Bull. Environ. Contam. Toxicol. (1994) 52:878–885

Environmental Contamination and Toxicology

Isolation, Culture, and Toxicity of the Cyanobacterium (Blue-Green Alga) *Microcystis aeruginosa* from a Freshwater Source in India

P. V. L. Rao, R. Bhattacharya, S. D. Gupta

Division of Pharmacology and Toxicology, Defence Research and Development Establishment, Gwalior-474002, India

Received: 1 June 1993/Accepted: 15 October 1993

Toxic cyanobacteria (blue-green algae) found in eutrophic freshwater, municipal and residential water supplies are an increasing environmental hazard in several parts of the world (Carmichael 1986; Codd and Poon 1988). Lethality to farm animals and liver damage to humans also have been associated with consumption of drinking water contaminated with cyanobacteria (Carmichael et al.1990; Falconer 1989). Two main types of toxins produced by cyanobacteria are known, namely cyclic peptide hepatotoxins and alkaloid neurotoxins. In the freshwater habitat, ***Microcystis aeruginosa*** is the most common hepatotoxin-producing cyanobacterium. The hepatotoxins,termed microcystins, found to date,are all cyclic heptapeptides (Sivonen et al. 1992);they are also cytotoxic and cause extensive hemmorrhage in the liver and some are known to be potent liver-tumor promoters (Nishiwaki-Matsushima et al. 1992). Except for a report by Mittal et al. (1979) about respiratory allergy in humans caused by some cyanobacteria, there are no confirmed deaths of animals or human intoxication attributed to cyanobacteria in India. So far no study has been done in India to evaluate the potential health hazard from toxic cyanobacteria in freshwater sources. The present study reports for the first time the isolation, culture and determination of toxicity of a strain of ***Microcystis aeruginosa*** collected from a freshwater pond in Banaras, India.

MATERIALS AND METHODS

The toxic strain of ***Microcystis aeruginosa*** reported in the present study was isolated from an individual

Correspondence to: P. V. L. Rao

colony from surface water of a freshwater pond in Banaras, India. Isolation and purification was done in Jaworski medium as described by Rippka (1988). Repeated washings with sterile medium and repeated passages yielded a unialgal strain which grows in the form of single cells. Subsequently, unialgal cultures were grown in four different media viz. MA medium as reported by Ichimiura (1979), CB (Shirai et al. 1989), ASM-1 (Gorham et al. 1964) and Jaworski media. In all experiments,150 ml of culture media was put into a 750 ml Erlenmeyer flask . Static cultures were maintained at $26\pm1^{o}C$ under 16:8 hr light:dark regime (1000 lux intensity). Growth was measured optically at 660 nm with a Spectronic 1201 spectrophotometer.

For comparison of growth in different media, an initial inoculum of 2×10^4 cells/ml was used. Dry matter content was determined at regular growth intervals by filtering 5-10 ml of batch-cultured samples through Whatman filter pads. The filters were dried overnight at 105^{o} C and the dry matter content was calculated by difference. Cells grown in CB medium were used in all subsequent experiments.

Bioassay for ***M. aeruginosa*** cell toxicity was done by the method of Ohtake et al. (1989). Briefly, cells grown in CB medium at late exponential growth were harvested by centrifugation. After three consecutive freeze-thaw cycles the cells were disrupted by sonication at 50 W for 3 min and centrifiuged at 30,000 X g for 15 min. The resultant supernatant was filtered through a membrane filter (0.45 µm) and lyophilized. The lyophilized cell extract was dissolved in 0.9% saline and injected intraperitoneally (ip) in male albino Swiss mice (20-25g) to determine acute (24 hr) LD 50 by Dixon's up and down method for small samples (Dixon 1965). Animals were divided equally into three groups of 12 animals each and administered as below ;

Group I - Saline (control)
Group II - 1 LD 50 toxin (lyophilized cell extract)
Group III - 2 LD 50 toxin (lyophilized cell extract)

Blood was collected from the mice in heparinized tubes from the retro orbital plexus, before sacrificing six animals each at 1 and 4 hr intervals, following exposure. Livers were excised, washed free of extraneous material and weighed. Liver/body weight index (LBI) was determined as liver weight X 100 /body weight. Standard procedures were followed to measure the activities of hepatic and serum alanine amino transferase (ALT) (Reitman and Frankel 1957), hepatic alkaline phosphatase (ALP) (Wright et al. 1972) and glutathione

(GSH) content (Ellman 1959). Data were subjected to Student's "t" test and the statistical significance was drawn at $p > 0.05$ level.

RESULTS AND DISCUSSION

The growth rates of ***M. aeruginosa*** in different media, as determined by optical density measurements, are shown in Fig. 1. As MA and CB media are rich in nutrients as compared to ASM-1 and JA, the growth was more rapid on these two media. Exponential growth was observed from 4-5 days and late exponential growth was observed up to 10-12 days in CB, MA and ASM-1 media. Growth rate declined in JA after 10 days of culture. In all the media no appreciable growth was observed after 25 days of culture. Dry weight measurements of growth on different media are shown in Fig.2. Maximum biomass of 4.16. 3.30, 2.30 and 2.16 g/l was obtained on CB, MA, ASM-1, and JA media, respectively, after three weeks of growth. In terms of growth rate, the four media can be rated in decreasing order as follows CB > MA > ASM-1 > JA.

The acute i.p. LD 50 of ***M. aeruginosa*** was 431 (370-501) mg/kg (dry weight). Clinical signs of toxicity which occurred within 1 hr of treatment included restlessness, labored ventilation, incoordination of movement, spasmodic leaping and splaying of hind limbs. Usually death occurred between 4 to 24 hr and was preceded by severe convulsions. Upon necropsy the animals showed grossly distended liver engorged with blood. The effect was more pronounced in the 2 LD 50 group irrespective of the duration of exposure, as evidenced by a significant increase in LBI (Table 1). One LD 50 group did not show any change over the saline-treated group. Table 2 shows the serum ALT and hepatic ALT, ALP and GSH activities in various treatment groups. The serum enzyme of hepatic origin, ALT, showed a significant increase in activity in both the 1 and 2 LD 50 groups at 1 and 4 hr intervals, with a corresponding decrease in enzyme activity in the liver. However, at 4 hr, ALT measured in the liver tended to normalize, indicating rapid recovery of the hepatic tissue. The other liver-specific enzyme, ALP, also exhibited a marked decrease in activity in both 1 and 2 LD 50 groups. In the 2 LD 50 group the effect was more deleterious over the 1 LD 50 group when observed at 4 hr. None of the treatments induced any change in the hepatic GSH level (Table 2).

The purpose of the present study was to isolate a cyanobacterium occurring in a freshwater source and determine the toxicity status of the isolated strain.

FIG.1.

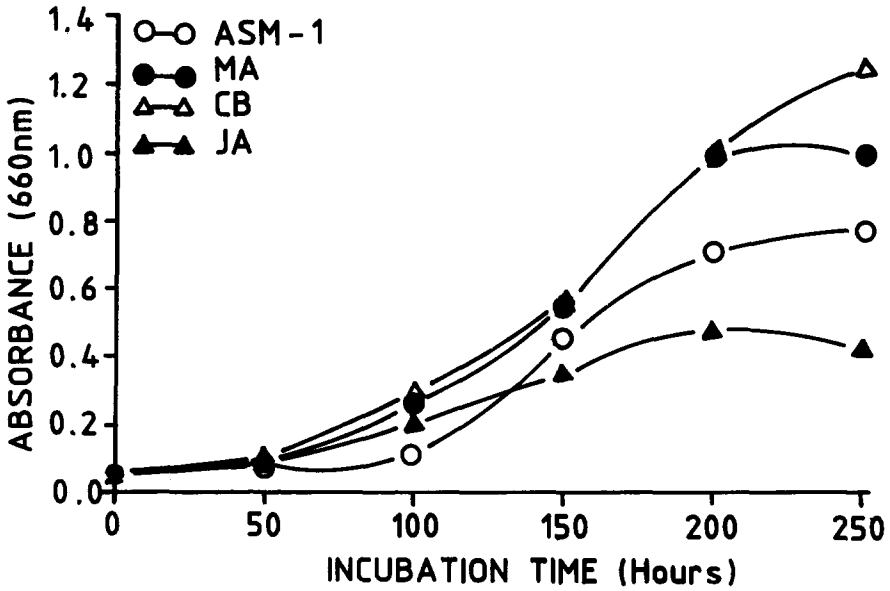

FIG.2.

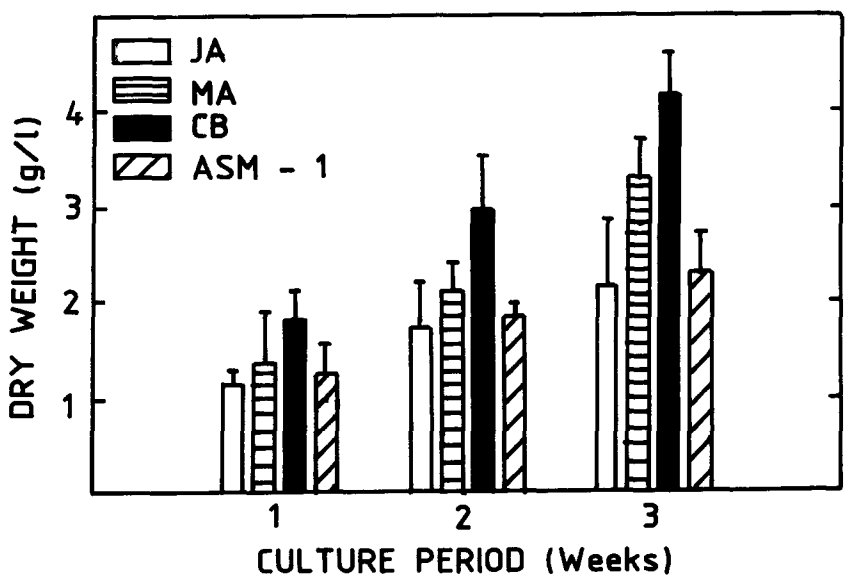

Figure 1. Growth patterns of ***Microcystis aeruginosa*** in JA, MA, CB , and ASM-1 media. The values are mean of five determinations each.

Figure 2. Dry weight determinations of ***Microcystis aeruginosa*** grown in JA , MA , CB and ASM-1 media. The Values are mean ± SE of five determinations each.

Table 1. Effect of ***Microcystis aeruginosa*** cell extract (toxin) on mouse liver/body weight index. (Values are mean ± SE, n=6)

Group	Liver/body weight index 1 hr	4 hr
Saline (Control)	4.03±0.22	4.10±0.37
Toxin 1 LD 50	4.78±0.33	4.60±0.30
Toxin 2 LD 50	5.33±0.33*	5.31±0.21*

* Means significantly different from control ($p < 0.05$)

Repeated subcultures and isolation resulted in non-axenic unialgal culture of ***Microcystis aeruginosa***. On the basis of LD 50, ***M. aeruginosa*** isolated in the present study was less toxic as compared to the toxicity (LD 50 30-50 ug/kg) of similar strains reported elsewhere (Carmichael et al. 1990). Toxicity of a species is dependent on many factors, including the environmental factors and time of collection (Codd and Poon 1988). The toxic and non-toxic forms of the same species may occur in the same water source. Microcystin is the common generic name given to the toxins produced by Microcystis species. Twenty-nine microcystins thus reported so far in the literature (Sivonen et al. 1992) are all hepatotoxic in nature. Death due to these hepatotoxins generally is caused by intrahepatic hemmorrhage and hypovolaemic shock (Theiss et al. 1988; Carmichael 1992). The liver is considered to be the major site of action of the toxin, and increased liver weight as observed in our study also was reported by Falconer et al. (1981) and Slatkin et al. (1983). We noted significant decreases in the levels of liver enzymes (ALT,ALP), with a corresponding increase (ALT) in blood. Jackson et al. (1984) also showed the increase in serum enzymes of hepatic origin AST, ALT, GLDH in sheep lethally poisoned by microcystin. A similar increase in levels of liver enzymes in blood, particularly y-glutamyltranspeptidase and alanine aminotransferase in a human population, during a toxic bloom in a reservoir of a city, was reported by Falconer et al. (1989).

The present study has shown the occurrence of toxic cyanobacterium in a freshwater source for the first time in India and the hepatotoxic nature of the strain.

Table 2. Effect of *M. aeruginosa* cell extract (toxin) on mouse serum and hepatic enzyme levels. (Values are mean ± SE , n=6)

Group	Serum		Hepatic					
	ALT[a]		ALT[a]		ALP[b]		GSH[c]	
	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr
Saline (control)	5.06 ± 0.31	5.13 ± 0.39	3.63 ± 0.16	3.78 ± 0.23	0.82 ± 0.02	0.79 ± 0.03	6.74 ± 0.54	6.65 ± 0.40
Toxin 1.0 LD50	7.58* ± 0.72	7.30* ± 0.66	1.62* ± 0.10	2.97 ± 0.39	0.40* ± 0.05	0.53* ± 0.02	6.22 ± 0.63	6.85 ± 0.73
Toxin 2.0 LD50	7.08* ± 0.42	6.93* ± 0.59	1.62* ± 0.11	2.51 ± 0.20	0.33* ± 0.03	0.24** ± 0.01	5.78 ± 0.81	7.34 ± 0.91

[a] n mol hydrazone formed min^{-1} mg $protein^{-1}$

[b] μg Pi formed min^{-1} mg $protein^{-1}$; [c] μ mol g^{-1}

* $p < 0.05$ vs saline (control) group ; ** $p < 0.05$ vs 1.0 LD50 Toxin group

Further studies are in progress on the isolation and characterization of the toxin produced. On the basis of reports that microcystin and other hepatotoxins from freshwater cyanobacteria are potent liver-tumor promoters (Nishiwaki-Matsushima 1992) and the lack of information on their occurrence, necessitates further studies on the prevalence of toxic cyanobacteria in Indian freshwaters.

Acknowledgments. We thank Prof H.D.Kumar, Banaras Hindu University, for providing facilities for collection of samples at Banaras and Dr. R.V. Swamy, Director, DRDE, for encouragement in this work.

REFERENCES

Carmichael WW (1986) Algal toxins. In: Callow JA (ed) Advances in Botanical Research, Academic Press, London pp 47-101

Carmichael WW, Mahmood NA, Hyde EG (1990) Natural toxins from cyanobacteria (blue-green algae).In : Hall S, Strichartz G (eds) Marine toxins - Origin, Structure and Molecular Pharmacology, ACS Symposium Series, Washington, DC, pp 87-106

Carmichael WW (1992) Cyanobacteria secondary metabolites - cyanotoxins. J Appl Bact **72**:445- 459

Codd GA, Poon GK (1988) Cyanobacterial toxins. In: Rogers LA, Gallor JR (ed) Biochemistry of Algae and Cyanobacteria. Clarendron Press, Oxford, pp 283-296

Dixon WJ (1965) The up and down method for small samples. J Am Stat Assoc **60**:967-978

Ellman GL (1959) Tissue sulphydryl groups. Arch Biochem Biophys **82**:70-77

Falconer IR, Jackson ARB, Landley J, Runnegar MT (1981) Liver pathology in mice in poisoning by the blue-green alga ***Microcystis aeruginosa***. Aust J Biol Sci **4**:179-187

Falconer IR (1989) Effects on human health of some toxic cyanobacteria (blue-green algae) in reservoirs, lakes and rivers. Tox Assess **4**:175-184

Gorham PR, McLachlan J, Hammer UT, Kim WK (1964) Isolation and culture of toxic strain of ***Anabena flos-aquae*** (Lyngb) de Breb. Verein Limnol **15**:796-804

Ichimura T (1979) Media for blue-green algae.In: Nishizawa M, Chihara M (ed) Methods in Algology, Kyoritsu, Tokyo, pp 294-304

Jackson ARB, McInres A, Falconer IR, Runnegar MTC (1984) Clinical and pathological changes in sheep experimentally poisoned by the blue- green alga ***Microcystis aeruginosa***. Vet Pathol **21**:102-113

Mittal A, Agarwal MK, Schivpuri DN (1979) Respiratory allergy to algae-clinical aspects. Ann Allergy **42** : 253-256

Nishiwaki-Matsushima R, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishiwaka T, Carmichael WW, Fujiki H (1992) Liver tumor promotion by cyclic peptide microcystin-LR. J Cancer Res Clin Oncol **118**:420-424

Ohtake A, Shirai M, Aida T, Mori N, Harada K, Matsuura K, Suzuki M, Nakano M (1989) Toxicity of *Microcystis* species isolated from natural blooms and purification of the toxin. App Environ Microbiol **55**:3202-3207

Reitman S, Frankel S (1957) A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. Am J Clin Pathol **28**: 56-63

Rippka R (1989) Isolation and purification of cyanobacteria. Methods Enzymol **167**:3-27

Shirai M, Matsumara K, Ohotake A, Takamura Y, Aida T, Nakano M (1989) Development of a solid medium for growth and isolation of axenic microcystin strain (cyanobacteria). App Environ Microbiol **55**:2569-2571

Sivonen K, Namikoshi N, Evans WR, Fardig M, Carmichael WW, Rinehart KL (1992) Three new microcystins, cyclic heptapeptide hepatotoxins, from Nostoc sp strain 152. Chem Res Toxicol **5**:464-469

Slatkin DN, Stoner RD, Adams WH, Kycia JH, Siegelman HW (1983) Atypical pulmonary thrombosis caused by a toxic cyanobacterial peptide. Science **220**:1382-1385

Theiss WC, Carmichael WW, Wyman J, Bruner R (1988) Blood pressure and hepatocellular effects of the cyclic heptapeptide toxin produced by ***Microcystis aeruginosa*** strain PCC - 7820. Toxicon **26**:603-613

Wright PJ, Leathwood PD, Plummer DT (1972) Enzymes in rat urine alkaline phosphatase. Enzymologia **42**:317-332