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Letter to the Editor

Sensitization Of A Child To Cyanobacteria After Recreational Swimming In A Lake

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Capsule Summary: We report a case of an 11 year-old white female who developed an allergic reaction after swimming in Lake Ontario, Canada. Specific IgE reactivity to various species of cyanobacteria extracts was found to be increased in the patient's serum. This case emphasizes the importance of cyanobacteria as an unrecognized allergen and that recreational exposure of sensitized individuals to freshwater cyanobacteria can lead to severe allergic reactions.

Key words

Allergy, sensitization, cyanobacteria, phycocyanin, microcystin

To the Editor:

Here, we report a case of an 11 year-old white female who developed an allergic reaction manifesting as severe facial swelling with periorbital edema and an erythematous pruritic blistering rash over her arms and hands (see Figure 1) after swimming in Lake Ontario at Presquile State Park and playing on the beach making bird's nests out of algae. Her mother subsequently confirmed with Canadian environmental health officials that the lake had an overgrowth of freshwater cyanobacteria. Skin prick testing to common aeroallergens were negative. Interestingly, despite being non-atopic, a serum sample sent to our laboratory was found to have increased specific IgE levels to cyanobacteria extract. This study's objective was to determine whether the patient's specific IgE to cyanobacteria was functionally relevant and to compare the allergenicity of various cyanobacteria species.

Cyanobacteria (a.k.a blue green algae) are a phylum of bacteria that grow in brackish and fresh water environments and are capable of producing toxins. Apart from their toxicity, cyanobacteria have been demonstrated to be sensitizing in susceptible individuals[1]. Sensitization have been attributed to several cyanobacteria species including: *Arthrospira*, *Oscillatoria*, *Lyngbya*, *Anabaena*, *Synechocystis*, *Aphanizomenon flos-aquae* and *Microcystis*[1–5]. More recently, severe cases of anaphylaxis have been reported following ingestion of the spirulina tablets containing cyanobacteria specie *Arthrospira*[2,3]. In all of these cases, the causes of sensitization have been diverse in terms of the cyanobacteria species implicated raising the possibility of cross-reactivity of various cyanobacteria species. In Lake Ontario for example, a number of cyanobacteria species have been detected including *Synechococcus, Oscillatoria, Anabaena, Aphanizomenon* and *M. aeruginosa* the latter being the most dominant species[6]. Previously, phycocyanin of *M. aeruginosa* was shown to be responsible for causing sensitization in a subset

of chronic rhinitis patients and the toxicity of these organisms is inversely proportional to their allergenicity[1,7]. Because all cyanobacteria species produce phycocyanin, we asked whether the patient was sensitized to one or more species of cyanobacteria. We performed an IgE specific ELISA using serum from the patient and extracts from a variety of laboratory strains of cyanobacteria. The results indicate varying degrees of cross reactivity with the majority of cyanobacteria species (see Figure 2A). While extracts from the microcystin non-producing strain of *M. aeruginosa* [MC(-)] (#2) and *Synechococcus sp.* (#5) showed the most reactivity, the extracts from *Nostoc* (#3) and *Scytonema ssp.* (#8) did not possess any IgE reactivity. Interestingly, IgE specific reactivity was also observed to *Arthrospira sp.* (#10) which, as previously mentioned, has been associated with anaphylaxis[2,3].

M. aeruginosa phycocyanin shows greater than 70% sequence similarities with phycocyanin from various species of cyanobacteria (see Supplemental Material, Table E1). To test if the allergen in these species is the same as the allergen found in MC(-), we performed an ELISA inhibition assay, using plates coated with *Synechococcus* sp. lysate (solid phase) and the MC(-) lysate as the serum inhibitor (solution phase). The results show that the MC(-) extract dose dependently inhibited binding of *Synechococcus sp.* lysate to specific IgE in the patient's serum indicating that the same allergenic peptide, phycocyanin, is present in both lysates (see Figure 2B). In order to demonstrate the functional relevance of the allergenic peptide, we assessed whether both the *Synechococcus* sp. and MC(-) lysates could release mediators using a rat basophil leukemic cell line that expresses high affinity human IgE receptor. As shown in Figure 2C, the patient's serum released approximately 7% and 10% of the total β-hexosaminidase content for *Synechococcus* sp. and MC(-) respectively, which was significantly greater than that released by

the control serum. This result indicates that both lysates could induce β-hexosaminidase release and confirms the functional relevance of specific IgE responses to these lysates.

Although *M. aeruginosa* has been isolated in Lake Ontario[6], we were unable to obtain any test samples from the same lake where the child played. Therefore, to test if the patient could potentially be sensitized by exposure to natural sources of cyanobacteria, axenic cultures of *M. aeruginosa* and *Oscillatoria sp*p. were isolated from environmental samples collected from Loch Norse (Northern Kentucky) and Great Lake St. Mary (Northern Ohio) respectively (see Supplemental Material, Figure E1A). While a specific IgE ELISA performed with *Oscillatoria sp.* showed immunoreactivity, similar to the laboratory strains (see Figure 2A), the *M. aeruginosa* species from Great Lake St. Mary did not show any reactivity (see Supplemental Material, Figure E1B). Further analysis revealed that this *M. aeruginosa* species produced a high amount of microcystin (271 ng/mL) which may have inhibited its allergenicity[7]. These results indicate that specific IgE in the patient serum reacts not only to extracts of laboratory strains but also to naturally occurring strains of cyanobacteria. The identity of the species causing the patients symptoms remains unclear, but assumptions can be made from the cyanobacteria (*M. aeruginosa)* samples isolated from Lake Ontario in the summers of 2001, 2003 and 2004. In the summer of 2004, data collected along the shores of Lake Ontario show the presence of high levels of phycocyanin and low levels of microcystin[8]. In addition, sequence analysis studies have identified unusual microcystin sequence features that may inhibit the production of microcystin in this particular strain[6]. Under these circumstances, it is reasonable to speculate that the *M. aeruginosa*, from Lake Ontario, produce less toxin and more phycocyanin rendering them more allergenic[7].

Thus, we conclude that this patient elicited functionally relevant specific IgE reactivity to various cyanobacteria species consistent with a previous exposure(s) leading to sensitization.

Cross-reactivity to multiple cyanobacteria species and selective inhibition assays indicate that phycocyanin is the major protein eliciting these allergic reactions. As cyanobacteria may act similarly to anemorphilous pollenating plants by being dispersed large distances resulting in remote outdoor and indoor exposure, sensitization in this child could have resulted from numerous potential sources. For example, cyanobacteria has been found to be more abundant in dust from the homes of children with asthma compared to the dust from homes of non-asthmatics children suggesting a possible association between cyanobacteria and asthma[9]. To our knowledge, this is the first case report confirming the functional relevance of cyanobacteria sensitization in a child after recreational exposure and that phycocyanin is the likely sensitizing protein. This case emphasizes the importance of cyanobacteria as an unrecognized ubiquitous allergen in allergic and non-allergic individuals and that recreational exposure of sensitized individuals to freshwater cyanobacteria can lead to severe allergic reactions. Further investigation into the prevalence of cyanobacteria sensitization in the general population is warranted given the high levels of cyanobacteria found in house dust samples and the amount of recreational time spent by Americans around fresh water lakes.

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METHODS

Reagents

Goat anti-human IgE and AP-conjugated rabbit anti-goat IgG were purchased from KPL (Gaithersburg, MD). Micro-titer ELISA plates from Corning (Corning, NY); EMEM from ATCC (Manassas, VA); G418 and pNPP substrate from Sigma-Aldrich (St. Louis, MO). Serum was collected from the patient and 3 non-atopic healthy control subjects, which were pooled and used as control sera in every experiment.

Cultivation of cyanobacteria and preparation of crude cell extracts

Toxic [MC(+)] and nontoxic [MC(-)] strains of *Microcystis aeruginosa* (The toxic strain has the gene coding for microcystin while the non-toxic strain does not), *Nostoc, Synechocystis., Synechococcus, Pseudanabaena, Oscillatoria, Scytonema, Lyngbya and Arthrospira* were obtained from The Culture Collection of Algae at the University of Texas (Austin, TX, USA). Growth of cyanobacteria cultures and preparation of crude cell extracts has been described elsewhere[7].

IgE-specific ELISA

IgE-specific ELISA was performed using a previously described method[7]. Briefly, 96-well polystyrene microtiter plates were coated with 100 µl/well of 10 µg/mL cyanobacterial lysates in 50 mM carbonate buffer, pH 9.6. The plates were incubated for 2 hr at room temperature (RT) and then overnight at 4°C. After overnight incubation, the wells were then emptied, washed 4 times for 5 min each with 0.05% Tween-20 in 0.01 M pH 7.4 PBS (PBS-T), and blocked for 1 hour with blocking buffer (2% BSA in PBS-T). One hundred µL of patient and control sera (diluted 1:10

with blocking buffer) were added onto the wells and further incubated for 1 hour at RT and then overnight at 4°C. The wells were washed 4 times with blocking buffer and incubated with goat anti-human IgE (diluted 1:1000 with blocking buffer) for 2 hours at RT, then washed again and incubated with alkaline phosphatase conjugated rabbit anti-goat IgG (1:1500). The plates were developed using p-nitrophenyl phosphate (pNPP) substrate and read at 405 nm using Multiskan Ascent 96 Plate Reader (Thermo scientific, Madison, WI).

ELISA inhibition

Serum from the patient was pre-absorbed at 4°C, overnight, with increasing concentrations of MC(-) lysate (solution phase). The MC(-) lysate-sera mixture was then added to wells coated with MC(-) and *Synechococcus sp.* lysates (solid phase). Subsequent steps of the inhibition assay was performed as described for the IgE-specific ELISA above.

Hexosaminidase Release Assay

A β-hexosaminidase release assay, which is a surrogate assay for measuring histamine release, was used to identify functional activity of *M. aeruginosa* and *Synechococcus sp.* extracts. Rat basophil leukemia cells (RBL SX-38; kindly provided by Dr. J-P Kinet, Harvard Medical School) cultured in complete medium (EMEM supplemented with 10 % fetal bovine serum) were seeded onto 96-well plates. The complete procedure for performing the assay has previously been described [7].

Statistical Analysis

All data are reported as the sample mean ± the standard deviation. A paired student's t-test was used to compare IgE reactivity of patient's serum to various cyanobacteria species while an unpaired Student's t-test was used to calculate the difference between patient and control sera in the β-hexosaminidase release assay.

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209 **Figure Legends**

210 **Figure 1.** Erythematous blistering rash on the child's hand after exposure to cyanobacteria.

211

212 **Figure 2.** ***(A*** IgE-specific ELISA using extracts from various cyanobacteria species [1-Toxic
213 [MC(+)], and 2-nontoxic [MC(-)] strains of *Microcystis aeruginosa, 3-Nostoc sp., 4-*
214 *Synechocystis sp., 5-Synechococcus sp., 6- Pseudanabaena sp., 7-Oscillatoria sp., 8-Scytonema*
215 *sp., 9-Lyngbya sp. and 10-Arthrospira sp.*] incubated with serum from patient (P) and pooled
216 control sera (N). Results from the patient/control sera (P/N) values for ELISA were then
217 calculated. Asterisk ($p<0.001$) and hashtags ($p<10^{-7}$) indicate a statistical significant difference
218 between patient's serum compared to pooled control sera. ***(B)*** ELISA inhibition of the
219 *Synechococcus sp.* lysate using *M. aeruginosa* [MC(-)] extract. ***(C)*** β-hexosaminidase release
220 assay using patient serum and extracts from *Synechococcus sp.* and *M. aeruginosa* [MC(-)].
221 Asterisk ($p<0.001$) and hashtags ($p<0.01$) indicate a statistical significant difference between
222 patient's serum compared to pooled control sera.

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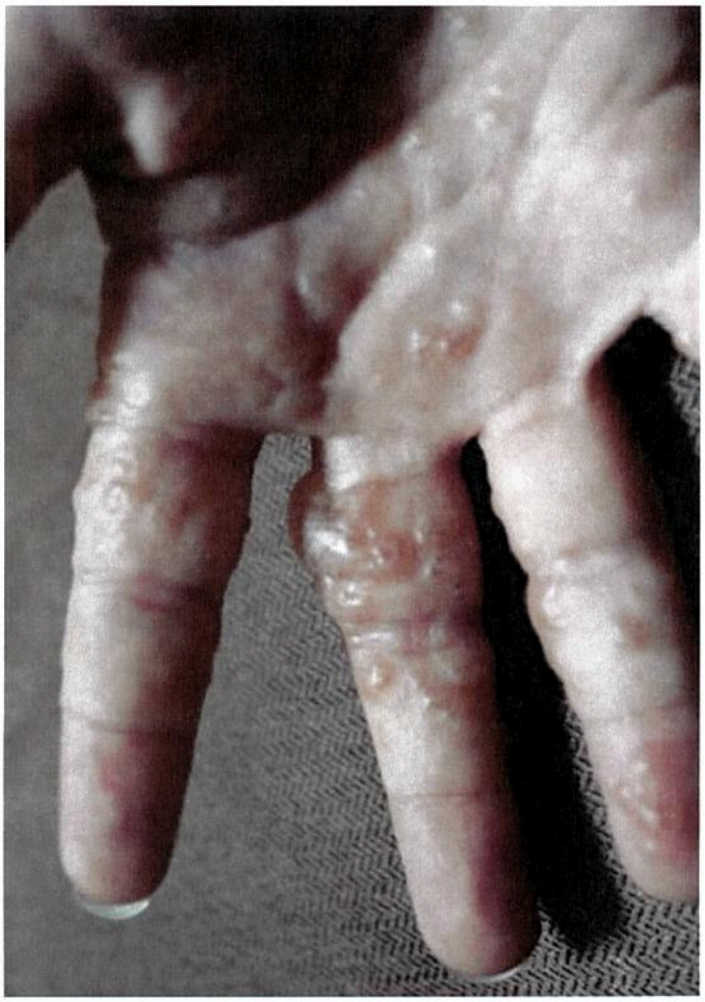

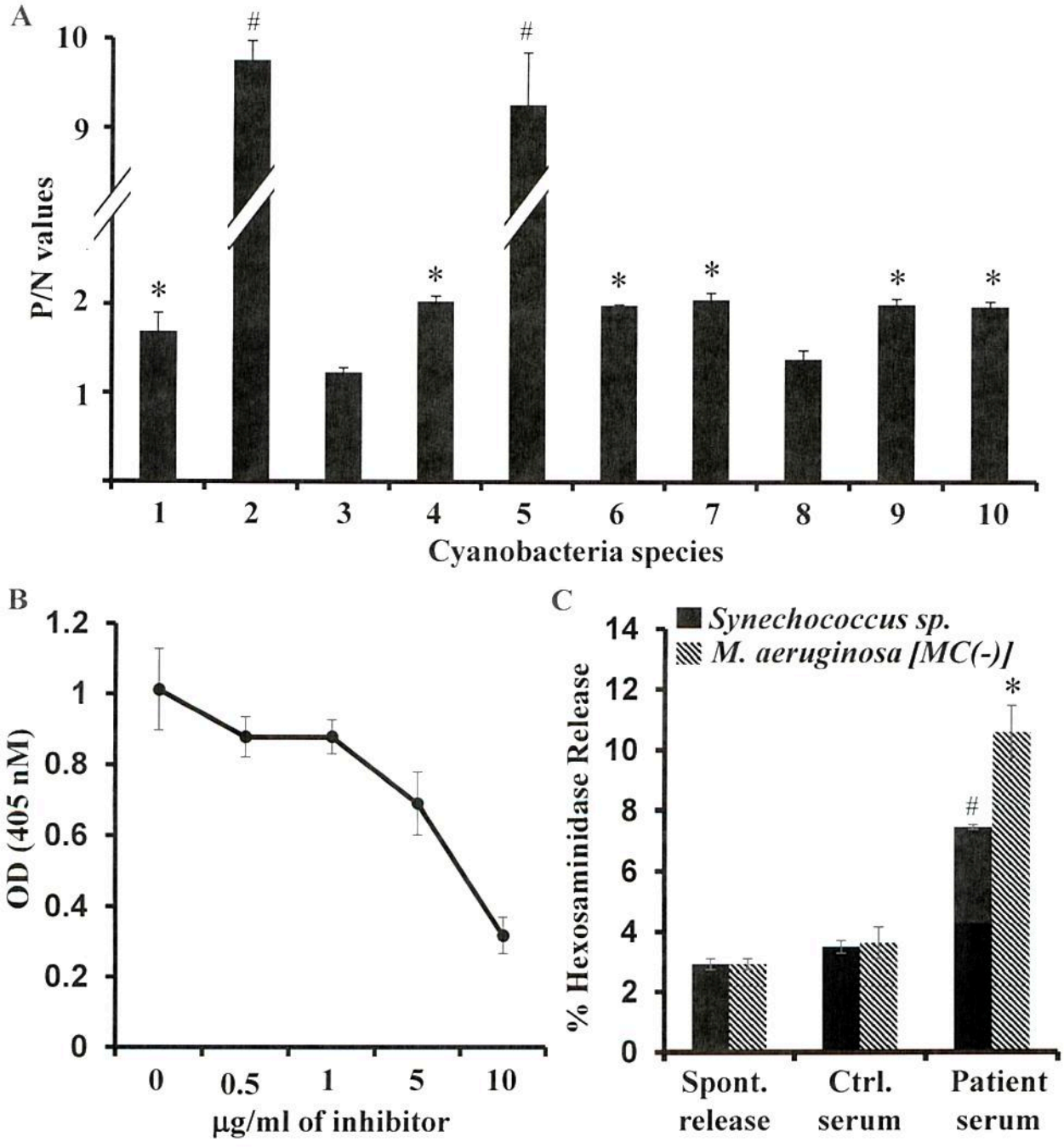
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Synechococcus sp.
M. aeruginosa [MC(-)]
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Spont. release
Ctrl. serum
Patient serum