCYST or nodularin variants and 50 μL of MCYST−HRP conjugate.

Analysis of MCYST-LR in Naturally Contaminated Samples with Idiotype Antibody (pAb1/r)-Based and Anti-anti-idiotype Antibody (pAb3/r)-Based ELISA. Three algal samples obtained from Lake Mendota (Madison, WI), 10 algal samples and 3 water samples obtained from Lake Winnebago (Oshkosh, WI), and 3 liver samples from sea otter supplied by Dr. Milton R. Smith (U.S. Department of the Interior, National Biological Service National Wildlife Health

Center, Madison, WI) were tested for the efficacy of pAb3-based ELISA for MCYST-LR. Algae and water samples were diluted with 0.01 M PBS to proper concentrations before they were subjected to cd-ELISA. Liver samples were subjected to a cleanup procedure before ELISA. Briefly, 7.5 g of liver was ground with 15 mL of 0.1 N NH_4HCO_3 in a Waring blender for 2 min and then centrifuged (17000*g*) at 4 °C for 20 min. The pH of the supernatant fluid was adjusted to 7.0 with 0.1 N HCl, and 1.5 mL of the solution was aspirated into a 15 mL test tube to which 5 mL of distilled water was added. The mixture was then added to a C_{18} reversed-phase Sep-Pak cartridge (Waters Associates/Millipore, Milford, MA) that had been sequentially washed with 5 mL of methanol and 10 mL of distilled water. The cartridge was then washed with 5 mL of distilled water. Microcystins were eluted from the column with 5 mL of CH_3OH/H_2O (60/40 v/v) and then diluted with PBS for cd-ELISA as described above, except that K-B substrate solution was used instead of OPD solution.

Protein Phosphatase Inhibition Assay. Protein phosphatase 1 (PP1), the catalytic subunit of rabbit skeletal muscle PP1 as expressed in *Escherichia coli*, was used in the following study (Zhang et al., 1992). Inhibition assay for MCYST-LR was conducted according to the protocols described by An and Carmichael (1994). PP1 activity was determined by measuring the rate of liberation of *p*-nitrophenol from substrate *p*-nitrophenol phosphate (*p*NPP) at 37 °C in the microtiter plate with an ELISA reader as described above. The reaction mixture (60 μL per well per assay) contained 0.4 μg of PP1, 5 mM *p*NPP, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, 50 mM Tris-HCl, pH 8.0, and BSA at a level of 0.5 mg/mL. To test the effect of MCYST-LR and pAb2 on PP1 activity, various concentrations of MCYST-LR or affinity-purifed pAb2 were present in the 60 μL incubation mixture. Triplicate analyses were made.

Effect of pAb1/r and pAb3/r on the Inhibitory Effect of MCYST-LR on PP1. An *in vitro* assay system for PP1 was used to test the effect of pAb1/r and pAb3/r on the inhibitory effect of MCYST-LR on protein phosphatase activity. To avoid the interference of phosphate ions on the assay, the antibodies were dialyzed against distilled water overnight in a cold room before the assay. pAb1/r or affinity-purified pAb3/r at different concentrations were preincubated with MCYST-LR at room temperature for 45 min using the same incubation mixture as described above except in the absence of enzyme and substrate. PP1 and substrate *p*NPP were added in sequence to the incubation mixture to start the assay.

RESULTS

Production and Characterization of Anti-idiotype Antibodies (pAb2/m) in Mouse. Anti-idiotype antibodies (pAb2/m) were produced in the ascites of BALB/c mice immunized with affinity-purified Fab fragment of rabbit pAb1/r. The pAb2 were further purified by ammonium sulfate precipitation and pAb1/r affinity purification steps. A cd-ELISA was used to determine whether the mouse pAb2/m has an epitope that resembles MCYST-LR for its binding to pAb1. Figure 1 shows that pAb2/m indeed can compete with MCYST-LR−HRP conjugate for its binding with the solid-phase pAb1. However, a large amount of pAb2/m was needed for such competition. The concentrations causing 50% inhibition (IC_{50}) of binding of MCYST−HRP conjugate to the solid-phase pAb1/r by free MCYST-LR and pAb2/m were found to be 1.4 and 157 nM, respectively. Attempts to use pAb2/m-coated ELISA plates and pAb1/r as the antibody for an indirect competitive ELISA of MCYST were unsuccessful.

Production and Characterization of Anti-anti-idiotype Antibodies (pAb3/r) in Rabbits. pAb3/r were generated in two rabbits after initial immunization and one booster injection (fourth week) with affinity-purified pAb2/m. Serum from the seventh week bleeding was further purified and characterized. The pAb3/r were purified by ammonium sulfate precipitation and MCYST-LR affinity column steps and then analyzed by cd-ELISA using OPD as the horseradish peroxidase substrate. Figure 2 shows that pAb3 have characteristics similar to those of pAb1/r, with high specificity for MCYST-LR. The IC_{50} values of binding of MCYST-LR−HRP to the solid phase pAb1/r and affinity-purified pAb3/r by MCYST-LR were 1.9 and 0.66 ng/mL, respectively.

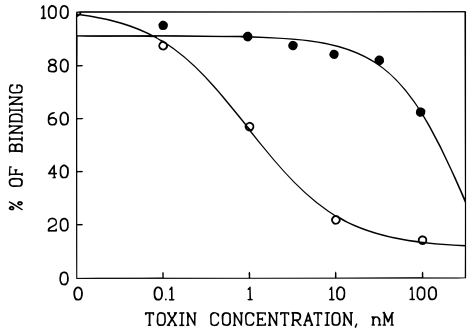

Figure 1. Inhibition of the binding of MCYST-LR−HRP to solid phase pAb1/r by MCYST-LR (○) or pAb2/m (●). In this assay, microtiter plate wells were coated with 100 μL of idiotype pAb1/r (8 μg/mL) and then reacted with a constant amount of MCYST-LR−HRP (0.6 μg/mL, 50 μL/well) together with either various concentrations of pAb2/m (0.02−28.6 μg/mL) or free MCYST-LR (0.01−100 ng/mL, 50 μL/well). On a molar basis, the concentrations causing 50% inhibition by free toxin and pAb2/m were 1.4 and 157 nM, respectively.

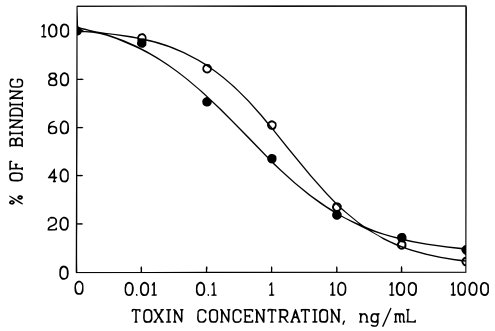

Figure 2. Effect of MCYST-LR on the binding of MCYST-LR−HRP to solid phase pAb3/r (●) or pAb1/r (○) in a cd-ELISA. The microtiter plate wells (100 μL per well) were coated with either pAb3 (3.5 μg/mL) or pAb1/r (8 μg/mL) and then reacted with a constant amount of MCYST-LR−HRP (0.6 μg/mL, 50 μL/well) together with various concentrations of MCYST-LR (0.01−1000 ng/mL, 50 μL/well). The IC_{50} values of binding of MCYST-LR−HRP to the solid phase pAb1/r and pAb3/r by MCYST-LR were 1.9 and 0.66 ng/mL, respectively.

Results for the cross-reactivities of various MCYST variants with pAb3/r are shown in Figure 3. Like pAb1/r, pAb3/r also cross-react with different MCYST analogues. The antibodies were most reactive with MCYST-LR and less so with MCYST-RR, MCYST-YR, and NODLN; the IC_{50} values for the binding of MCYST-LR−HRP to the solid-phase pAb3/r by MCYST-LR, MCYST-RR, MCYST-YR, and nodularin were 0.7, 24.4, 19.2, and 24.3 ng/mL, respectively.

Analysis of MCYST in Blue-Green Algae by pAb3/r-Based cd-ELISA. To test the efficacy of pAb3/r-based cd-ELISA for MCYST, 19 samples of algae, liver, and water were subjected to both pAb3/r-based and pAb1/r-based cd-ELISA. Results of these analyses are shown in Table 1. The correlation coefficient of a linear regression between pAb3/r-based cd-ELISA and pAb1/r-based cd-ELISA data was 0.88 ($y = 0.85x + 102$ ppb, $p < 0.0001$; y = pAb1-based ELISA; x = pAb3-based

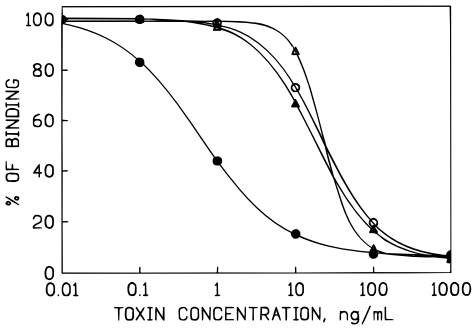

Figure 3. Cross-reactivity of pAb3/r with different MCYST variants in a cd-ELISA. The microtiter plate wells were coated with 100 μL of pAb3/r (3.5 μg/mL); MCYST-LR−HRP (0.03 μg/well) was used as the marker enzyme. Fifty microliters of toxin was used in each assay. The concentrations causing 50% inhibition of binding of MCYST-LR−HRP to the antibodies by MCYST-LR (●), MCYST-RR (○), MCYST-YR (▲), and NODLN (△) in the ELISA were 0.7, 24.4, 19.2, and 24.3 ng/mL, respectively.

Table 1. Comparison of MCYST-LR Levels in Naturally Contaminated Samples Analyzed by pAb1-Based and pAb3-Based cd-ELISA

	concn of microcystin (ng/mL)	
sample	Ab3-based ELISA	Ab1-based ELISA
1. algae 1[a]	11.3	124.5
2. algae 2	193.6	735
3. algae 3	21.6	129.2
4. algae 5	26.5	50.2
5. algae 6	3.8	46.4
6. algae 7	948.7	627.4
7. algae 575	920.7	842.9
8. algae 11	710.7	799.4
9. algae 12	97.7	142.1
10. algae 15	920.5	792.7
11. algae 18	467.3	343.2
12. algae 19	1096.9	1397
13. algae 20	857.5	735.3
14. water 1	ND[b]	ND
15. water 2	ND	ND
16. water 3	ND	ND
17. liver 1	1.7	ND
18. liver 2	1.4	6.7
19. liver 3	ND	ND

[a] Samples 1−3 and 4−13 were obtained from Lake Mendota and Lake Winnebago, respectively. Sea otter liver samples were supplied by Dr. Smith (see text). [b] ND, nondetectable.

ELISA). A good correlation was observed between the data obtained from pAb3/r-based and pAb1/r-based ELISA, although we find that the toxin values obtained from pAb3/r-based ELISA are usually lower than those from pAb1/r-based ELISA.

Inhibitory Effect of MCYST-LR and pAb2/m on Recombinant PP1 *in Vitro*. Results for the inhibitory effect of MCYST-LR and pAb2/m on PP1 using *p*NPP as substrate are shown in Figure 4. The dephosphorylation of the substrate by the recombinant PP1 was inhibited by MCYST-LR at various concentrations tested. pAb2/m generated in the present study could not mimic the inhibitory effect of MCYST-LR on PP1.

Effect of pAb1/r and pAb3/r on the Inhibitory Effect of MCYST-LR. To test whether the pAb1/r and pAb3/r can neutralize the toxicity of MCYST-LR, a quantitative assessment of the effect of MCYST-LR on the PP1 in the presence and absence of pAb1/r and pAb3/r was carried out *in vitro*. Results shown in Figure 5 indicate that about 80% of PP1 activity was restored when the reaction mixture containing 8 nM MCYST-LR was preincubated with 4.2 μM anti-MCYST-LR pAb1 or 45 μM pAb3/r, respectively. pAb3/r obtained from rabbit immunized with pAb2/r as immunogen also could reverse the inhibitory effect of MCYST on PP1. However, pAb3/r appear to be less effective than pAb1/r.

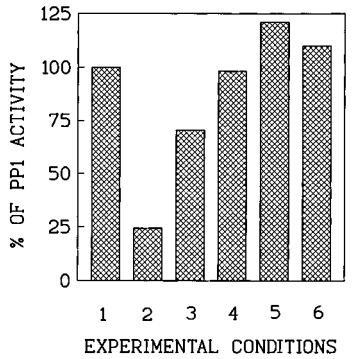

Figure 4. Effect of free MCYST-LR and pAb2/m on PP1 activity. Various concentrations of MCYST-LR and pAb2/m were preincubated with PP1 (0.4 μg/assay) in reaction buffer at room temperature for 10 min before the addition of substrate *p*NPP (final concentration of 5 mM) to start the reaction. Each assay volume was 60 μL. Experiments 1, 2, 3, and 4 contained 0, 15, 7.5, and 1.5 nM MCYST-LR, respectively. Experiments 5 and 6 contained 1 and 0.5 μM affinity-purified pAb2/m in the 60-μL incubation mixture.

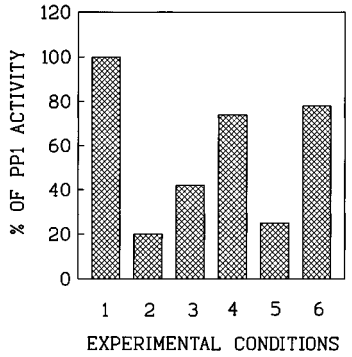

Figure 5. Neutralization of the inhibitory effect of MCYST-LR to PP1 by pAb3/r and pAb1/r. MCYST-LR (8 nM) was incubated with various concentrations of pAb3/r or pAb1/r in the incubation mixture at room temperature for 45 min, and then 0.4 μg of PP1 in 10 μL of enzyme dilution buffer (50 mM Tris-HCl at pH 7.4, 1 mM $MnCl_2$, 2 mM dithiothreitol, 1 mg of BSA/mL) was added. The substrate *p*NPP was added 10 min later to start the reaction. All of the experiments contained 8 nM MCYST-LR in the reaction mixture except the control (experiment 1). Experiments 1 and 2 contained no antibodies. Experiments 3 and 4 contained 22.5 and 45 μM pAb3/r, respectively. Experiments 5 and 6 contained 0.42 and 4.2 μM pAb1/r, respectively.

DISCUSSION

Recent development for the wide use of immunoassays for many low molecular weight haptens, including mycotoxins, phycotoxins, and many other agricultural and environmental toxicants, has led to increased demand for immunochemical reagents. Anti-idiotype antibodies provide an alternative approach to obtain these reagents. In the present study, we have demonstrated that anti-idiotype antibodies for MCYST-LR can be elicited in mice by immunization with affinity-purified pAb1/r against MCYST-LR. Data obtained from ELISA showed that the pAb2/m generated are able to compete for the binding of MCYST−HRP conjugates to idiotype antibodies. In addition, the purified Fab of pAb2/m was shown to be a good immunogen in generat-

ing pAb3/r, which were subsequently demonstrated to be useful for analytical purposes. These data suggest that some population of the generated, purified pAb2/m has an internal image of MCYST-LR.

Although data obtained from cd-ELISA indicated that the pAb2/m inhibited the binding of MCYST-LR to the original idiotype antibodies (pAb1/r), the apparent affinity of the purified pAb2/m for pAb1/r was about 112 times less than the binding of MCYST-LR to the pAb1/r. On a molar basis, IC_{50} values for the binding of MCYST-LR−HRP to pAb1/r by the MCYST-LR and pAb2/m were 1.4 and 157 nM, respectively. These data are somewhat suprising because anti-idiotype antibodies are generally of very high affinity and hence the lower IC_{50} values. However, Jerne (1974) postulated that the anti-idiotype antibodies (Ab2) generated from idiotype antibodies represent a heterogeneous population, including Ab2α, Ab2β, and Ab2γ of which only Ab2β bears an internal image of the orignial immunogen. Thus, it is possible that only a small pAb2/m subpopulation generated in the present study is the Ab2β type. Assuming that pAb2/m obtained from the present study have affinity for the pAb1/r similar to that of MCYST-LR, the IC_{50} data suggest that only about 1% of the population was Ab2β type. This observation is similar to some data obtained from studies of the anti-idiotype antibodies for several other hapten-type toxins (Chanh et al., 1992; Hsu and Chu, 1995).

The pAb2/m generated in the present study could not mimic the biological function of MCYST-LR in a colorimetric PP1 assay. However, when the rabbit pAb2 (pAb2/r) generated from a monoclonal antibody (mAb1/m) that has a higher affinity for MCYST-LR (about 20 times higher than pAb1/r used in the present study; Huang and Chu, 1996) was tested, an inhibition of PP1 activity was demonstrated (B. H. Liu and F. S. Chu, unpublished observations, 1996). Thus, idiotype antibodies (Ab1) with high affinity to the original immunogen may be necessary for the generation of Ab2 showing detectable biological function. Nevertheless, even using the new antibodies, as much as 2.5 μM rabbit pAb2 was still required to give the same inhibitory effect on PP1 as 15 nM MCYST-LR (Liu and Chu, unpublished observations, 1996). In the case of okadaic acid (OA), another potent protein phosphatase inhibitor, Shestowsky et al. (1993) demonstrated that the anti-anti-idiotype antibodies raised against anti-OA monoclonal antibodies were able to inhibit PP1 and PP2A catalytic subunits in a phophatase radioassay. OA inhibited PP1 and PP2A catalytic activity effectively at concentrations as low as 19 and 0.2 nM, respectively, but the IC_{50} values for inhibition of PP1 and PP2A by anti-idiotype antibodies (Ab2) were 1100 and 145 nM, respectively. These investigators concluded that Ab2 were not as potent as free OA for the inhibition of protein phosphatase. Because the pAb2 is approximately 150 times the size of MCYST-LR, steric or conformational hindrance may greatly affect the interaction between the pAb2 and the reaction site of PP1.

Recent studies have shown that there is covalent binding between MCYST-LR and PP1 after an initial noncovalent interaction (Moorhead et al., 1994; Robinson et al., 1991). Mackintosh et al. (1995) found that Cys^{273} of PP1 binds covalently to the methyldehydroalanine (Mdha) residue of MCYST and that the IC_{50} values for inhibition of the Cys^{273} mutants by MCYST increase 10−20-fold. These observations suggest that the covalent bond between MCYST and PP1 is critical for the inhibition of PP1 activity. While antibodies such as pAb2/m or pAb2/r could interact noncovalently with PP1 through competition with MCYST at the binding site, it is unlikely that they will bind covalently with PP1. The initial noncovalent binding between the pAb2 and the enzyme would be overwhelmed by the subsequent covalent interaction between the toxin and the enzyme.

Although pAb2/m appear to have little use in the immunoassay of microcystins at present, pAb2/m have been found to be good immunogens in generating pAb3 in rabbit. Thus, it is possible that Ab2/m could be developed as a vaccine. The sensitivity of the pAb3/r-based ELISA for MCYST was found to be comparable to or better than that of the original pAb1/r-based ELISA. However, we found that pAb3/r is highly specific for MCYST-LR. The relative cross-reactivities of pAb1/r to MCYST-LR, MCYST-YR, MCYST-RR, and NODLN were reported to be 100, 126, 65, and 48, respectively (Chu et al., 1989). In contrast, the relative reactivities of pAb3/r with MCYST-LR, MCYST-YR, MCYST-RR, and NODLN are found to be 100, 2.9, 3.6, and 2.9, respectively. These results could be due to the affinity chromatography purification of pAb3/r involved in the antibody preparation, a protocol that did not apply to the preparation of the pAb1/r in the previous study (Chu et al., 1989). A population of pAb3/r with high specificity for MCYST-LR was selected in this study.

To ascertain the feasibility of using the pAb3/r-based ELISA for analysis of MCYST-LR in naturally contaminated samples, 19 samples of algae, liver, and water were analyzed. With the exception of a few samples, data obtained from the pAb3/r-based ELISA were consistent with those obtained from the pAb1/r-based ELISA. Because the pAb3/r are highly specific for MCYST-LR, inconsistent data for several samples were likely due to the presence of other MCYST variants in the samples. Both affinity-purified pAb3/r and pAb1/r were able to neutralize MCYST-LR's inhibitory effect on PP1 *in vitro*, but the amount needed for such an effect by pAb3/r was 10 times more than that of pAb1/r. In addition, affinity purification of pAb3/r was necessary for the cd-ELISA. In conclusion, although the present studies have demonstrated the feasibility and methodology of producing anti-idiotype and anti-anti-idiotype antibodies against MCYST, to render useful pA2/m and pAb3/r for more practical applications, further studies are warranted to generate antibodies with high affinity to MCYST, including monoclonal antibodies.

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