Kinetics of Distribution of Microcystin LR in Serum and Liver Cytosol of Mice: An Immunochemical Analysis

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The kinetics of distribution of microcystin LR (MCYST-LR) in the serum and liver of mice dosed with the toxin (35 μg/kg) was analyzed by a direct competitive ELISA that has an analytical recovery of 67–109.9% of the toxin added to serum and liver cytosol (1–100 ng/mL). MCYST-LR was detected in serum (7.8 ng/mL) and liver (45 ng/mL) 15 min postinjection and peaked at 2 h (62.7 ng/mL) in serum and at 12 h (250.5 ng/mL) in liver cytosol. A considerable amount of toxin was still found in serum (37.5 ng/mL) and cytosol (228.6 ng/mL) at 24 h postinjection. Between 15 min and 24 h, approximately 4.4–35.2% and 16–89.5% of the injected MCYST-LR was found in serum and liver cytosol, respectively. Reduced [^{3}H]MCYST-LR was used simultaneously with the ELISA analysis. An excellent correlation ($r = 0.99$ at $p < 0.0001$) between data obtained from both methods was found. The presence of MCYST-LR in liver cytosol was also confirmed by immunochromatography, which detected 98% of toxin injected into the HPLC column. An immunoreactive peak (shoulder) was also found. In vitro analysis of protein phosphatase 2A activity of liver cytosol revealed that the enzyme activity started to decrease at 1–2 h postinjection. Maximum decrease in enzyme activity occurred at 6–12 h after the animal received the toxin.

INTRODUCTION

Microcystins (MCYSTs) are a group of cyclic heptapeptide hepatotoxins produced by several species of blue-green algae such as *Anabaena flos-aquae, Microcystis aeruginosa*, and *Oscillatoria agardhii* (Botes et al., 1984; Carmichael, 1986). Microcystin LR (MCYST-LR), the leucine–arginine (LR) variant of the cyclic peptide, is the major toxin in this group and the most toxic, with a LD_{50} (ip) of approximately 50 μg/kg in mice (Botes et al., 1984; Carmichael, 1986, 1988; Carmichael et al., 1988a,b). This group of phycotoxins has been shown to cause intermittent poisoning to wild and domestic animals and liver damage, gastroenteritis, diarrhea, and dermatitis in humans (Gorham and Carmichael, 1988). Recent studies demonstrated that MCYST-LR is a potent inhibitor of protein-phosphatases 1 and 2A (PP1 and PP2A) both in vitro and in vivo (Honkanen et al., 1990; Ichinose et al., 1990; MacKintosh et al., 1990; Matsushima et al., 1990; Yoshizawa et al., 1990) as well as a potent tumor promoter in rats (Falconer, 1991; Nishiwaki-Matsushima et al., 1991, 1992). Since protein phosphorylation and dephosphorylation play an important role in cell regulation, the cancer-promoting activity of MCYST-LR was considered to be due to its inhibitory effect on the protein phosphatase.

Using radiolabeled MCSYTs, including ^{125}I-labeled MCYST-YM (Falconer, 1986), ^{14}C-labeled MCYST-LR (Brooks and Codd, 1987), and [^{3}H]MCYST-LR (Pace et al., 1991; Robinson et al., 1989, 1991), the kinetics of disposition of the toxin in various organs and tissues of mice and rats has been investigated. The toxin was found to be tightly bound to the liver cytosol. However, correlation of the binding of the toxin to the cytosol with simultaneous decrease of PP1 or PP2A activities at sublethal doses of toxin has not been studied. The use of radioactive toxin in analysis also suffers from the disadvantage of being either not very sensitive, as in the case of ^{14}C, or not the original toxin, as in the case of ^{3}H- or ^{125}I-labeled toxin. With the availability of specific antibody against MCYST-LR as well as an effective enzyme-linked immunoassay for the toxin (Chu et al., 1989, 1990), the present study was carried out. The objectives of the present study were (a) to establish an effective ELISA protocol for monitoring MCYST-LR in serum and liver cytosol, (b) to study the kinetics of distribution of MCYST-LR and its metabolites in serum and liver cytosol by ELISA as well as by a radiotracer method and by combination of HPLC and ELISA methods (immunochromatography), and (c) to determine kinetically the effect of MCYST-LR on the liver protein phosphatase activity. Detailed various approaches and results obtained from the present study are reported herein.

MATERIALS AND METHODS

Materials. Microcystin LR, bovine serum albumin (BSA), and 30% hydrogen peroxide were obtained from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Specific polyclonal antibodies against MCYST-LR, tritiated reduced MCYST, and horseradish peroxidase (HRP)-labeled MCYST-LR were prepared as previously described (Chu et al., 1989, 1990). Tissue solubilizer (SOLVABLE) was purchased from DuPont (Boston, MA).

o-Phenylenediamine (OPD) was purchased from Idexx (Portland, ME). Microtiter ELISA plates were purchased from Nunc (Roskilde, Denmark). Precoated reversed-phase thin-layer chromatography plates (RPTLC, Si C_{18F}) were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Sep-Pak C_{18} reversed-phase cartridges were from Waters Associates (Milford, MA).

Animals. Male ICR mice (Harlan-Sprague-Dawley Inc., Madison, WI), weighing 20–27 g, were maintained under controlled temperatures (20–25 °C) and a 12-h light/dark cycle. The animals were allowed free access to Purina Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water. All mice were acclimatized for 3–5 days before experiments. Female albino rabbits were purchased from Smith's Rabbitry (Seymour, WI).

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Establishment of Sample Preparation Protocol. *Recovery of MCYST-LR Added to Blood.* For the recovery study, microcystin LR (MCYST-LR) was added to the whole blood (rabbit) at levels between 0.2 and 100 ng/mL and stirred in a cold room for 30 min. The blood samples were used directly or fractionated as follows: (a) separated into plasma and cell pellet by centrifugation at 5000*g* (Sorvall Model RC-5, DuPont) for 20 min and (b) separated in the cold room (6 °C) into serum and cell clot. Part of the plasma and serum samples were kept in the freezer (−20 °C) before analysis. Part of the blood samples were kept in the freezer overnight, defrosted, and centrifuged 5000*g* for 20 min to remove the cell debris.

One milliliter each of the above test samples was diluted 5-fold with distilled water. After adjustment to pH 3.0 with 0.1 N HCl, the sample was loaded to a C_{18} reversed-phase Sep-Pak cartridge that had been pretreated sequentially with 5 mL of methanol and 30 mL of distilled water. The cartridge was then washed with 15 mL of acidified distilled water (pH 3.0) and 5 mL of 20% MeOH, followed by elution with 5 mL of various concentrations of aqueous methanol solution (60–100% MeOH). The eluents were dried with air and reconstituted with 0.01 M phosphate buffer–saline (PBS) for ELISA analysis.

Recovery of MCYST-LR Added to Liver Cytosol. Similar to the analysis of blood and serum, hepatic cytosol from mouse was subjected to a Sep-Pak C_{18} cartridge to remove interference before analysis. Microcystin-LR was added to the cytosol preparation at concentrations between 1 and 50 ppb for this test. In general, 6 mL of diluted sample (1 mL of hepatic cytosol plus 5 mL of distilled water) with pH adjusted to 3.0 were loaded onto the Sep-Pak cartridge that had been previously washed with methanol (MeOH) and distilled water. After a wash with 20 mL of distilled H_2O (pH 3.0), the cartridge was washed with 5 mL each of 20, 75, and 100% methanol. The concentration of MCYST-LR in each of these fractions was analyzed by ELISA and HPLC.

Kinetic Distribution of MCYST-LR and Radiolabeled Reduced MCYST-LR. Two groups of male ICR mice (total 36 mice) were intraperitoneally (ip) injected either with 35 μg/kg of body weight of MCYST-LR (group A) or with 35 μg/kg of MCYST-LR plus 3×10^5 disintegrations per min (dpm) of purified [^{3}H]MCYST-LR (group B). Two mice from each group were anesthetized with ether and then killed at the following intervals: 0, 1, 15, 30, and 45 min and 1, 2, 6, 12, and 24 h postinjection. A minimum of 900 μL of blood was drawn from each mouse via heart puncture using a 22G needle and 2.5-mL syringe. Serum was separated from the blood by centrifugation at 3000*g* for 20 min in an Eppendorf microcentrifuge and frozen for subsequent tests. Livers (weight 0.7–0.72 g/mouse) were removed, placed in an ice-cold phosphate buffer (pH 7.4) bath, and homogenized with a Potter–Elvehjem tissue homogenizer in 5 mL of buffer A (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 22 mM EGTA, 2 mM 2-mercaptoethanol, 10% glycerol, and 0.25 M sucrose) at 0–4 °C. The liver slurry was then centrifuged at 100000*g* for 5 h. The supernatant fluids were separated into small portions (300 μL/vial) and kept frozen at −70 °C for subsequent tests. Serum and liver cytosol samples from two control mice (received no toxin) were also collected.

Quantitation of [^{3}H]MCYST-LR in Serum and Liver. In the radiotracer study, serum and liver cytosol collected from the [^{3}H]MCYST-LR-treated mice were digested with tissue solubilizer before scintillation counting. In general, 60–500 μL of sample was incubated with an equal volume of tissue solubilizer at 50 °C in a shaking water bath for 1 h. After reaction, 0.1 mL of 100 mM EDTA and 0.3 mL of 30% H_2O_2 were added and incubated at 50 °C for another hour to decolorize the sample. After cooling, 10–15 mL of cocktail (Ready solv, Beckman) was added for radioactivity counting. The radioactivity was counted in a Beckman Model LS-5801 liquid scintillation spectrometer. All of the counts were corrected to disintegrations per min (dpm) by a channel ratio method by using known standards of tritiated toluene.

Direct Competitive ELISA. The protocol for the direct ELISA was essentially the same as that described by Chu et al. (1989). Polyclonal antibody against MCYST-LR was diluted (1:1000) with 0.01 M phosphate buffer saline (PBS). Each well of a 96-well microtiter plate was coated with 100 μL of diluted polyclonal antibody. After incubation at 4 °C overnight, the plate was washed with PBS–Tween (0.05% Tween 20 in PBS, 350 μL/well), incubated with BSA–PBS (0.01% BSA in PBS, 200 μL/well) at 37 °C for 30 min, followed by washing with PBS–Tween four times. Standard MCYST-LR (50 μL/well) at different concentrations, PBS, or sample solution together with MCYST-LR–HRP conjugate (50 μL/well) was then added and incubated at 37 °C for 60 min. After the plate was washed with PBS–Tween four times, 100 μL of freshly prepared OPD substrate solution (40 mg of OPD and 0.04 mL of 30% H_2O_2 in 100 mL of substrate buffer, i.e., 0.05 M citric acid plus 0.1 M Na_2HPO_4 at pH 5.0) was added, and plates were incubated at room temperature for 15–20 min. The reaction was stopped by addition of 100 μL of 1 N HCl to each well. The absorbance at 490 nm was determined in a microplate reader (Thermomax, Molecular Devices, Menlo Park, CA).

High-Performance Liquid Chromatography (HPLC). Following C_{18} reversed- phase Sep-Pak cartridge cleanup, limited samples of the 75% methanolic eluate were subjected to HPLC using a Spherical C_{18} reversed-phase column (5 μm, 3.9 mm × 15 cm, Waters Associates) under the conditions previously described (Chu et al., 1989). A Beckman Model 100A HPLC system equipped with a Model 421A pump and a Waters Associates Model 440 detector was used. The column was run isocratically at a flow rate of 1 mL/min with a solvent system of H_2O/CH_3CN (25/75 v/v) containing 0.1% trifluoroacetic acid. The elution pattern was obtained by measuring absorbance at 254 nm. In general, 50 μL of sample in methanol was injected onto the column. Quantitation was done by comparing the peak areas of the unknowns with a standard curve, which was established by injecting various concentrations of MCYST-LR (50–250 ng/injection) onto the column.

Immunochromatography. For immunochromatography, fractions (0.5 mL each) collected from HPLC separation were tested with direct competitive ELISA. By comparison of the MCYST-LR standard, the concentration of MCYST-LR could be estimated directly from the standard curve. Quantitation could also be done by comparing the peak area of the immunochromatogram of the unknown and the peak area of the immunochromatogram of the standard.

Analysis of Protein Phosphatase 2A Activity in Liver Cytosol. The protein phosphatase 2A activity of hepatic cytosol of mice from each time point was determined according to the method described previously (Lin and Chu, 1994). Briefly, 5 μL of cytosolic fraction diluted 10^1–10^4-fold with buffer A was incubated with 1 μg of ^{32}P-labeled substrate (histone H1) at room temperature for 1 h. The incubation mixture (60 μL) was then subjected to SDS–PAGE to separate the phosphoprotein. The radiolabeled protein band was detected by autoradiography. After the gel was sliced, the radioactivity in the ^{32}P-labeled protein band was determined. The PP2A activity in the liver cytosols was determined by comparing the differences between radioactivity in the substrate in the absence of PP2A (cytosol) and radioactivity of the substrate with the enzyme (cytosol).

RESULTS

Establishment of ELISA Protocol for the Analysis of MCYST-LR in Serum and Liver Cytosol. *Standard Curve for ELISA of MCYST.* A standard curve for direct competitive ELISA of MCYST-LR is shown in Figure 1. The percent of binding of MCYST-LR in the samples to the solid-phase antibody was calculated from the following formula: % of binding = $(A_s/A_o) \times 100$, where A_s and A_o are the absorbances of the sample and blank well (no toxin added), respectively. Under the experimental conditions, the A_o values for the blank were generally about 0.9–1.2. The standard curve fits a typical competitive curve (sigmoid type) with a 50% inhibition at 0.68 ng of MCYST/mL and a linear response of inhibition in the range 0.1–1 ng/mL (5–50 pg/assay). Assuming the concentration of MCYST causes a 10–15% inhibition of binding of MCYST-HRP to the coated antibody as the minimum detection level, the minimum detection level for MCYST-LR in the ELISA would then be about 10 pg/assay (0.2 ng/mL).

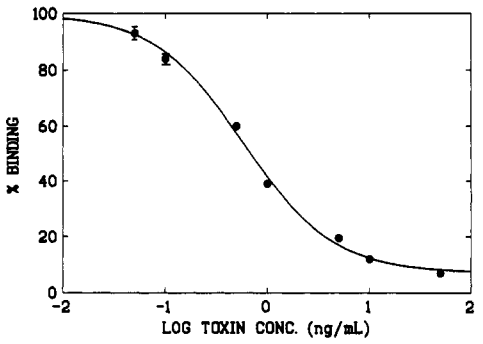

Figure 1. Standard curve for direct competitive ELISA of MCYST-LR in PBS. MCYST-LR-HRP was used in the direct competitive ELISA. Fifty microliter portions of different concentrations of standard MCYST-LR in PBS were used in each assay. All data were obtained from the average of three sets of experiments. The percent of binding was calculated from the formula of $(As/A_0) \times 100$, where A_s and A_0 are the absorbance of the sample and blank well (no toxin added), respectively. A_0 was 1.001. MCYST-LR concentrations (0.05–50 ng/mL) shown on the x-axis are in log scale.

Treatment of Blood with Reversed-Phase Sep-Pak C_{18} Cartridge. Before analysis of the sample, the effect of control blood on the ELISA was tested. Considerable amounts of interfering materials were found in the blood sample, as indicated by the the high absorbance readings. With no cleanup, the percent of binding of the control blood samples was between 118 and 139 for 1:100 and 1:2 dilutions. However, the interfering materials were removed by treatment of the blood with a reversed-phase Sep-Pak C_{18} cartridge. Most of the interfering materials were removed after the cartridge was washed with water and 20% methanol. The percent of binding for the materials eluted from the cartidge for the control sample with 75% MeOH at 1:2, 1:10, 1:100, and 1:1000 dilutions were found to be 79, 89, 92, and 97%, respectively.

Analytical Recovery of MCYST-LR Added to Different Blood Fractions. Results of the analytical recovery of MCYST-LR added to blood by the direct ELISA are given in Table 1. There was a significant loss of MCYST after storage of the whole blood samples in the freezer, defrosting, and centrifugation; only 26–74.1% of MCYST-LR was found in the blood samples. Such loss is probably due to the association of MCYST with cellular components of blood which were later removed by centrifugation. However, good recovery was found for plasma and serum samples; 67–109% and 63.1–103.5% of the added MCYST-LR were found in the plasma and serum fraction samples, respectively. These data suggest that serum or plasma samples should be used for the analysis of MCYST-LR in blood. To avoid any interaction of MCYST-LR with cellular components, the blood sample should be centrifuged immediately after collection from the experimental animal to separate the serum or plasma.

Quantitation of MCYST-LR in Liver Cytosol by Direct ELISA. Using the same protocol, the analytical recovery of MCYST-LR added to the liver cytosol was tested. Between 79 and 99% (10–100 ng/mL) of the added toxin was found (Table 1). The results indicated that the ELISA protocol used for serum samples could be applied to the detection of MCYST-LR in liver cytosol.

Kinetics of Distribution of MCYST-LR in Serum and Liver Cytosol. Results for the kinetics of appearance of MCYST-LR in serum and kinetics of accumulation in liver cytosol samples, as monitored by direct competitive ELISA, are shown in Figure 2. The uptake of the toxin in the serum was rapid. The toxin was detectable (7.8 ng/mL) 15 min postinjection, reached the maximum level (62.7 ng/mL) in serum at 2 h, and declined thereafter. Microcystin LR was still detectable (37.5 ng/mL) in the serum 24 h postinjection. Assuming a total of 4 mL of blood in a mouse, about 4.4–35.2% of the injected toxin was found in the serum samples over the period tested. A time-dependent appearance of MCYST in serum was observed during the first hour. Least-squares linear regression analysis of these data (inset of Figure 2) revealed that the toxin appeared in the serum at a rate of 0.87 ng mL^{-1} min^{-1}.

Data on the kinetics of accumulation of MCYST-LR in liver cytosol were consistent with the appearance of this toxin in serum (Figure 2). Although a detectable level of MCYST-LR (45 ng/mL) was found in liver cytosol as early as 15 min postinjection, accumulation of the toxin in the liver reached the maximum (250.5 ng/mL) at 12 h postinjection, which was about 10 h later than in serum. Since 2.5 mL of cytosol preparation was obtained from one liver of a mouse dosed with 700 ng of MCYST-LR, about 89% of injected toxin was found in the cytosolic fraction. At 24 h postinjection, about 71% of MCYST-LR was still detectable. Like the appearance of MCYST in serum, a time-dependent accumulation of the toxin in liver cytosol was also observed during the first hour. Least-squares linear regression analysis of these data (inset of Figure 2) revealed that the toxin accumulated in the liver cytosol at a rate of 3.08 ng mL^{-1} min^{-1} in the first hour.

Monitoring of MCYST-LR in Mouse Serum and Liver Cytosol by the Radiotracer Method. The kinetics of appearance of MCYST-LR in the mouse serum and liver cytosol were also estimated by radiolabeled reduced tritiated MCYST-LR (total injection 3×10^5 dpm/mouse). The results, expressed as radioactivity of [^{3}H]MCYST-LR, are shown in Figure 3. At 15 min postinjection, 4075.5 dpm/mL serum was detected. This would be equivalent to about 5.36% of the injected radiolabeled toxin as estimated from a total of 4 mL of serum in a mouse. The radioactivity reached the maximal level at 2 h and declined later. As much as 5.36–39.2% of the radiolabeled toxin was present in the serum sample.

The uptake of [^{3}H]MCYST-LR in liver was detected at 15 min and increased significantly during the first 60 min. The maximal level was observed at 12 h and then leveled off. At 60 min, the average amount of radioactivity in liver cytosol was 70 356 dpm/mL cytosol, which is equivalent to 58% of the total injected reduced tritiated MCYST-LR (total 1.76×10^5 dpm in 2.5 mL of cytosol). At maximal accumulation time (12 h), the radioactivity was 106 092 dpm/mL cytosol in liver. In the time periods tested, about 17–88% of the injected reduced [^{3}H]MCYST-LR appeared in the cytosol. Again, a time-dependent appearance/accumulation of the radioactivity in serum and liver cytosol was observed during the first hour. Least-squares linear regression analysis of these data (inset of Figure 3) revealed that the radioactivity appeared in the serum and liver cytosol at rates of 369 and 1400 dpm mL^{-1} min^{-1}, respectively.

The correlation of the data obtained from the ELISA and radiotracer methods, as shown in Figure 4, indicated that there was no significant difference between the data obtained from both methods for the uptake of MCYST-LR. At each tested time interval, the correlation coefficient for data obtained at each time interval for both methods of serum and liver cytosol samples was 0.99 at $p < 0.0001$.

HPLC Analysis of MCYST-LR in Hepatic Cytosolic Fraction. Immunochromatography, an approach to identify immunoreactive compounds, involves using an immunochemical method (such as ELISA) to quantify the

Table 1. Analytical Recovery of MCYST-LR Added to Whole Blood and Liver Cytosol by Direct Competitive ELISA

MCYST-LR added (ng/mL)	plasma		serum		whole blood		liver cytosol	
	%	SD	%	SD	%	SD	%	SD
0.2	169 (10.4)[a]	0.06	N/A[b]		79 (0.02)	5.2	N/A	
1	67.4 (5.7)	0.01	63.1 (2.8)	0.08	41.6 (2.6)	0.08	N/A	
5	82.4 (2.9)	0.03	84.8 (17.1)	0.2	26.8 (5.7)	0.2	N/A	
10	88.8 (6.3)	0.02	80.2 (4.7)	0.14	26.5 (6.8)	0.3	79 (0.02)	5.2
50	N/A		N/A		N/A		97.4 (0.22)	4.3
100	109.9 (4.6)	0.07	103.5 (7.5)	0.02	74.1 (18.8)	0.3	98.9 (0.11)	2.3

[a] Values in parentheses represent coefficient of variation (CV) (%). [b] N/A, not analyzed.

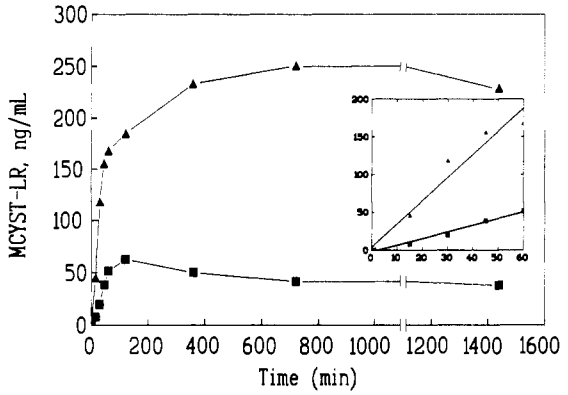

Figure 2. Kinetics of appearance of MCYST-LR in mouse serum and liver cytosol. Mouse serum and liver cytosol samples were analyzed for MCYST-LR with a direct competitive ELISA after C_{18} reversed-phase cartridge cleanup. The concentrations of samples at each time point were plotted as ng/mL vs time (min). The square and triangle represent serum (■) and liver cytosol (▲), respectively. The inset represents data from the first hour as analyzed by least-squares linear regression. The legends in the insets are the same as in the main panel. Values for slope, p, and r (correlation coefficient) for cytosol and serum are 3.08, 0.0006, and 0.9797 and 0.87, 0.0001, and 0.9915, respectively.

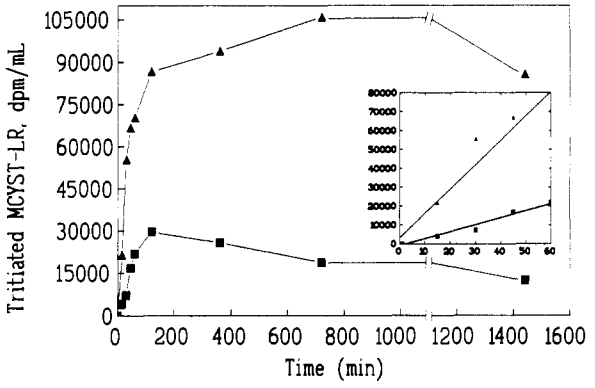

Figure 3. Kinetics of appearance of radioactivity in mouse serum and liver cytosol after receiving [^{3}H]MCYST-LR. Mouse serum and liver cytosol samples were digested with tissue solublizer before scintillation counting. The levels of MCYST-LR in the samples at each time point were plotted as dpm/mL vs time. The square and triangle represent serum (■) and liver cytosol (▲), respectively. The inset represents data from the first hour as analyzed by least-squares linear regression. The legends in the insets are the same as in the main panel. Values for slope, p, and r (correlation coefficient) for cytosol and serum are 1390, 0.0016, and 0.9668 and 369, 0.0003, and 0.9861, respectively.

unknowns after separation by chromatography. This approach has advantages of both methods. In addition, it can also identify new immunoreactive compounds and metabolites. A typical immunochromatogram of a liver cytosol sample from a mouse that received MCYST-LR is shown in Figure 5. In this experiment, 50 μL of a 12-h-postinjection mouse liver cytosol sample (cleaned up by C_{18} Sep-Pak) was injected into the HPLC column. The cytosol sample showed a small peak (about 0.005 absorbance unit at 254 nm) with the same retention time as the MCYST-LR standard (retention time 11.5 min, data not shown). However, because there was a large amount of impurity present in the sample, it was very difficult to estimate the peak areas (curve A, Figure 5). Nevertheless, when the collected fractions were analyzed by direct ELISA

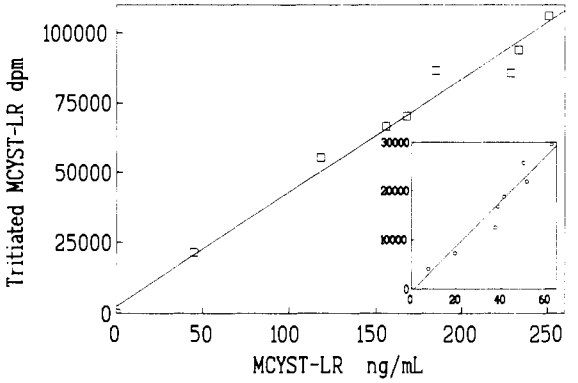

Figure 4. Correlation of data obtained from ELISA and radiotracer. The data obtained from ELISA and radiotracer at each time point were calculated to determine the correlation relationship by computer program, Inplot4 (Graphpad). The data had no significant difference ($r = 0.99$ at $p < 0.0001$). The main graph is the correlation of cytosol samples; the inset is the correlation of serum samples.

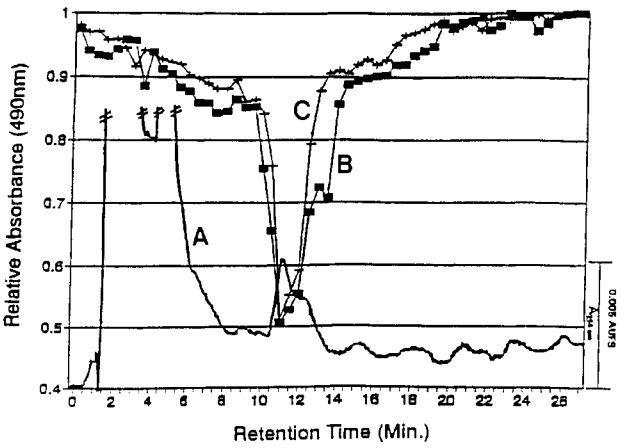

Figure 5. Immunochromatography of mouse liver cytosol. Regular HPLC elution pattern (positive peaks, absorbance at 254 nm) and ELISA chromatogram of sample (negative peaks, absorbance at 490 nm) and ELISA chromatogram of a standard MCYST-LR (negative peak) are shown in curves A, B, and C, respectively. Either 50 μL of mouse liver cytosol (12-h postinjection sample, equivalent to a total of 241.5 ng of MCYST-LR), which had been subjected to cleanup by C_{18} cartridge, or 50 μL of standard MCYST-LR (250 ng total) was injected into the HPLC column. The HPLC elution pattern was obtained by monitoring the absorbance at 254 nm. The immunochromatograms were obtained by plotting the relative A (490 nm), blank (PBS, $A_{max} = 0.889$) of ELISA. Fractions, collected every 0.5 min, were analyzed by direct competitive ELISA. The solid squares (■, B) represent liver cytosol and the crosses (+, C) standard MCYST-LR.

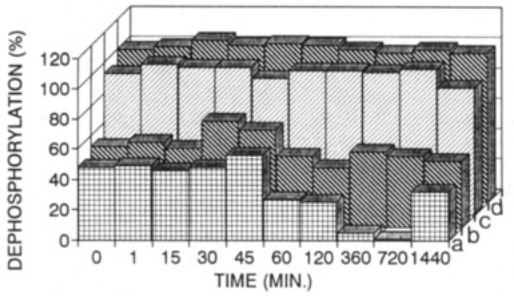

Figure 6. Protein phosphatase 2A activity in liver cytosol of mice receiving MCYST-LR. Liver samples collected at different time intervals after injection with 35 μg/kg MCYST-LR were used for the preparation of cytosols. In general, 20 μL of liver cytosol (0.28 g/mL) at various dilutions (1:10 to $1:10^4$) was used in each assay. Each bar represents the average data from four experiments. A dose-dependent inhibition was difficult to observe at high concentrations of cytosol (1:10 and 1:100 dilutions). Inhibition of PP2A activity was detected in the samples 60 min after dosing when the assay was carried at cytosol dilution of $1:10^3$ and $1:10^4$. The control (receiving no toxin) is shown as zero time. At 12 h in $1:10^4$ dilution, the PP2A activity was near zero. The cytosol dilutions were 10^1-fold (row d), 10^2-fold (row c), 10^3-fold (row b), and 10^4-fold (row a) from back to front, respectively.

to give an immunochromatogram (curve B, Figure 5, or top negative peak; curve C, 250 ng of pure MCYST), even if the collected fractions were diluted 10 times before ELISA, the concentration of MCYST-LR could readily be determined. About 245 ng of MCYST-LR was detected in this peak when compared to the standard curve of MCYST-LR. Almost 97% of the injected MCYST-LR (concentration of MCYST-LR in the sample before HPLC was determined by ELISA) was found by the HPLC–ELISA methods. It is interesting to note that a shoulder immunoreactive peak with apparent retention time of 13.5 min was found in the cytosol.

Association of MCYST-LR with Protein Phosphatase 2A in Vivo in Mice Liver Cytosol. In vitro analysis of PP2A activity in the liver cytosol of an ICR mouse that had received MCYST-LR revealed that the enzyme was inhibited by microcystin LR when the cytosol was diluted 10^4 times (data not shown). At this dilution, the liver concentration was 28 μg/mL because 2.5 mL of the undiluted enzyme preparation was originally from one mouse liver weighing 0.7 g. A time course for the PP2A activity in mouse liver cytosol of mice that had been injected with 35 μg of MCYST-LR/kg of body weight was also carried out. The protein concentrations of the cytosol obtained from liver at different time intervals were very consistent. No significant differences of the total protein concentration for various samples were found. The absorbances at 280 nm of the diluted solution were within the range 0.28–0.31. In the assay, 20 μL of cytosol obtained from each time interval was diluted with buffer A from 1:10- to $1:10^4$-fold; 20 μL of each of the diluted samples was incubated with 1 μg of ^{32}P-labeled substrate at room temperature for 1 h. Results of the protein phosphatase 2A activity in mouse liver cytosol at different dilutions and various time intervals are given in Figure 6. Significant inhibitory effect (30% dephosphorylation at 60 min vs 50% at zero time) by MCYST was observed 60 min postinjection for cytosol diluted 10^4-fold (row a). The PP2A activity decreased to near zero for this dilution at 12 h postinjection. No such decrease in PP2A activity was found for the cytosol from a control mouse at this dilution. At 24 h, an increase of PP2A activity was observed; this might be due to the decrease in MCYST-LR in liver (catabolism). Inhibition was not observed when the crude enzyme was tested at 10^1 and 10^2 dilutions (rows c and d). This is probably due to a high concentration of total proteins that interfered with enzyme assay.

DISCUSSION

Previous investigations in our laboratory have led to a simple and sensitive direct competitive ELISA for the determination of MCYST-LR in water and algal blooms (Chu et al., 1989, 1990). The adaptation of the ELISA for the analysis of MCYST-LR in blood and liver cytosol was investigated in the present study. We found that both types of sample, after cleanup with a C_{18} reversed-phase cartridge and dilution with buffer, could be used directly in the ELISA. The method is simple, rapid, and sensitive. The effectiveness of this method was evident from good analytical recovery of MCYST-LR added to blank serum (63–103%) and blank liver cytosol (79–99%) and from the data obtained from the application of this method in the kinetic distribution of MCYST-LR in mouse serum and liver following injection of the toxin. In the distribution studies, an excellent agreement between the ELISA data and the radiotracer method was obtained.

Both ELISA data and radiotracer results are similar to the observation obtained by Robinson et al. (1989), who found that the chemically tritiated reduced MCYST-LR exhibited the same retention time and ultraviolet absorption profile as the unlabeled toxin, indicating that the [^{3}H]MCYST-LR and the MCYST-LR have the same characteristics. Both ELISA and radiolabeled methods showed the catabolism of MCYST-LR by liver because there was a slight decrease in toxin concentration at 24 h postinjection. Results obtained by both methods revealed accumulation of a large amount of MCYST-LR in the liver, which is the target organ of intoxication. These results are similar to the report of Robinson et al. (1990), who found that about 60–71% of [^{3}H]MCYST-LR was deposited in mouse liver from 15 to 60 min postinjection. Also, in a study using rat hepatocyte suspensions and perfused livers, Hooser et al. (1991) found that as much as 65–77% of the radiolabeled MCYST was in the cytosolic fraction. Thus, our data support previous findings that MCYST-LR was strongly bound to the cytosol. However, an immunoreactive peak (shouder) with an apparent retention time of 13.5 min was found in the cytosol (Figure 5). The nature of this peak is unknown. Further identification of this minor peak is necessary.

Since MCYST-LR has been found to be a potent PP1 and PP2A inhibitor, we also examined whether the binding of the toxin to the liver cytosol correlated with the decrease in PP activities in the liver by analyzing the cytosol PP2A activity kinetically after the animal received the toxin. Whereas the inhibitory effect was not found when a high cytosol concentration was used, a significant decrease of PP2A activity was found when optimal cytosol concentration was used in the assay. Because MCYST-LR is lethal to mice at levels of 75–100 μg/kg (intraperitoneal injection, Hermansky and Stohs, 1990; Slatkin et al., 1983) and about 75% of the injected toxin accumulates in the liver (Hooser et al., 1991), a dose (35 μg) that resulted in minimal toxic effects but was sufficient for measuring the distribution of the toxin in serum and liver was selected in the present study. This is the first time that an in vivo decrease of PP2A was demonstrated at this sublethal level. Results obtained from this study were consistent with those obtained by the ELISA and radiotracer methods, namely, the higher the concentration of MCYST-LR present in the liver, the stronger the inhibitory effect of PP2A activity. Kinetically, the highest inhibition was observed at 6–12 h after dosing, a period of maximal accumulation of the toxin in the cytosol.

In conclusion, the present results indicate that the ELISA could be used as an effective method for monitoring human and animal exposure to microcystin. The immunochromatography approach could be used for further confirmation of the identity of MCYST-LR and its metabolites in body fluids and tissues. We confirmed the binding of MCYST to cytosol and demonstrated that such binding is consistent with its inhibition of PP2A activity even at a sublethal dose. Our observation reiterates that inhibition of phosphatase may modulate the toxicity of microcystin LR when PP2A is involved in dephosphorylating regulatory phosphoproteins that control many cellular functions (Cohen, 1989; MacKintosh, 1990).

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