

Cyanobacteria: An unrecognized ubiquitous sensitizing allergen?

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ABSTRACT

This study was designed to investigate the prevalence of skin sensitization using detoxified cyanobacterial reagents in a chronic rhinitis population. Subjects ≥ 6 years of age who presented for allergy consultation to a community allergy practice and required skin-prick testing (SPT) to common seasonal and perennial aeroallergens were enrolled after signing an informed consent. Detoxified cyanobacteria species were used for skin testing. Skin testing of unexposed, nonsensitized control subjects using these detoxified cyanobacterial skin test reagents was performed to identify irritant threshold responses. All subjects signed an Institutional Review Board–approved informed consent before participation. Two hundred fifty-nine patients ranging in age between 7 and 78 years old underwent testing. The majority were white female patients and over two-thirds (73.4%) were atopic. Seventy-four (28.6% of the population) patients were SPT⁺ to at least one of the cyanobacteria species. Positive SPTs were present in 86% of patients to *Microcystis aeruginosa* and 12% of patients to *Aphanizomenon-flos aquae*. There was a strong association between severity of atopy (number of positive SPTs), having allergic rhinitis and sensitization to one or more cyanobacteria species ($p < 0.001$). This is the first study to show that cyanobacterial allergenicity resides in nontoxin-containing components of this organism.

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Prokaryotic planktonic organisms are the oldest photosynthetic, oxygenic life forms that gave sharp rise to atmospheric oxygen ~ 2.5 billion years ago.^{1,2} They continue to contribute up to 30% of the yearly oxygen production on earth.^{1,2} The largest group of photosynthetic prokaryotes (cells without membrane-bound organelles) are classified as cyanobacteria. This group comprises ~ 165 genera and 1500 species.^{1,2} Cyanobacteria are found in all illuminated environments on earth, including hot springs, ice fields, soil, and even animal fur and play key roles in the carbon and nitrogen cycles of the biosphere.³ Two nontoxic species, *Arthrospira platensis* (*Spirulina*) and *Aphanizomenon-flos aquae*, are mass produced for consumption worldwide as protein-rich nutritional supplements.⁴ However, ~ 40 cyanobacterial species produce toxins, called cyanotoxins, with dermatotoxic, neurotoxic, and/or hepatotoxic properties, and have been implicated in animal and, rarely, human deaths.^{5,6} Because they are Gram-negative, they also contain lipopolysaccharide (LPS) or endotoxin as a major component of their cell walls.⁷ Inhaled and oral sensitization in humans to cyanobacteria has not been explored

because of their potential for combined exotoxin and endotoxin adverse effects. Because cyanobacteria occur as blooms on fresh and brackish water surfaces, routes of human exposure could occur through inhalation, direct oral ingestion, by eating contaminated food, or by simple dermal contact. Their presence indoors is most likely caused by contamination from outdoor sources as well as aerogenic dissemination from indoor water sources such as aquaria or humidifiers.⁸ Ingestion could occur by swallowing water from contaminated bodies of water or by eating or by consuming contaminated blue-green algae food supplements.^{6,9}

Previous studies have investigated the irritant response of detoxified cyanobacteria strains in normal volunteers by patch testing.^{10–12} Mittal *et al.* showed positive IgE-mediated skin tests to toxic strains of common aerogenic algae in the Delhi area. To date, there are no data available regarding whether nontoxic components in cyanobacteria are capable of sensitizing exposed chronic rhinitis individuals over time because of the lack of readily available skin-prick test (SPT) reagents devoid of toxins.¹³ Therefore, the purpose of this preliminary study was to investigate the prevalence of cyanobacteria skin sensitization using detoxified cyanobacterial reagents in a chronic rhinitis population.

METHODS

Study Population

After signing an Institutional Review Board–approved informed consent, subjects ≥ 6 years of age underwent SPT to seasonal and perennial aeroaller-

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gens and extracts of detoxified cyanobacterial species. Subjects with dermatographia or if taking medications that prohibited skin testing were excluded. A chart review was conducted for each subject to obtain information regarding potential sources of indoor cyanobacteria exposure.

Preparation of Skin and *In Vitro* Test Reagents

Removal of Exotoxins. Each cyanobacterial strain was detoxified by precipitating the protein from 1 g of the respective cyanobacterial lyophilized cells in 50 mL of 100% methanol and mixed for 10 minutes before centrifuging for 10 minutes at $10,000 \times g$. This process was repeated three times and the final product was dried over filtered nitrogen. The final methanol analyte of each cyanobacteria protein contained $\leq 10\%$ methanol.^{14,15} ELISA and high-performance liquid chromatography methods were used to ensure that <0.5 ng of hepatotoxin or microcystin (as microcystin LR, YR, RR, and LA), obtained from the *Microcystis aeruginosa* strain PCC7820, were not present.^{15,16}

Preparation of Skin Test Reagents. Dried, detoxified cyanobacteria were overlaid with 99% acetone, vortexed, and left over night at 4°C . After centrifugation at $10,000 \times g$ for 30 minutes, the supernatant was decanted and the cyanobacterial pellet was retained and air-dried. Aliquots were suspended in phosphate-buffered saline in a 1:20 w/v dilution, extracted by centrifugation in an ice bath for 15 minutes, vortexed, and left overnight at 4°C . After centrifugation, the supernatant was sterilized using a $0.22\text{-}\mu\text{m}$ filter (Millipore Corp., Bedford, MA), and aliquoted. Protein content (data not shown for each cyanobacteria strain) was assayed by the bicinchoninic acid protein method to confirm that sufficient protein was extracted.¹⁷ The range of protein content for each extract varied from 0.11 to 7.08 mg/mL.

SPT Diagnostic Testing. Cyanobacteria skin testing included nine species ubiquitous in freshwater blooms, soil, and air samples: *M. aeruginosa*, *A. platensis* (*Spirulina*), *Aphanizomenon-flos aquae*, *Oscillatoria*, *Synochocystis*, *Psuedanabaena*, *Scytonema*, *Lyngbya*, and *Synochococcus* administered as 1:20 w/v dilutions. Cyanobacterial SPTs were applied along with a panel of common aeroallergens, a negative saline, and positive histamine HCl (10 mg/mL) control. A positive SPT was defined as a wheal 3 mm or more than the saline control with erythema. All patients were physician diagnosed with allergic rhinitis (AR), nonallergic rhinitis (NAR), or mixed rhinitis (MR) based on correlation of history with SPT responses or serum-specific IgE and an irritant index score.¹⁸ Before skin testing, 10 nonallergic individuals without known cyanobacteria

Table 1 Frequency and percentage of gender, race, atopy, and cyanobacteria for 259 patients

Demographics	n	Percent
Gender		
Male	101	39.0
Female	158	61.0
Race		
White	209	80.7
AA/Asian	50	19.3
Atopy		
Yes	190	73.4
No	69	26.6
Cyanobacteria SPT*		
Positive	74	28.6
Negative	185	71.4

*Sixty-four positive to *Microcystis aeruginosa*, nine positive to *Aphanizomenon-flos aquae*, three positive to *Synochocystis*, one positive to *Lyngbya*.

AA = African-American; SPT = skin prick testing.

exposure underwent titration SPT to each reagent to identify irritating threshold responses.

Statistical Analysis

The Pearson's chi-square test and Fisher's exact test were used to test the association between cyanobacterial SPT and atopy, total positive test number of SPT, rhinitis type, and home exposures. The SAS9.2 (by SAS Institute, Inc., Cary, NC) was used and values of $p < 0.05$ were considered statistically significant.

RESULTS

The demographic characteristics of all patients' SPTs to cyanobacteria are summarized in Table 1. A total of 259 patients between 7 and 78 years old, 14 of whom were between the ages 7 and 18 years, underwent testing. The majority were white female patients and over two-thirds (73.4%) were atopic. Seventy-four (29% of the population) patients were SPT⁺ to at least one of the cyanobacterial strains. Predominant positive SPTs were to *M. aeruginosa* (86% of SPT⁺ subjects) and *Aphanizomenon-flos aquae* (12% of SPT⁺ subjects). There was no correlation between positive SPT to cyanobacteria and age, gender, or race. There was a strong association between being atopic and eliciting a positive SPT to one or more cyanobacterial species (Table 2; $p < 0.001$), which was associated strongly with the degree of atopy defined as the number of positive SPTs to common aeroallergens (Table 3; $p < .001$). Not surprisingly, skin sensitization to cyanobacteria was more prevalent in patients with a physician diagnosis of AR compared with MR and NAR diagnoses

Table 2 Frequency table for atopy and algae-positive skin-prick testing (SPT) for 259 patients *p* value is testing the association between atopy and algal-positive SPT*

Cyanobacteria	Atopy		Total
	Yes	No	
Positive	68	6	74
Negative	122	63	185
Total	190	69	259

* $p < 0.001$; *p* value is testing the association between atopy and algal-positive SPT.

Table 3 Frequency table for total positive test number of skin-prick tests (SPT) to aeroallergens and cyanobacterial skin sensitization ($n = 259$)

Cyanobacteria	Total Positive SPT of Common Aeroallergens			Total
	≤ 2	3~7	> 7	
Positive	11	41	22	74
Negative	78	49	58	185
Total	89	90	80	259

$p < 0.001$; *p* value is testing the association between total positive SPT to common aeroallergens and cyanobacteria sensitization.

Table 4 Frequency table for rhinitis types and cyanobacterial skin sensitization ($n = 234^*$)

Algae	Rhinitis			Total
	AR#	NAR	MR	
Positive	58	7	6	71
Negative	97	43	23	163
Total	155	50	29	234

*Twenty-five subjects were not given a diagnosis of rhinitis. # $p < 0.01$; *p* value is testing the association between chronic rhinitis subtypes and cyanobacteria sensitization.

AR = allergic rhinitis; NAR = nonallergic rhinitis; MR = mixed rhinitis.

(Table 4; $p < 0.01$). No significant association between cyanobacteria sensitization and nasal eosinophils was observed in a subset of patients ($n = 87$; data not shown).

Interestingly, a significant association between active smokers and cyanobacterial sensitization was found (Table 5; $p < 0.01$). Home characteristics were not associated with cyanobacterial sensitization (Table 5; $p > 0.05$).

DISCUSSION

Our investigation is the first to show that cyanobacterial allergenicity resides in a nontoxin-containing component of the organism. Our study did not investigate the possibility that an allergenic epitope could also exist in the toxic fraction of these organisms. The association between cyanobacterial sensitization was greatest in AR patients who had a higher percentage of positive SPT responses to seasonal and perennial aeroallergens. The positive correlation between smoking and cyanobacterial skin sensitization requires further investigation to better understand this possible relationship.

Animal hypersensitivity models have been used to investigate the allergenicity of toxic microalgae.^{12,19} One model evaluated contact sensitivity in guinea pigs and rabbits and did not see any clear dose-response relationship¹⁹; the other found no relationship between microcystin-containing cyanobacteria and delayed contact sensitivity using the mouse ear swelling test.¹² However, it was shown that the nontoxic *Aphanizomenon* genera exhibited greater allergenicity than toxic cyanobacterial strains.¹⁹ Our results found the greatest percent of skin sensitization to *M. aeruginosa* and *Aphanizomenon-flos aquae*, partially supporting the previous report concerning *Aphanizomenon-flos aquae*.¹⁹

The presence of cyanobacterial activity in aerial biota of both outdoor and indoor environments is well documented by stationary and moving samplers.^{20,21} Airborne dispersal patterns of cyanobacteria are variable based on proximity to soil algae populations and meteorological conditions, particularly wind direction and elevation.²² In a more recent survey of the subtropical city of Varnasi, India, it was observed that soil algae were dominant sources of aeroalgal flora and air-borne cyanobacteria were more common in warmer months of the year.²³ Most case reports and field studies have focused on occurrence of toxic symptoms in humans.^{24,25} However, in two prospective cohort studies, respiratory symptoms associated with higher concurrent cyanobacterial water content were among the most prominent patient-reported symptoms.^{24,25} Only the study by Stewart *et al.* found a statistically significant relationship between respiratory symptom reporting and cyanobacteria biomass.²⁵

Oral contact with pathogenic or nonpathogenic cyanobacterial species is an obvious route of exposure. A recent risk analysis estimated the number of water-borne illnesses in the United States to be 19.5 million/year and the potential risk of cyanobacterial toxins is often considered a health concern.²⁶ More significant is that several "nontoxic" cyanobacterial species are regularly consumed as nutritional supplements. One of these, *Spirulina* (*Arthrospira platensis*), is produced in large outdoor ponds, dried in solar evaporators, and

Table 5 Frequency tables for home exposures and cyanobacteria ($n = 259$)

Cyanobacteria	Air Conditioning	Forced Air Heat	Humidifier	Plants	Aquarium	Smoking Tobacco
Positive	73/250 (29%)	70/243 (29%)	11/42 (26%)	21/69 (30%)	3/14 (21%)	9/59 (15%)
Negative	177/250 (71%)	173/243 (71%)	31/42 (74%)	48/69 (70%)	11/14 (79%)	50/59 (85%)
p Value	0.23	0.74	0.71	0.69	0.54	0.01

The p values are testing the association between home exposures and cyanobacteria.

consumed worldwide because of its immunomodulatory effect in allergic conditions including AR.^{27,28} Another edible species, *Aphanizomenon-flos aquae*, harvested from Klamath Lake, OR, experiences periodic extensive contaminating blooms of *M. aeruginosa* containing potent hepatotoxic microcystins.⁹ Because microcystins were as high 10.89 $\mu\text{g/g}$ in some consumer products, the Oregon Health Department adopted a regulatory standard of 1 $\mu\text{g/g}$ as a safe microcystin level for this product.⁹ The IgE-sensitizing potential using nontoxic *Spirulina* and *Aphanizomenon* strains had not been determined before this current investigation.

Demonstration of human allergenicity to several cyanobacterial strains determined in this investigation appears to contradict the hypothesis that most eukaryotic proteins lack bacterial homologs, suggesting that prokaryotic organisms are relatively nonallergenic.²⁹ However, a number of exceptions to this hypothesis exist among Gram-negative and -positive heterotrophic bacteria.^{30–33} Some bacterial exotoxins and endotoxins act as potent adjuvants and/or may enhance IgE-mediated histamine release.^{34,35} Assuming this biological principle to be correct, it is possible that cyanobacterial allergenicity can be partially explained by concurrent adjuvant effects of LPS constituents within the plasma membranes of these Gram-negative autotrophs. Although endotoxin potency is variable among cyanobacterial species and strains compared with classic Gram-negative heterotrophs (e.g., *Escherichia coli*), LPS could exert adjuvant effects by stimulating the innate immune system, leading to or augmenting adaptive IgE-specific immune responses.³⁶ For example, transient flu-like syndromes have occurred a few hours after taking a bath, showering, or exposure to humidifiers with water containing elevated levels of cyanobacterial-derived endotoxin.^{37,38}

There are several limitations of this study that warrant mentioning. First, this is a cross-sectional study of a population seeking consultation for chronic rhinitis symptoms and therefore may not reflect the prevalence of cyanobacterial skin sensitization in the general population. To maximize generalizability of our results, we serially tested subjects presenting for assessment of

chronic rhinitis symptoms and therefore captured patients with AR, MR, and NAR conditions. Although some positive skin tests were noted in the NAR ($n = 7$) and MR ($n = 6$) groups, the number of positive skin test reactions was significantly greater in the AR group ($n = 58$; $p < 0.01$). Second, although this study shows sensitization to several toxic and nontoxic genera of cyanobacteria, it does not establish clinical relevancy, which will require more extensive attention to routes of exposure, appropriate sampling of proximate water/soil sources, indoor and outdoor aerobiological sampling, and laboratory-controlled provocation tests.

In summary, the results of this study provide a direct means of evaluating human skin sensitization apart from the toxic effects of cyanobacteria. Ideally, long-term, controlled, cohort studies should be designed to establish the clinical relevance of this observation. Such studies will likely assume greater importance as increased human exposure to cyanobacteria parallels widespread implementation of future unique applications using these organisms (i.e., biofertilization, biofuels, hydrogen production, and space habitation).^{39–42}

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