Regular article

Microcystin-LR is not Mutagenic *in vivo* in the λ /*lacZ* Transgenic Mouse (MutaTMMouse)

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The water pollution of toxic cyanobacteria (blue-green algae) is causing a serious public health problem in many parts of the world. Microcystin-LR (MCLR) is a potent cyclic heptapeptidic hepatotoxin produced by the cyanobacterium *Microcystis aeruginosa*. MCLR presents acute and chronic hazards to human health and has been linked to primary liver cancer in humans chronically exposed to this peptide toxin through drinking water. To assess the *in vivo* mutagenicity of MCLR, the λ /*lacZ* transgenic mice (MutaTMMouse) were treated with MCLR (1 mg/kg per week $\times$ 4) and examined for mutant frequencies (MFs) in the *lacZ* and *cII* genes of liver and lungs. Micronucleus induction in peripheral blood cells was also assessed. Co-mutagenic effect of MCLR was studied in combination with *N*-nitrosodiethylamine (DEN). MCLR did not increase either MFs of the target genes in liver and lungs or micronucleus frequency in the peripheral blood cells of the λ /*lacZ* transgenic mouse. While DEN treatment increased MFs significantly, the co-administration of MCLR did not potentiate its mutagenicity. We conclude that pure MCLR has no *in vivo* mutagenicity as it failed to induce gene mutation and micronucleus in transgenic mouse. Its tumor promoting effect is independent of its interaction to DNA.

Key words: Microcystin-LR, *N*-nitrosodiethylamine, *lacZ*, *cII*, MutaTMMouse

Introduction

The water pollution of toxic cyanobacterial bloom (blue-green algae) is an increasing problem worldwide and worsens with eutrophication of drinking- and recreational- water reservoirs due to industrialization (1,2). Cyanobacteria produce lethal toxins, and often associated to death of livestock and cases of human illness caused by drinking water contaminated by these toxins, which have drawn the attention of the World

Health Organization (WHO) (3). Microcystins are the most common group of cyanobacterial toxins comprised of over 60 structurally related cyclic heptapeptides (4) with potent hepatotoxicity and tumor promotion ability (5,6). Among them, Microcystin-LR (MCLR) is the most frequent secondary metabolite produced by *Microcystis aeruginosa* (2,7). MCLR presents acute and chronic hazards to human health (8,9). Although human illnesses attributed to microcystins include gastroenteritis and allergic/irritation reactions, the primary target of the toxin is the liver (10–14). It has been suspected to be involved with promotion of primary liver cancer in humans chronically exposed to doses of these peptide toxins through drinking water (15,16).

Algal toxins were reported to cause chromosomal breakages in human lymphocytes *in vitro* (17). Genotoxicity of cyanobacterial extract has been demonstrated by SOS chromotest with *Escherichia coli* PQ37 and the comet assay with human lymphocytes (18) and in four strains of *Salmonella typhimurium* (TA97, TA98, TA100 and TA102) in Ames test with or without S9 mix (19). In the same study, however, pure MCLR did not show any mutagenicity in all these strains. MCLR was reported to damage the mitotic spindle apparatus and thus induces polyploidy and apoptosis and necrosis in Chinese hamster ovary (CHO-K1) cells (20), and to induce gene mutation with base substitution in human RSa cells (21). Recently we have demonstrated mutagenic and clastogenic activities of MCLR in human lymphoblastoid cells (TK6) after 24 h treatment *in vitro*

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(22). Because MCLR induced mainly LOH type mutations rather than point mutations, mutagenicity of MCLR might be exerted by a clastogenic mechanism. Nevertheless, *in vivo* genotoxicity of this cyanotoxin is less convincing and relatively undescribed. Therefore, the present study was conducted to evaluate the *in vivo* mutagenicity of MCLR using transgenic (TG) mouse mutation assay. TG system has been shown to be useful for studying chemical mutagenesis and clastogenesis *in vivo* (23–25). This is largely attributed to its ability to detect tissue-specific gene mutations. TG assay also permits analysis of mutation at the molecular level and allows examination of the relation between mutagenesis and carcinogenesis *in vivo* in detail (25). Since MCLR has been found to promote tumor initiated with *N*-nitrosodiethylamine (DEN) in rats (5), the co-mutagenic (potentiation) effects of MCLR in combination with DEN was also studied.

Materials and Methods

Chemicals: MCLR and DEN were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MCLR was dissolved in saline immediately before use. Phenyl- β -D-galactoside (P-gal) was purchased from Sigma.

Treatment of MutaTMMice: Male MutaTMMice (5–6-week old, ca. 25 g body weight) supplied by Covance Research Products (PA, USA) were acclimatized for 1 week before use. The animals were divided into 4 groups of 5 mice each and administered with weekly doses of either vehicle (saline), MCLR (1 mg/kg, 1/10 of LD₅₀ in mice), DEN (25 mg/kg, 1/4 of LD₅₀ in mice) or DEN + MCLR (25 mg/kg and 1 mg/kg, respectively) for 4 weeks. Saline and MCLR were administered intragastrically while DEN was intraperitoneally injected. No apparent sign of toxicity was observed in any mice.

Micronucleus assay in peripheral blood cells: Forty eight hours after the first treatment, 5 μ L of peripheral blood was collected from the tail vein without anti-coagulant. The blood thus collected from each animal was placed on an acridine orange-coated glass slide, covered with a cover slip, and supravitaly stained (26). Type I, II, and III reticulocytes (RETs) with red fluorescent reticulum in the cytoplasm were scored under a fluorescent microscope. One thousand RETs were examined per animal within a few days after the slide preparation. The number of RETs with micronucleus (MNRETs) was recorded.

Mutation assay: 1) Tissue collection: Mice were killed by cervical dislocation 7 days after the last treatment. Liver and lungs were removed, immediately frozen in liquid nitrogen, and stored at -80°C until DNA extraction. MFs of *lacZ*/*cII* transgenes derived from liver and lungs were determined as described

previously (27–29). DNA sequencing of mutants isolated from the control and MCLR treated animals were carried out as described below.

2) Sequence analysis of *cII* gene: The *cII*-mutant plaques were transferred into a microtube containing 50 μ L SM buffer and 5 μ L chloroform. The λ phage *cII* region was amplified directly from mutant plaque solution by Taq DNA polymerase (Takara Shuzo, Tokyo, Japan) with primers P1, 5'-AAAAAGGGCATCAAATTAACC-3'; and P2, 5'-CCGAAGTTGAG-TATTTTGTG-CTGT-3'. Amplification was done by the Minicycler PTC-150-25 (MJ Research, Inc., MA, USA) under the following thermal cycling: 95°C 5 min $\rightarrow$ (95°C 20 s, 53°C 30 s, 72°C 40 s) $\times$ 30 cycles $\rightarrow$ 72°C 10 min. Amplification of 446 bp PCR product was checked by 2100 Bioanalyzer using lab DNA chips (Agilent Technologies, USA) and purified with a microspin column (Amersham Pharmacia, Tokyo, Japan) before being used for a sequencing reaction with the Ampli Taq cycle sequencing kit (PE Biosystems, Tokyo, Japan). The sequencing reaction was performed by Minicycler PTC-150-25 with 25 cycles of denaturing at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min, with the primer P1. The reaction product was purified by ethanol precipitation and analyzed by the ABI PRISM[®] 310 Genetics Analyzer (PE Biosystems, Tokyo, Japan).

3) Statistical analysis: The results of the different treatment groups were compared using Students' *t*-test. Significance was indicated by *P* values <0.05 .

Results

Micronucleus induction in peripheral blood:

Results of the micronucleus test 48 h after the first administration of chemicals in the MutaTMMice is shown in Fig. 1. The mean frequency of MNRETs did not increase significantly ($P>0.05$) in any of the treatment group in comparison with that of the control group.

Mutant frequency of *lacZ* and *cII* genes: The mutant frequencies (MFs) observed in the DNA preparations extracted from the liver and lung tissues 7 days after the last treatment are shown in Table 1. In MCLR-treated mice, the MFs of *lacZ* and *cII* genes in liver were not different significantly ($P>0.05$) from that of the background levels. Although a slight increase was observed in the lungs, it was not statistically significant ($P>0.05$). DEN treatment significantly ($P<0.05$) increased MFs of both the target genes in both liver and lungs (for *lacZ* gene 6.1 fold and 3.7 fold respectively and for *cII* gene 11.0 fold and 4.6 fold, respectively). We did not observe significant difference in MFs between DEN-treated and DEN + MCLR co-treated animals.

***cII* mutation spectrum:** Thirty four MCLR-in-

Fig. 1. Incidence of MNREs in the peripheral blood of MutaTMMouse 48 h following treatment with MCLR (1 mg/kg), DEN (25 mg/kg) and DEN (25 mg/kg)+MCLR (1 mg/kg).

duced and 46 DEN-induced mutants together with 42 spontaneous mutants from the liver were subjected to sequence analysis. The mutation spectra are summarized in Table 2. Spontaneous mutations consisted mainly of base substitutions (37/42). Among them, G:C to A:T transitions (21/26) predominated and most of them (17/21) occurred at CpG sites. DEN-induced mutations also consisted mainly of base substitutions (42/46). Compared to the control, G:C to A:T transitions were decreased in DEN treated group (50% versus 24% respectively) while A:T to T:A transversions were increased (2% versus 28%, respectively). However no obvious change was observed for incidences of mutations induced by MCLR including transitions (62% versus 62%) and transversions (27% versus 21%).

Table 1. MFs in the *lacZ* and *cII* gene from liver and lung of MutaTMMouse treated with MCLR (1 mg/kg), DEN (25 mg/kg) and DEN (25 mg/kg)+MCLR (1 mg/kg)

Organ	Treatment	<i>lacZ</i>				<i>cII</i>			
		Total plaques	Mutants	MF ($\times 10^{-6}$)	Mean $\pm$ SD	Total plaques	Mutants	MF ($\times 10^{-6}$)	Mean $\pm$ SD
Liver	Control	3311250	138	41.7	43.8 $\pm$ 11.7	3486000	69	19.8	20.5 $\pm$ 8.2
	MCLR	4053750	173	42.7	40.3 $\pm$ 13.7	4282500	94	21.9	21.1 $\pm$ 3.5
	DEN	3175000	963	261.7	268.4 $\pm$ 62.4*	3,495,000	788	225.5	226.6 $\pm$ 54.2*
	DEN+MCLR	2122500	472	222.4	206.9 $\pm$ 83.4†	2,149,500	391	181.9	176.4 $\pm$ 77†
Lung	Control	3823750	144	28.8	32.1 $\pm$ 13.9	4305000	110	25.6	25.7 $\pm$ 4.3
	MCLR	3823750	134	35.0	35.7 $\pm$ 4.89	2468250	93	37.7	36.9 $\pm$ 21.3
	DEN	3622500	416	114.8	117.5 $\pm$ 17.2*	2874000	332	115.5	118.1 $\pm$ 10.1*
	DEN+MCLR	2576250	264	102.5	109.9 $\pm$ 44.7†	1,136,250	141	124.1	132.2 $\pm$ 20.6†

*Compared to the control group $P < 0.05$

†Compared to the DEN-treated group $P > 0.05$

Table 2. Summary of *cII* mutations in the liver of control, MCLR- and DEN-treated MutaTMMice

Mutation class	Liver					
	Control	CpG	MCLR (%)	CpG	DEN (%)	CpG
Base	37 (89)		28 (82)		42 (91)	
Transitions	26 (62)		21 (62)		20 (43)	
G:C to A:T	21 (50)	17 (40)	20 (59)	17 (52)	11 (24)	6 (13)
A:T to G:C	5 (12)		1 (3)		9 (20)	
Transversions	11 (27)		7 (21)		22 (48)	
A:T to T:A	1 (2)		1 (3)		13 (28)	
A:T to C:G	4 (10)		2 (6)		2 (4)	
G:C to T:A	4 (10)		4 (12)		7 (15)	
G:C to C:G	2 (5)		0 (0)		0 (0)	
−1 Frameshift	1 (2)		2 (6)		1 (2)	
+1 Frameshift	3 (7)		4 (12)		0 (0)	
Deletion	0 (0)		0 (0)		1 (2)	
Insertion	0 (0)		0 (0)		0 (0)	
Complex	1 (2)		0 (0)		2 (4)	
Total	42 (100)		34 (100)		46 (100)	
MF ($\times 10^{-6}$)	43.8		40.3		268.4	

Discussion

The occurrence of toxic cyanobacterial blooms found in eutrophic, municipal, and residential water supplies is an increasing public health problem. Frequent deaths of domestic and wild animals are caused by drinking water contaminated by lethal toxins produced by cyanobacteria. MCLR is the most commonly encountered and among the most toxic algal cyclic peptide hepatotoxins. Epidemiological studies have indicated a close relationship between primary liver cancer in human and cyanobacteria contaminated drinking water (15,16). While there are several reports showing the *in vitro* genotoxicity of MCLR (21,22) or cyanobacterial extract (18,19), the evidence for the *in vivo* genotoxicity of this toxin is less convincing. Therefore, the main objectives of this study were to assess the *in vivo* genotoxicity of MCLR (if any) and its role in potentiation of DEN induced mutations for its suggested tumor promoting effects. To meet out these objectives male MutaTMMouse were administered with MCLR alone or in combination with DEN and examined for two end points- point mutation in transgenes, and micronucleus induction in peripheral blood cells. Considering the strong correlation between organ specific genotoxicity and organ specific carcinogenicity, the assessment of genotoxicity in multiple organs *in vivo* may indicate its target organ in humans and provide useful information for the evaluation of chemical safety. In the present research, hence, two target organs -liver and lungs- were examined for the evidence for mutagenicity.

Intraperitoneal injection of the raw cyanobacterial extracts containing several other microcystins besides MCLR induced micronucleus in the mouse bone marrow cells (19) and degradation and fragmentation of DNA in the liver cells (30). In the present study a pure MCLR (1 mg/kg = 1/10 of LD₅₀) was used, but no mutagenicity was observed. In another study, neoplastic nodule formation has been observed in the livers of mice received 100 intraperitoneal injections of sublethal doses of MCLR (20 µg/kg) over a period of 28 weeks (31). In the same study, oral administration of relatively higher doses of MCLR (80 µg/kg) under similar experimental conditions did not induce characteristic chronic injuries. Similarly, as suggested by the authors, fragmentation of DNA observed in hepatocytes of mice treated with the extract or MCLR (0.5–2.0 folds of LD₅₀ doses) might be a consequence of endonucleolytic DNA degradation associated with cytotoxicity, rather than by a direct toxin-DNA interaction (30). In support to this, recently Zegura *et al.* (32) have reported that the genotoxicity of MCLR could be mediated by reactive oxygen species. So it may be inferred that some other mutagenic toxins present in the extracts or different routes of administration might be responsible for the positive results observed in those studies. However,

MCLR treatment caused enhanced formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine in a time- and dose-dependent manner *in vitro* in primary cultured hepatocytes and *in vivo* in rat liver cells that could involve in the formation of hepatic tumors during long-term exposure to this cyanobacterial hepatotoxin (33). In contrast, in our study, under present experimental conditions, MCLR failed to induce mutation in both target genes (*lacZ* and *cII*) in liver and lungs of TG mouse. The *in vivo* micronucleus test in peripheral blood cells also yielded negative results. These results indicate that MCLR is capable of inducing neither point mutation nor chromosomal breakage *in vivo* in mouse organs.

It is widely believed that MCLR has tumor promoting effect (5,6). To test the possible potentiating effect of MCLR on mutagenicity of DEN, in our study, mice were simultaneously treated with DEN (25 mg/kg) and MCLR (1 mg/kg) once a week for four weeks. Relative to control mice, no significant increase in micronucleus frequency was observed either in DEN- or DEN+ MCLR-treated mice. This is in consistent with the negative results observed with DEN as previously reported (24). Further, simultaneous administration of MCLR with DEN did not increase MF caused by DEN in either of the target genes, although DEN treatment resulted in a significant increase in MFs in both *lacZ* and *cII* genes from liver and lungs. This indicates that the tumor promoting effects of MCLR is independent of mutagenicity of DEN. Because MCLR is known as an inhibitor of protein phosphatase 1 and 2A (5,34), the tumor promoting activity might be exerted by a disturbance of protein phosphorylation. Okadaic acid, which is known as a tumor promoter and a strong inhibitor of protein phosphatases (35), has similar mutagenic properties as MCLR (non-mutagenic in *Salmonella* and mutagenic in mammalian cells (36,37)). It is possible that tumor promoting activity of both compounds has a common mechanism through the inhibition of protein phosphatases.

In conclusion, pure MCLR has no *in vivo* genotoxicity as it is failed to induce gene mutation and micronucleus in transgenic mouse. Also lack of potentiation of DEN induced mutations in transgenes, as observed in the present study, indicates that the tumor promoting effects of MCLR is independent of its interaction to DNA.

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