

Allergenic (Sensitization, Skin and Eye Irritation) Effects of Freshwater Cyanobacteria—Experimental Evidence

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ABSTRACT: Many studies have been published on the human health effects of cyanobacterial toxicity. As a public health concern, we have to mention that the development of contact dermatitis, asthmalike symptoms, and symptoms resembling hay fever during bathing in cyanobacterial blooms have been also described. *Microcystis aeruginosa*, *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*, and *Cylindrospermopsis raciborskii* are the most common species found in Hungarian freshwaters. A sensitization test on albino guinea pigs, and intradermal reactivity, and ocular irritation test on albino rabbits were carried out with freeze-dried algal suspension in physiological salt solution. The sensitivity of guinea pigs is similar to that of humans. *Microcystis*, *Anabaena*, *Cylindrospermopsis*, and *Aphanizomenon* bloom and strain samples were examined in sensitization and irritation tests and no correlation was found between the toxin content and the allergenic character. The most toxic one (*Microcystis aeruginosa*) was not the most allergenic sample, but the nontoxic *Aphanizomenon* was the most allergenic one. The axenic strains were not allergenic at all. The pure microcystin LR was only slightly allergenic even in high concentration (1.5 mg/ml). Water and lipid soluble fractions were obtained by water and chloroform extraction of lyophilized algal suspensions. The chloroform fraction was bound on C18 cartridges and eluted by methanol in nine fractions. Only one of the lipid soluble fractions was skin irritative whereas the strongest irritative effect was shown by the water soluble fraction. © 2001 by John Wiley & Sons, Inc. *Environ Toxicol* 16: 512–516, 2001

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INTRODUCTION

Cyanobacterial blooms and scums occur in the same season as the recreational bathing in freshwaters. Hence a need to experimentally examine the allergenic and irritative effects of cyanobacteria. There are some data

on contact irritative and allergenic effects of cyanobacteria (Billings, 1981). Turner et al. (1990) described cases of pneumonia in soldiers caused by inhalation during canoeing in cyanobacterial blooms. In Australia, 852 persons were involved in a prospective epidemiological study (Pilotto et al., 1997) in which the relationship between the symptoms of diarrhoea, vomiting, hay fever, and eye irritation occurring within 7 days after bathing in freshwaters containing cyanobacterial

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blooms, and the toxin content, the number of cyanobacteria, and the time spent in the water was examined. Significant correlation was found between the symptoms mentioned above and the number of cyanobacteria, and the time spent in the water. In a retrospective epidemiological study in Hungary, it was found that in 1994, many children suffered from skin and eye irritation in a heavy bloom of *Microcystis aeruginosa* (Törökné, unpublished data).

There is a great need to complement the data in case studies in the literature with experimental evidence of cyanobacterial allergenic or irritative effects on animals. That is why we chose the usual methods (ISO standards) used in examining compounds for allergenic or irritative effects in guinea pigs or rabbits.

MATERIALS AND METHODS

Materials

Natural cyanobacterial bloom samples were collected by plankton net of mesh size 20 μm from different ponds and lakes in Hungary and one sample was received from Lake Kinneret, Israel. The samples were lyophilized and stored at 24°C until use. The samples were almost monospecific, only 1–2% of green algae and some diatoms accompanied them. The samples 8 and 9 were monospecific laboratory cultures. The samples used are shown in Table I. Axenic cyanobacterial strains were also examined, which were received from the University of Helsinki, Viki Research Centre (Kaarina Sivonen) (Table II). Microcystin-LR (1.5 $\mu\text{g/mL}$) obtained from the University of Dundee, UK was used in the sensitization test.

Methods

Minimal lethal doses (MLD) were determined by the method of Falconer (1993) in intraperitoneal mouse assay.

TABLE I. Natural samples of cyanobacterial blooms used

Genus	Source of Samples
1. <i>Anabaena</i>	Reservoir Fehérvárcsúrgó
2. <i>Aphanizomenon</i>	Reservoir Zámoly
3. <i>Microcystis</i>	Pond Diósjenő
4. <i>Microcystis</i>	Reservoir Komrávölgy
5. <i>Microcystis</i>	Lake Velencei
6. <i>Microcystis</i>	Reservoir Fehérvárcsúrgó
7. <i>Microcystis</i>	Pond Dinnyés
8. <i>Cylindrospermopsis raciborskii</i>	Lake Balaton
9. <i>Aphanizomenon ovalisporum</i>	Lake Kinneret, Israel

TABLE II. Axenic cyanobacterial strains used

Genera	Strain
1. <i>Anabaena</i>	123
2. <i>Anabaena</i>	202 A1
3. <i>Oscillatoria</i>	97
4. <i>Oscillatoria</i>	2B
5. <i>Microcystis</i>	269
6. <i>Microcystis</i>	199

The microcystin content of the samples was determined as follows: 5 mg lyophilized cyanobacterial material was extracted in 1 mL of methanol : 0.002 M KH_2PO_4 in the ratio of 2 : 1. Microcystin-LR, RR, YR (MC-LR,RR,YR) were determined by the HPLC method. Instrument: Merck Hitachi D7000, column: LiChroCART 125-4 Purospher RP18e (5 μm), pre-column: LiChroCART 4-4 purospher RP18e (5 μm), liquid phase: 0.002 M KH_2PO_4 , pH = 6.4/MeOH, gradient 30–60% of MeOH, detector UV Merck Hitachi L 7400, wavelength: 238 nm, injection volume 10 μL , pump: Merck Hitachi L 7001. Microcystin-LR,RR,YR (Calbiochem, USA) standards were used.

For the determination of cylindrospermopsin, 10 mg lyophilized material was extracted with 200 μL pure water and the HPLC method was used as follows: HP 1090, column: LiChroCART 125-4 Purospher RP18e (5 μm), pre-column: LiChroCART 4-4 purospher RP18e (5 μm), liquid phase: MeOH / H_2O gradient 1–10%, detector: DAD, wavelength: 262,290, injection volume 25 μL . Cylindrospermopsin purified by the Kossuth Lajos Scientific University, Debrecen, Hungary was used as a standard.

Maximization sensitization test was carried out on albino guinea pigs according to ISO 10993-10:1995(E) 6.2. The lyophilized cyanobacterial material was suspended in pyrogen-free distilled water in 1 mg/mL concentration. The suspension was infused for 2 hours and was then centrifuged at 3000 rpm for 10 minutes. NaCl was then added to the supernatant to make it a physiological solution and was used immediately for testing. In a preliminary test, the concentrations to be used in the main test, were determined. In the main test, 0.1 mL test solution was injected intracutaneously on the shaved backs of the animals for the intradermal induction phase with the highest concentration that did not cause extensive destruction of the skin and did not otherwise adversely affect the animals in the preliminary test. One mg/mL concentration was used in all the experiments. Seven days after completion of the intradermal induction phase, the test material was administered topically to the intrascapular region of each animal using 20 mm $\times$ 40 mm filter paper, so as to cover

the intradermal injection sites. It was secured with an occlusive dressing. After 48 hours, the dressing and the patches were removed. At least 14 days after completion of the topical induction phase, all the test and control animals were challenged by administering the test material by topical application to one flank of each animal using appropriate patches soaked in the test material. It was then secured with occlusive dressing. After 24 hours, the dressing and patches were removed. The skin reactions (erythema and oedema formations) were observed in the 10 test and 5 control animals. One sample was tested on each animal and 10 animals were used to test each sample. The controls were treated with saline. The percentage of sensitized animals was determined and categorized as shown in Table III.

The intracutaneous (intradermal) irritation test was done on albino Californian rabbits according to ISO 10993-10:1995(E) 5.4. 0.2 mL of the stock solution of cyanobacterial material (1 mg/mL) was injected intradermally and the skin reactions such as erythema and oedema were classified after 24, 48, and 72 hours on the point system as represented in Table IV. According to the severity of oedema and erythema, points are given for the symptoms. Three animals were used for each sample with five intradermal injections on one side in the back and five injections on the other side in the back as controls with saline solution. So 15 sites were examined for a sample.

In the eye irritation test (ISO 10993-10:1995(E) 5.3), 0.1 mL stock solution was instilled into the lower conjunctival sac of one eye of each albino rabbit four times a day with 30 minutes between the treatments. The whole procedure was repeated on the next day in the same way. The other eye received the control of physiological salt solution. Three replicates of different animals were used. If the symptoms score eye irritation in more than one rabbit, then the material was rated as an eye irritative agent.

Different extractions were made on *Aphanizomenon ovalisporum* nonaxenic strain from Lake Kinneret, Israel. One part of the lyophilized material was extracted by water, evaporated, and used for the skin

TABLE III. Categories in sensitization test

Percentage of Sensitized Animals (%)	Category	Qualifying
0–8	I.	Not defined allergen
9–28	II.	Slight allergen
29–64	III.	Moderate allergen
65–80	IV.	Strong allergen
81–100	V.	Extremely strong allergen

TABLE IV. Categories in intradermal test

Category	Average Points
Negligible	0–0.4
Slight	0.5–1.9
Moderate	2–4.9
Severe	5–8

irritation test. The other part of freeze-dried material was extracted by chloroform, evaporated and this sample was divided into two parts again. The first was dissolved in acetonitrile, and after centrifugation was applied to a C18 cartridge and eluted with methanol (used for HPLC). This procedure resulted in six fractions. The other part of the lipid soluble fraction was dissolved in chloroform, centrifuged, and applied to a C18 cartridge. Then it was eluted by chloroform, acetonitrile, and methanol to provide three other fractions.

RESULTS

The natural samples contained different amounts of microcystins and cylindrospermopsin as shown in Table V. The correlation coefficient between the results of ip. mouse assay and MC content is -0.665 .

In the axenic strains, only the microcystin-LR was determined as shown in Table VI.

As can be seen, all types of natural bloom samples with different species and toxin content were used in the sensitization and irritative tests. Similarly, the axenic strains had different toxin contents and toxin types.

As shown in Table VII, all the natural samples caused allergenic effects in guinea pigs. There is no correlation

TABLE V. Minimal lethal doses and toxin content in natural cyanobacterial samples

Genus	Ip. Mouse Assay (MLD) (mg/kg)	Microcystin-LR, RR,YR (mg/g)
1. <i>Anabaena</i>	150	0.3
2. <i>Aphanizomenon</i>	>800	Not detected
3. <i>Microcystis</i>	100	0.43
4. <i>Microcystis</i>	800	0.1
5. <i>Microcystis</i>	25	2.21
6. <i>Microcystis</i>	50	0.88
7. <i>Microcystis</i>	20	1.71
8. <i>Cylindrospermopsis raciborskii</i>	400	0.015 cylindrospermopsin
9. <i>Aphanizomenon ovalisporum</i>	Not determined	0.8 cylindrospermopsin

TABLE VI. Toxin content in the axenic strains

Genus	Microcystin-LR (mg/g)	Toxic Effect in Mice
1. <i>Anabaena</i> (123)	Not determined (anatoxin-a)	Neurotoxic
2. <i>Anabaena</i> (202A1)	10.2	Hepatotoxic
3. <i>Oscillatoria</i> (97)	10.1	Hepatotoxic
4. <i>Oscillatoria</i> (2B)	Not detected	Nontoxic
5. <i>Microcystis</i> (269)	Not detected	Nontoxic
6. <i>Microcystis</i> (199)	7.9	Hepatotoxic

between the toxin content and the allergenic reaction or irritative effect. The most toxic sample (5) was not the most allergenic, but the nontoxic *Aphanizomenon* caused the most serious allergenic effect. It is interesting to note that there is also no relationship between the allergenic effect and the irritative effect.

In spite of the experience of the natural samples, there was no allergenic effect with the axenic strains.

On the basis of the preliminary test for MC-LR, 1.5 µg/mL was used in the main test. It was found that even though such a high concentration of MC-LR was used, the allergenic effect was slight with 22% of animals affected.

Aphanizomenon ovalisporum with a moderate skin irritation effect was extracted as detailed in the methods. The results are summarized in Table VIII. The water soluble fraction had a slight skin irritative effect on albino rabbits. It is interesting to note that if the lipid soluble fraction was examined all together, then it caused negligible irritation, but if the fractionated lipid soluble fractions were used separately, then the 8th (acetonitrile soluble) fraction showed slight intradermal reactivity.

TABLE VIII. Effects of water and lipid soluble fractions in intradermal irritation test

Fractions	Points	Category
Water soluble	1.167	Slight
Lipid soluble all together	0.22	Negligible
8th fraction of lipid soluble	0.5	Slight

DISCUSSION

Our results of HPLC analyses of the toxin content correlates well enough (r : 0.665) with the toxic effects in mice. That there is no closer relationship among them may be explained if the samples can contain other toxins that were not measured chromatographically (Jungman, 1992, Törökne, unpublished data).

Our experimental data correlates well with Pilotto et al.'s (1997) findings as there is no correlation between the toxin content and the allergenic effect of cyanobacteria. There are no data in the literature on allergenic effects of axenic cyanobacteria. This is the first experimental evidence that axenic cyanobacteria have not caused allergenic effects on albino guinea pigs. Keleti and Sykora (1982) emphasized that the allergenic effect is more likely caused by lipo polysaccharides of cell walls, than the toxin content. Our experiments strengthen this statement but implicate the lipopolysaccharides of contaminant bacterial flora rather than cyanobacterial cell walls. The WHO Guidelines recommend that bathing in freshwater should be prohibited when cyanobacteria dominate the phytoplankton and the chlorophyll-a concentration exceeds 50 µg/L or the number of cyanobacteria is over 100 000 cell/mL (Chorus and Bartram, 1999). These recommendations are derived from toxicity data, and do not consider

TABLE VII. Allergenic and irritative effects of natural cyanobacterial bloom samples in guinea pigs

Genus	Microcystin-LR, RR,YR (mg/g)	Percentage of Sensitized Animals (Categories)	Skin Irritation	Eye Irritation
1. <i>Anabaena</i>	0.3	45 (III)	Slight	—
2. <i>Aphanizomenon</i>	Not detected	91 (V)	Negligible	—
3. <i>Microcystis</i>	0.43	30 (III)	Slight	+
4. <i>Microcystis</i>	0.1	50 (III)	Slight	—
5. <i>Microcystis</i>	2.21	67 (IV)	Negligible	+
6. <i>Microcystis</i>	0.88	30 (III)	Slight	+
7. <i>Microcystis</i>	1.71	67 (IV)	Slight	+
8. <i>Cylindrospermopsis raciborskii</i>	0.015	50 (III)	Not determined	Not determined
9. <i>Aphanizomenon ovalisporum</i>	0.8	Not determined	Moderate	Not determined

allergenic responses, which may occur at much lower cyanobacterial concentrations as a result of allergenicity of associated contaminant microorganisms. On the other hand, we should also consider the toxin content of the scums and blooms collecting on the shores, where mainly children play (Chorus, 1998). In conclusion, during cyanobacterial blooms and scums, it is necessary to consider the toxin content of the water and biomass and as well as to determine the allergenic effects.

CONCLUSIONS

- Natural cyanobacterial bloom samples can cause allergenic and irritative effects in albino guinea pigs and rabbits.
- Axenic cyanobacterial strains tested do not cause any allergenic effects in the experimental animals.
- Pure microcystin-LR even in high concentration (1.5 $\mu\text{g/ml}$) caused only a slight allergenic effect in guinea pigs.
- From the water and lipid soluble fractions, only the water soluble fraction caused a slight skin irritative effect in rabbits.

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