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ScienceDirect
Harmful Algae 7 (2008) 584–589
HARMFUL ALGAE
www.elsevier.com/locate/hal

Dermal toxicology of *Lyngbya majuscula*, from Moreton Bay, Queensland, Australia

Nick Osborne [a,b,c,*], Alan Seawright [b], Glen Shaw [d,e]

[a] *School of Population Health, The University of Queensland, Herston Road, Herston, Queensland 4006, Australia*
[b] *National Research Centre for Environmental Toxicology, The University of Queensland, 39 Kessels Road, Coopers Plains, Queensland 4108, Australia*
[c] *Allergy and Immune Disorders, Murdoch Childrens Research Institute, Flemington Road, Parkville, Melbourne, Victoria 3052, Australia*
[d] *School of Public Health, Griffith University, University Drive, Meadowbrook, Queensland 4131, Australia*
[e] *Australian Rivers Institute, Griffith University, 170 Kessels Road, Nathan, Queensland 4111, Australia*

Received 16 April 2007; received in revised form 31 May 2007; accepted 15 December 2007

Abstract

Lyngbya majuscula is a filamentous marine cyanobacterium with a worldwide distribution in temperate and tropical regions to a depth of 30 m. Over 70 chemicals have been isolated and characterised from this organism, many of which are biologically active. Previously, *L. majuscula* has been reported as implicated in negative health outcomes only in Hawaii and Okinawa. Recently large blooms of *L. majuscula* have occurred with increasing repetition in the Moreton Bay region as well as other areas along the Australian coastline.

Lyngbya toxin A (LA) and debromoaplysiatoxin (DAT) were found in samples of *L. majuscula* collected from Eastern Moreton Bay and North Deception Bay, Queensland, Australia, respectively. Samples of *L. majuscula* were also obtained from West Maui, Hawaii and the freshwater *Lyngbya wollei* from Florida. A quantitative measure of the irritant effects of the chemicals found in *L. majuscula* was made using a mouse ear swelling test. The relative toxicities of two purified toxins, LA and DAT, were examined. These were found to produce swelling to a similar extent. The time course of inflammation and histopathological results were also similar for the two purified toxins. Less than 1 μg per ear of either toxin or a mixture (1:1) of the two toxins caused a measurable increase in ear thickness. When toxins were combined (1:1) there was an additive, not synergistic effect. Increases in ear thickness occurred within 15 min. Crude extracts of *L. majuscula* from Moreton Bay were also applied to mice ears. The effect of crude extracts from Eastern Moreton Bay was not fully explained by the measured LA content, suggesting other toxin(s) and/or modulating factors were present. The toxic effects of *L. majuscula* containing DAT from North Deception Bay were explained by the concentrations measured. Some samples of *L. majuscula* containing no measurable quantities of LA or DAT were found to exert an inflammatory response. This response had a different time course to the response produced by LA or DAT.

Keywords: Cyanobacteria; Dermal; Irritant contact dermatitis; *Lyngbya majuscula*; Toxicology; Blue-green algae; Cyanobacterium

1. Introduction

Lyngbya majuscula (Dillwyn) was first implicated as causing dermatitis in the late 1950s after reports of acute contact dermatitis in Hawaii (Grauer and Arnold, 1961) and has been reported in Queensland, Australia (Osborne et al., 2007). Toxic factors were isolated from *L. majuscula* that demonstrated dermonecrotic activity following injection into the abdominal skin of mice and guinea pigs (Moikeha and Chu, 1971; Moikeha et al., 1971), and later debromoaplysiatoxin (DAT) and lyngbya toxin A (LA) were identified and characterised (Cardellina et al., 1979; Mynderse et al., 1977). LA was found to be structurally related to the toxic dermal irritant teleocidin, previously extracted from *Streptomyces* (Takashima et al., 1962). The aplysiatoxins had previously been found in the marine mollusc *Stylocheilus longicauda* (Kato and Scheuer, 1974).

Initial toxicological studies with purified DAT found it produced an irritant pustular folliculitis in humans and severe cutaneous inflammatory reaction in rabbits and mice (Solomon and Stoughton, 1978). Only very small quantities of purified toxin were needed to induce inflammatory reactions in humans

* Corresponding author at: Allergy and Immune Disorders, Murdoch Childrens Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Melbourne, Victoria 3052, Australia. Tel.: +61 3 9936 6506; fax: +61 3 9348 1391.
E-mail address: nicholas.osborne@mcri.edu.au (N. Osborne).

1568-9883/$ – see front matter
doi:10.1016/j.hal.2007.12.022

(500 ppm or 0.5 μg/ml in ethanol) and rabbits and mice (0.5 ppm). Exposure of humans to this cyanobacterium in the environment is associated with irritant contact dermatitis, as well as eye and respiratory irritation (Osborne et al., 2001).

More recently the oral toxicology of *L. majuscula* and purified toxin derived from this organism have been examined in mice (Ito and Nagai, 1998, 2000; Ito et al., 2002). LA had an intra-peritoneal (i. p.) lethal dose of 250 μg/kg in immature mice while no deaths occurred in mature mice at 300 μg/kg. No lethality occurred at per oral (p.o.) 1000 μg/kg. At p.o. 600 μg/kg enhanced mucous secretion and light erosion occurred after 10 min in the stomach and small intestine. Effective dose levels were found to be similar between LA and aplysiatoxin (AT) (Ito et al., 2002). The proposed mechanism of action of the toxins LA and DAT is via protein kinase C-mediated receptors (Mullin et al., 1990).

The aim of this study was to establish an animal model for dermal toxicity that would elucidate the toxicity of Queensland *L. majuscula* strains, to complement epidemiological studies (Osborne et al., 2007). It was proposed that purified LA and DAT would be used to establish a dose–response curve against which toxicology of Queensland *L. majuscula* could then be determined. The potency of LA and DAT in regard to dermal toxicity could then be established as well as determining if the anecdotal toxicity of *L. majuscula* from Queensland was due these two toxins. A comparison of differences in histopathology of LA and DAT was proposed in order to compare other states of dermal toxicity.

2. Methods

LA and DAT standards were obtained from Prof. Richard Moore, University of Hawaii. Crude extracts were produced from samples of *Lyngbya* obtained from Moreton Bay, Florida and Hawaii (Table 1). The cyanobacterium was stored in seawater until returned to the laboratory and frozen and stored (0 °C). On thawing, cyanobacterium was rinsed with distilled water and refrozen for lyophilisation. Frozen samples of *L. majuscula* (∼20 g) were lyophilised until dry and ground using a coffee grinder (Phillips, Sydney, Australia). Samples were extracted with 50 ml of acetone (EM Science, Gibbstown, USA) overnight, with 20 min of sonication during this time. The extract was filtered through glass fibre filter paper (Whatman, Maidstone, UK) under vacuum into a Büchner flask to remove particulate matter and solvent was removed by rotary evaporation (Büchi, Flawil, Switzerland) with temperature maintained at less than 40 °C in the heated water bath. Extracts were solubilised in 50% methanol and passed through a 0.45 um Millex-HA (Millipore, Milford, USA) syringe driven filter unit before testing for the presence of LA and DAT.

Table 1
Crude extracts of *Lyngbya* used in this study

ID	Date of collection	Location
07c		Honokowi, West Maui
20	25-Feb.-00	Bongaree, Deception Bay
12	28-Feb.-00	Bongaree, Deception Bay
15	15-Mar.-00	Amity Banks
7	14-Mar.-00	Amity Banks
16	23-Jun.-00	Amity Banks
97	12-Jun.-00	Weeki Wachee, Florida
17	05-Apr.-00	Pebble Beach, Deception Bay
14	14-Apr.-00	Godwin Beach, Deception Bay

Species *L. majuscula* and *L. wollei* samples were collected from different areas and times in an effort to have a range of toxin levels.

High performance liquid chromatography–triple quadrupole mass spectrometry (HPLC/MS/MS) (PerkinElmer Sciex Instruments, Waltham, USA) was used for this analysis. The HPLC column was an Altima C18 (Alltech, Nicholasville, USA) 150 mm × 4.6 mm. Flow rate was 0.8 ml/min. Isocratic elution was used and the mobile phase consisted of 65% solvent A (60% CH_3CN/0.1% formic acid), 35% solvent B (95% CH_3CN/0.1% formic acid). The toxins of interest eluted over 17 min. Injection volume was 10 μl. The eluted product was split 1:4 to the MS. LA was measured at molecular ion ($M + H^+$) 438.3 and the fragment at 410. DAT was measured with ions ($M + Na^+$) 615.3 and fragment 543.3.

Appropriate quantities of toxin in aqueous methanol were selected to give desired concentrations in 20 μl and dried under a stream of nitrogen. Dimethyl sulphoxide (AMRESCO, Solon, USA) was used to dissolve extracts and purified *L. majuscula* toxins at appropriate concentrations for application to mice ears. Solutions were prepared immediately prior to application to mice ears. All chemicals used were of analytical standard or higher.

Six to eight-week-old female specific pathogen free (SPF) BALB/c mice were obtained from University of Queensland, Australian National University, Curtain University and University of Adelaide animal breeding programmes. Mice were maintained under Queensland Health Ethics Committee guidelines (approval no. NRC 3/98/20). All mice were housed in plastic boxes with wood shavings at 21 ± 3 °C and 55 ± 10% humidity with a 12-h light/dark cycle in animal facilities at Queensland Health Scientific Services. Standard pelletised diet (Narco Pty, Brisbane, Australia) and tap water were supplied ad libitum. Mice weighed 18–21 g at the start of the study and were weighed daily during the trial period. Ink tail marks were used for identification.

2.1. Mouse ear swelling test (MEST)

Although the MEST was originally developed as a murine model of the elicitation phase of a hypersensitivity response (Gad, 1994; Gad et al., 1986), it is used here referring to the test of irritancy (Hayes and Meade, 1999; Klimuk et al., 2000). An increase in ear thickness of over 20% has been used as a positive result, and no negative control (DMSO alone on opposite ear) saw an increase greater than 20%. Baseline mouse ear thickness of both right and left ears was measured using a micrometer (Mitutoyo, Tokyo, Japan). Then 20 μl of DMSO vehicle was applied to the dorsal and ventral surfaces of left ear pinna using a pipette (Gilson, Middleton, USA) and plastic disposable tip. DMSO (20 μl) containing toxin was applied to the right ear. Both ears were measured hourly for the first 8 h and then daily for at least 14 days. The measure of ear swelling was taken at