Production and Characterization of Monoclonal Antibodies against Microcystin LR

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Microcystins are a family of cyclic polypeptides produced by different species of cyanobacteria (blue-green algae), which can form blooms in lakes and water reservoirs.[1] Their basic structure is a cyclic heptapeptide and their structural variations give rise to more than 50 types of microcystins known today (Fig. 1).[1] The most extensively studied form is microcystin-LR that contains L-leucine and L-arginine in the two main variant positions.

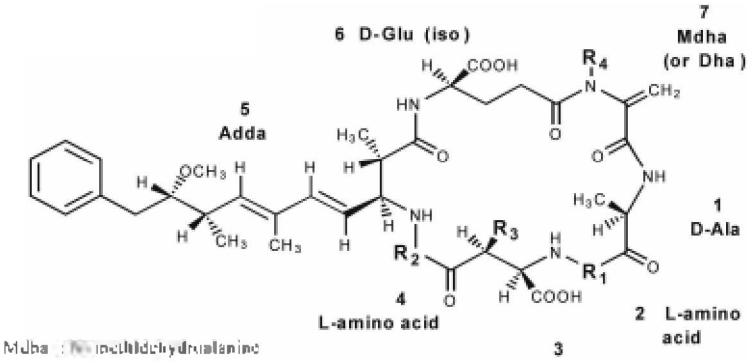
Microcystins and related polypeptides are potent hepatotoxins in fish, birds, and mammals.[2] The consequence of an acute poisoning by these compounds is a rapid disorganization of the hepatic architecture,[2,3] leading to massive intrahepatic hemorrhage, often followed by death of the animals by hypovolemic shock or hepatic insufficiency.[4] Matsushima *et al.*[5] have seen that microcystins penetrate with difficulty into the epithelial cells, which reflects tissue specificity, and their target cell is the hepatocyte. This cellular specificity and organotropism of microcystins is due to the selective transport system, the multispecific bile acid transport system, present only in hepatocytes.[3,6]

Microcystins are potent inhibitors of protein phosphatases 1 and 2A,[7-9] which are regulatory enzymes present in the cytosol of the mammalian cells. This action may explain the effects of microcystins as cancer promoters[10,11] and the promotion of primary liver cancer in humans exposed to long-term low doses of these cyclic peptide toxins through drinking water[12-14] as well as the cytoskeletal disruption and formation of plasma membrane blebs (blebbing) in hepatocytes.[3]

Since microcystins are potent hepatotoxins for humans and animals, the development of sensitive and reliable detection methods becomes of great importance. The efforts have been aimed at developing more sensitive screening methods to replace the nonspecific mouse bioassay, traditionally used for the identification of toxic strains of *Microcystis*.

Thus far, physicochemical techniques have been used as a sensitive method of analysis,[15-17] but this approach relies on the availability of toxin standards for comparison and is therefore only applicable to known toxins. It is also a relatively slow technique and requires expensive equipment and appropriate training.

The development of biological methods was first focused on enzyme-linked immunosorbent assays (ELISA). Using



	R_1	R_2	R_3	R_4	MW
Microcystin - LR	Leu	Arg	CH_3	CH_3	994
Microcystin - YR	Tyr	Arg	CH_3	CH_3	1044
Microcystin - RR	Arg	Arg	CH_3	CH_3	1037

Figure 1. Structure of microcystins. A characterisitic of microcystins and related cyanobacterial toxins is the hydrophobic amino acid Adda which contains in position 5 two conjugated double bonds. Numbers represent the positions of the corresponding amino acid.

polyclonal antibodies. the sensitivity limit was first ng/mL.[18] and later on it was 95 pg/mL.[19] Although the use of a monoclonal antibodies that selectively recognized microcystin can enhance the detection limit. the production of a monoclonal antibodies is not trivial. Microcystin seems to evoke a minimal immune response in animals.[20] presumably due to its low molecular weight. This paper deals with the establishment of monoclonal antibodies (Mabmcs) against a variant of microcystin: the microcystin-leucine-arginine variant (MCLR) and the characterization of the Mabmcs.

Experimental Section

Structures of microcystins and their derivatives used in these experiments are shown in Figure 1. Microcystis aeruginosa NIES 298, a cyanobacterium known to produce MCLR, was provided by National Institute of Environmental Research (Korea). One liter batch cultures of cells were grown in MA (Microcystis Aeruginosa) medium. Cultures were maintained at 20-25 °C under constant illumination by white fluorescent light incident on the surface of the growth flask. Cells were harvested by centrifugation (9000 × *g*. 5 min) and were lyophilized prior to storage at -20 °C. Microcystin-LR was purified by several steps of open column chromatography and identified by high performance liquid chromatography using Beckman equipment. The equipment included a 116 pump (SYSTEM GOLD Programmable Solvent Module 126). 126 Detector (SYSTEM GOLD Programmable Detector Module 166) and a multisolvent delivery system. Chromatograms were monitored at UV 238 nm. The column was a Ultrasphere 5 μm ODS (Beckman 4.6 mm × 25 cm). Methanol/0.02 M Na_2SO_4 (55 : 45) was used as a mobile phase at a flow rate of 2 mL/min.

To produce monoclonal antibodies against microcystin-LR, microcystin-LR was conjugated to keyhole limpet hemocyanin (KLH) in the presence of 1-ethyl-3,3'-dimethyl-aminopropyl-carbodiimide (EDAC). Hybridomas producing anti-MCLR Mabmcs were prepared by a standard method for immunization and cell fusion. BALB/c mice were immunized with MCLR-KLH. The initial injection used 0.2 mL of the conjugate solution and 0.2 mL of complete Freund's adjuvant. Booster injections used conjugate solution and incomplete Freund's adjuvant. The Mabmc was produced in BALB/c mice by the hybridoma cell line, SP_2/O-Ag14.

Two weeks after fusion, the hybridomas were screened for the production of anti-MCLR antibodies by an indirect ELISA, in which the MCLR-protein conjugates were coated onto plates. Hybridomas were estimated as positive for the generation of specific antibodies in case they were positive for MCLR-BSA, MCLR-KLH. The positive hybridomas were cloned several times by a limiting dilution method. Each of the established hybridoma cells producing the antibody were grown in medium supplemented with HT. Large quantities of antibodies were prepared from serum-free cultured supernatants of hybridomas by membrane ultrafiltration, ammonium sulfate precipitation, finally purifying using protein G column.

For ELISA. 96-well microtiter plates (Greiner Labortechnik, Polystyrene) were coated with Mabmc in 50 mM sodium-carbonate buffer at concentrations of 2 μg/mL and incubated at 37 °C for 1 hr. The competitive assay was performed in one step by incubating serial dilutions of free MCLR and MCLR-alkaline phosphatase conjugate (MCLR-ALP) on Mabmc-coated plates. The wells were incubated for 1 hr at 37 °C and washed three times with 0.2% Tween 20/PBS. After washing, 100 μL of substrate solution (KPL, Bluephos phosphatase solution A. B) was added and the plates were incubated for 30 min at room temperature. The reaction was stopped by the addition of 100 μL/well of 0.2 M EDTA (pH 8.0). Absorbance at 620-655 nm was measured by using an auto ELISA reader (Bio-rad model 550 reader).

Results and Discussion

To produce a good quality of mouse monoclonal antibody. it is very important to prepare antigen. microcystin LR in extremely pure form. To do this. we used a supercritical fluid extraction method[22,23] developed by our research group. Using supercritical carbon dioxide as a supercritical fluid, the microcystins were extracted from cyanobacterium. The microcystin LR was successfully extracted with a ternary mixture (90% carbon dioxide. 9.0% methanol, 1.0% water). This supercritical fluid extraction method has several advantages over solid-phase extraction sample preparation for the analysis of microcystins. Sample handling steps are minimized, thus reducing possible losses of analytes and saving analysis time. No organic solvent extractions are involved in this method and no clean-up steps are employed.

KLH-conjugated microcystin LR was used as an immunogen for the production of mouse monoclonal antibody. Immunization. cell fusion and screening of hybridoma cells producing anti-microcystin LR antibody were conducted according to a standard method.[24]

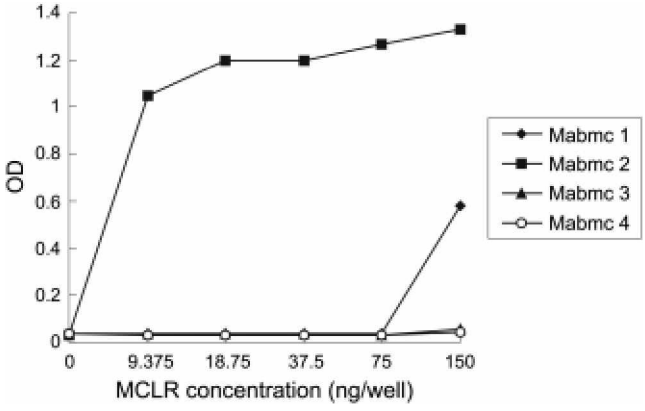


Figure 2. Reactivities of monoclonal antibodies to MCLR-conjugate. Each Mab at an appropriate concentration, mixed with indicated concentrations of MCLR. was added to MCLR-KLH-Coated wells of ELISA microtiter plates followed by the addition of HRP-labeled anti-mouse Ig G antibody. Mabmc 2 clones shows the highest reactivity to MC-KLH complex.

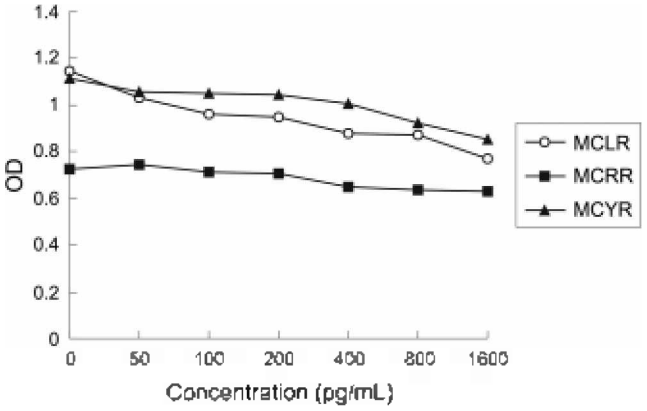


Figure 3. Specificity of monoclonal antibodies to different MC species. Known concentrations of different MCs were used in the competitive ELISA.

To ensure the specific and reversible binding of the monoclonal antibody, we performed indirect ELISA. We tested with MCLR-KLH covalently bound to 96-well plates. Two fusion experiments yielded a total four hybridoma secreting Mabmcs to MCs. They reacted with MCLR-KLH. The binding of Mabmcs to the MCLR-KLH coated to the plates was inhibited by free MCLR, and the concentrations required for complete competition were varied (Fig. 2). Mabmc 2 clones shows the highest reactivity to MC-KLH complex.

To estimate the epitope of the monoclonal antibodies produced in this study, microcystin LR, RR, YR were subjected to indirect competitive ELISA experiments. Microcystin RR and microcystin YR as well as microcystin LR showed a fairly good binding ability against our monoclonal antibody (Fig. 3). This result is suggesting that the epitope recognized by our momoclonal antibody is located around the Adda portion in the structure of microcystins (Fig. 1). Microcystin LR, RR, YR have a unique and common structural feature of hydrophobic b-amino acid abbreviated to Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid).

To decrease the risk of exposure to microcystins, monitoring the toxins in reservoirs and drinking water is essential. Currently, liquid chromatography[25] and mouse bioassay are most frequently used to detect the toxins in cyanobacterial water blooms.[26] However, it is difficult to detect microcystins directly in the water by these methods since the concentration of the toxins in the water was usually too low. The detection limit of our ELISA with our best monoclonal antibody, Mabmc 2 would be about 30 pg/mL of microcystin LR in water. If the ELISA can be applied to environmental water samples without reducing the high sensitivity, the trace amounts of microcystins in water would be directly detected by the ELISA. Our current trials suggest that the present ELISA can be applied to the direct determination of microcystins in freshwater samples,[27] as well be reported in detail elsewhere.

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