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Hemagglutination Method for Detection of Freshwater Cyanobacteria (Blue-Green Algae) Toxins

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Strains of the freshwater cyanobacteria (blue-green algae) *Anabaena flos-aquae* and *Microcystis aeruginosa* produced toxins that caused intermittent but repeated cases of livestock, waterfowl, and other animal deaths. They also caused illness, especially gastrointestinal, in humans. The most common group of toxins produced by these two species were peptide toxins termed microcystin, *M. aeruginosa* type c, and anatoxin-c. A method was found to detect the toxins which utilizes their ability to cause agglutination of isolated blood cells from mice, rats, and humans. The method could detect the toxin in samples from natural algal blooms, laboratory cultures, and toxin extracts. The method consists of: (i) washing lyophilized cyanobacteria cells with physiological saline (0.9% NaCl), (ii) centrifuging the suspension and then mixing portions of the cell-free supernatant with equal volumes of saline-washed erythrocytes in V-shaped microtiter plates, (iii) allowing the mixture to stand for 3 to 4 h, and (iv) scoring the presence of the toxin as indicated by blood cell agglutination. Nontoxic strains, as determined by intraperitoneal mouse bioassay of cyanobacteria or green algae, did not produce an agglutination response.

Over the last 100 years, about 12 species belonging to 9 genera of freshwater cyanobacteria (blue-green algae) have been implicated in case histories of animal poisonings. In most reports, however, the species responsible have been *Microcystis aeruginosa* Kütz emend Elenkin, *Anabaena flos-aquae* (Lyngb.) de Bréb, and *Aphanizomenon flos-aquae* (L.) Ralfs. These bloom-forming species have caused deaths of livestock and other animals in western Canada, midwestern United States, South America, Europe, Asia, South Africa, and Australia. These poisonings are of concern to veterinarians, wildlife biologists, environmentalists, and public health officials. There are several reviews on toxic cyanophytes; the most recent are by Moore (21), Collins (8), and Gorham and Carmichael (14, 15).

Structurally, the known toxins produced include alkaloids, peptides, and pteridines. Although the greatest and most dramatic animal losses have been due to alkaloid neurotoxins, deaths due to the peptide toxins are widespread and therefore of greater concern for animal and human health. It is now known that the peptide toxins are produced by strains of both *M. aeruginosa* and *Anabaena flos-aquae*. Some of the *Anabaena* toxins have been given names based on their gross toxicological signs when injected intraperitoneally into mice, rats, and chicks. At present, there are six toxins produced by different strains of *A. flos-aquae*; these are termed anatoxin (antx)-a, -a(s), -b, -b(s), -c, and -d (7). Of these, only antx-a has undergone extensive toxicological and pharmacological examination. It is a bicyclic tertiary amine (9) having potent pre- and postsynaptic effects on the nicotinic receptor (5, 25). Antx-b and -d also have gross neuromuscular activity, but their pharmacology and structure have not been investigated. Antx-c is a mixture of peptide or peptide-containing toxins which are produced by many strains of *A. flos-aquae*.

M. aeruginosa also appears to produce a toxin like antx-c, currently called *M. aeruginosa* type c toxin. Although their amino acid sequences are uncertain, it is known that all of these toxins are small peptides, perhaps cyclic, with a molecular weight ranging from about 500 to 1,700 (3, 14). Based on current evidence, antx-c and *M. aeruginosa* type c are related and perhaps are the same as the peptide toxin, termed microcystin, which was described for *M. aeruginosa* strain NRC-1 (4). Signs of poisoning in affected animals drinking from waters infested with these toxins have consistently indicated that the liver is engorged with blood and that punctate hemorrhages are present (11, 16, 19, 24, 26). Such effects are also observed with laboratory animals given intraperitoneal and oral dosages of freeze-dried clonal isolates or the toxin extract. Histopathology of mouse livers indicates that severe

erythrocyte accumulation occurs in the hepatic lobule with chronic or acute dosages of lyophilized cells containing these toxins or of the toxin extracts. Erythrocyte accumulation occurs in the area of the afferent portal veins (14, 16, 26; W. W. Carmichael, unpublished data).

Although almost all reports to date concerning toxicities from freshwater cyanobacteria involve livestock, waterfowl, and other small animals, there is evidence for their role in certain waterborne human illnesses. These gastrointestinal illnesses can result from accidental drinking of raw surface water (10) or water from municipal water supplies (2, 20; H. T. P. Sargunar and A. A. A. Sargunar, Proc. 4th Int. Symp. Mycotoxins and Phytotoxins, 1979, GIO). The mouse bioassay is currently the best available test for detection of toxicity in freshwater cyanobacteria, but other assay methods that are more sensitive and more discriminating among the different toxin types are needed. The consistently observed heavy accumulation of blood in the hepatic units of livers affected by these peptide or peptide-containing toxins resembles signs of poisoning previously reported for certain lectins (17, 18, 22). Since one of the main properties of lectins is their ability to agglutinate cells, especially erythrocytes, it was felt that this may also be part of the action for cyanobacteria toxins. Erythrocyte agglutination was therefore tried as a detection method for these toxins.

MATERIALS AND METHODS

Table 1 lists laboratory-cultured strains of green and blue-green algae used. Algal bloom samples were collected directly from a surface concentration or by concentration of the algae in the water by using standard plankton nets. All field samples, except those from Hastings Lake, were collected by persons from Alberta Environment, Division of Pollution Control. These field samples of concentrated and unconcentrated algal blooms were coarse filtered to remove vascular plant debris and then lyophilized.

Laboratory strains of cyanobacteria (nonaxenic) were maintained in screw-top test tubes and 25-ml Delong flasks, containing ASM-1-TR culture medium (6), at 22 ± 1°C with continuous illumination from Vita-Lite fluorescent tubes. Light intensity was approximately 100 $\mu E/m^2/s$ of photosynthetically active radiation. Cultures, grown in 1-liter Delong flasks, were lyophilized near the end of their log phase of growth (10^5 to 10^6 cells or filaments per ml).

Lyophilized cell material (100 mg) was suspended in 0.9% NaCl (physiological saline) to a concentration of 20 mg/ml. This concentration was chosen because it is in the top range for a natural water bloom (heavy cyanobacteria blooms are designated as greater than 3 g [dry weight] per liter [W. W. Carmichael and P. R. Gorham, unpublished data]) and provided enough material for a mouse bioassay. The suspension was washed twice with saline, using gentle centrifugation. After the last wash, the supernatant was used for the agglutination test.

Blood was obtained by cardiac puncture (using a heparinized syringe) from Sprague-Dawley rats (200 to 300 g) and ICR Swiss mice (20 to 25 g). For some tests, human type O rr and type AB RR packed erythrocytes, obtained from the University of Alberta Hospital blood bank, were also used. All samples were washed three times with physiological saline and resuspended to a concentration of 1.0%. Erythrocytes could be stored at 5°C for up to 4 weeks, using a 1:1 mixture of washed blood plus Alsever preservative (13). Hemagglutination tests were run with Cooke microtiter V plates. Four concentrations, in duplicate, of sample supernatant–1.0% erythrocytes were used: 1:1, 1:2, 1:4, and 1:10. Volumes were kept constant with saline. Volumes used were: 1:1, 100 μl of sample and 100 μl of blood; 1:2, 50 μl of sample, 50 μl of saline, and 100 μl of blood; 1:4; 25 μl of sample, 75 μl of saline, and 100 μl of blood; 1:10, 10 μl of sample, 90 μl of saline, and 100 μl of blood. The microtiter plates were covered and allowed to sit for 3 to 4 h at room temperature. A positive response (++) was scored when clumping of erythrocytes cells was observed in the test well. A partial positive (+−) was scored when about one-half of the cells had settled to the bottom of the test well and the rest remained diffused. A negative response (−−) was noted when a well-defined spot of erythrocytes was present at the bottom of the well, along with a clear solution on top (Fig. 1B).

Acute toxicity of the cultures and samples of different blooms was tested by intraperitoneal injection of

TABLE 1. *Laboratory cultures of freshwater cyanobacteria and green algae used for testing erythrocyte hemagglutination*

Culture strain	Toxin type[a]
Anabaena flos-aquae	
NRC-44-1	a
M-20	a
NRC-525	a(s)
A-52	b
S-UTH-1	b(s)
S-23	d
F-1	c
A-113	c
A-120	c
A-128	c
M-13	c
M-5	c
Microcystis aeruginosa	
NRC-1-SS-17	Microcystin
A-143	*M. aeruginosa* type c
A-207	*M. aeruginosa* type c
A-272	*M. aeruginosa* type c
S-33	*M. aeruginosa* type c
S-61	*M. aeruginosa* type c
A. flos-aquae 1444	NT
Lyngbya boregerti M-9	NT
L. versicolor 1092	NT
Lyngbya sp. 622	NT
Tolypothrix sp.	NT
Cosmarium sp.	NT
Euglena sp.	NT
Oedogonium sp.	NT
Scenedesmus sp.	NT
Selenastrum capricornutum	NT

[a] As determined by intraperitoneal mouse bioassay (milligrams of lyophilized cells per kilogram of body weight). *NT*, Nontoxic at 1,500 mg/kg.

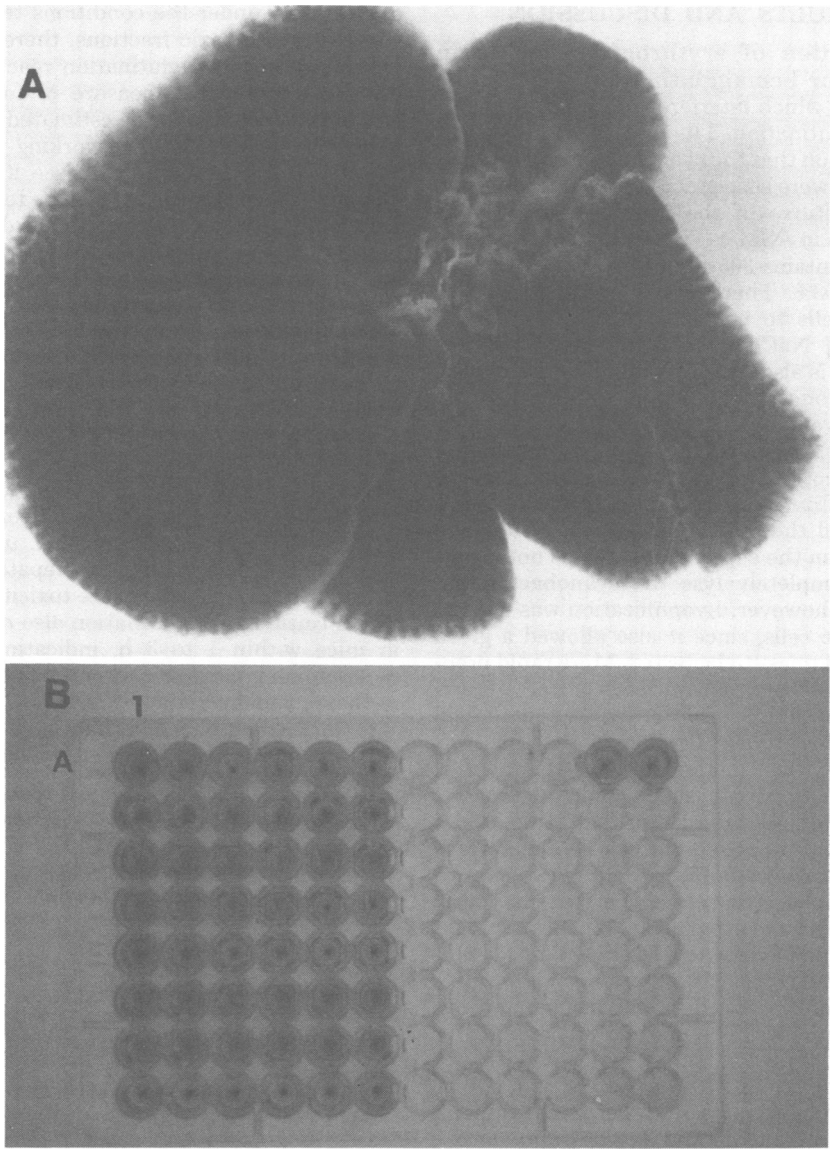

FIG. 1. *(A) Excised mouse liver after an intraperitoneal acute dose (approximately 30 mg/kg) of lyophilized M. aeruginosa producing antx-c. The liver can also be swollen with blood and appear much darker than in this photograph. Similar signs would be seen using extracted toxins. (B) Cooke microtiter plate showing the range of responses seen with different cyanophyte strains and concentrations of sample. Replicates are seen as rows 1 and 2, 3 and 4, and 5 and 6. Highest concentration, i.e., 100-μl sample, is in rows 1 and 2. For example, ++ is recorded under rows C, F, and G for all concentrations. A −− response (nontoxic strain or sample) can be seen for all of row H. An example of the endpoint of a response can be found between D3,4, and D5,6. A11,12 is a negative control (erythrocytes plus saline).*

male mice weighing 20 to 25 g. Several dilutions of each sample were tested, using three to five mice each, until a point was reached at which all the mice lived. The 50% lethal dose (milligrams per kilogram of body weight) was then estimated as the midpoint between the concentration at which the mice lived and the concentration at which they all died. Survival times in the mice for antx-c microcystin- or microcystis type c-producing strains were all about 2 to 3 h. Liver damage was indicated by a swollen, enlarged, reddened condition plus red spots revealing erythrocyte accumulation in the hepatic units (Fig. 1A).

RESULTS AND DISCUSSION

Preparation of erythrocytes and toxin extracts for hemagglutination. Two factors were found which interfered with proper erythrocyte agglutination. The first involved the salt concentration that the cyanobacteria, algal cells, or extracts were suspended or dissolved in. Laboratory cultures of the cyanobacteria strains were grown in ASM-1-TR medium. This culture medium contains 305 mg of inorganic salts per liter, or 0.03%. Therefore, it was necessary to wash all cells to be tested with physiological saline (0.9% NaCl) to prevent lysis of erythrocytes. This is also true for water bloom samples, since salt concentrations in a typical eutrophic lake can average about 500 to 700 mg/liter, or 0.5 to 0.7%. Second, it was found that antx-c toxins are not secreted as much as antx-a; therefore, lyophilized cells were used for the test since this allowed the maximum amount of toxin to be present in the supernatant. It was not necessary to completely lyse the cyanobacteria or algal cells, however. Lyophilization was used to prepare the cells, since it also allowed a given weight of biomass to be tested. More rapid preparation of cells such as sonication or freeze-thawing of a field sample also worked. In a rapid field analysis where quantification of the cells was not needed, these methods of cell treatment or others such as osmotic shock could be used.

Results of all blood agglutination tests. Tables 2 and 3 present the results for agglutination tests using laboratory cultures and water-bloom samples, respectively, of selected toxic cyanobacteria and other algae. Whenever mouse toxicity could be detected, i.e., liver damage plus death within 1 to 3 h, there was a positive agglutination produced in rat, mouse, and human erythrocytes. The method was specific for microcystis type c or antx-c toxins and did not give false positives with antx-a, -b, or-d. Toxic fractions from extracts of *M. aeruginosa* microcystin or type c toxin and *A. flos-aquae* antx-c gave a positive erythrocyte agglutination, whereas nontoxic fractions gave a negative agglutination (data not shown). This provided evidence that it was the intraperitoneal mouse toxic fraction of the cells which caused the hemagglutination. The endpoint for any hemagglutination response was about 2.5 mg of cells per ml (Tables 2 and 3). Therefore, to check the toxicity of an algal bloom, the concentration should be about 2 to 3 g/liter, a heavy bloom. Lower algal bloom concentrations could be tested provided the material was concentrated with a dip net or by another method that provided enough cells for the agglutination assay.

The agglutination assay described here is not quantitative under the conditions tested. Even with extracted toxic fractions, there is enough variability in the agglutination reaction that a reliable quantitative measure of toxicity, i.e., 50% lethal dose, cannot be estimated for a given culture or sample. We are working to improve the methods of extraction to see if there is a quantitative use for the assay. The test reported here is sensitive for a toxin produced by strains of these cyanophytes which causes death in mice within 1 to 4 h. The toxin has the ability to cause tissue damage, particularly in the liver, giving a characteristic accumulation of erythrocytes in the hepatic units. The toxin also has erythrocyte-agglutinating properties. Quantities of the toxin are released into the environment both naturally and after lyophilization of the algae, allowing a positive agglutination reaction. There is evidence from toxin extractions of different strains that more than one toxic component is produced (11, 14, 26; Carmichael, unpublished data). This indicates that the hepatotoxin may not be the only cause of acute toxicity. Extracts which cause hemagglutination also cause death in mice within 1 to 3 h, indicating that the hepatotoxin is a major part of the toxic principle in these cyanobacteria.

It can be reemphasized that, in all cases studied, a positive hemagglutination indicates the

TABLE 2. *Hemagglutination results from laboratory cultures of selected cyanobacteria*

Culture[a]	Hemagglutination response of supernatant concn (mg [dry wt] of cells/ml)				Mouse toxicity[b] (mg/kg)
	10	5	2.5	1	
A. flos-aquae					
NRC-44-1	−−	−−	−−	−−	Antx-a
M-20	−−	−−	−−	−−	Antx-a
NRC-525	−−	−−	−−	−−	Antx-a(s)
S-UTH-1	−−	−−	−−	−−	Antx-b(s)
A-52	−−	−−	−−	−−	Antx-b
S-23	−−	−−	−−	−−	Antx-d
A-113	++	++	+−	−−	100
A-120	++	++	+−	−−	100
A-128	++	+−	+−	−−	50
M-13	++	++	++	−−	50
M-5	++	++	++	−−	50
F-1	++	++	+−	−−	300
M. aeruginosa					
NRC-1-SS-17	++	++	++	−−	50
A-143	++	++	−−	−−	100
A-207	++	++	++	+−	500
A-272	++	++	−−	−−	100
S-33	++	++	+−	−−	100
S-61	++	+−	−−	−−	100

[a] All other cultures listed in Table 1 showed no agglutination or mouse toxicity.

[b] Expressed as intraperitoneal 50% lethal dose. Data are for signs of poisoning with antx-c, microcystin, or microcystis type c toxin. Antx-a, -b, -a(s), -b(s), and -d all have neurotoxic properties; therefore, toxicity is not reported here.

TABLE 3. *Hemagglutination results from field material collected in July and August 1979*

Collection site	Bloom composition[a]	Sample concn (mg [dry wt] of cells/ml)				Mouse toxicity[b] (mg/kg)
		10	5	2.5	1	
North Dakota	100:0:0:0	++	++	++	−−	1,000
Hastings Lake[c] (July 22)						
1	70:5:20:5	++	++	++	−−	—[d]
2	85:Tr:3:12	++	++	+−	−−	350
3	45:46:1:7	++	++	+−	−−	350
4	88:2:1:9	++	++	+−	−−	350
5	86:Tr:0:14	++	−−	+−	−−	200
6	72:8:8:13	++	++	+−	−−	200
7	90:2:4:4	++	+−	−−	−−	200
8	79:21:Tr:0	++	++	+−	−−	200
9	92:Tr:8:0	++	++	+−	−−	350
10	98:1:1:0	++	+−	−−	−−	200
Hastings Lake (July 31)						
1	78:14:7:1	++	+−	−−	−−	350
2	70:21:4:5	++	++	+−	−−	125
3	73:2:11:15	++	++	+−	−−	125
4	73:6:12:8	++	+−	−−	−−	125
5	82:Tr:8:10	++	−−	−−	−−	125
8	83:13:3:1	++	++	++	+−	125
9	82:11:6:2	++	+−	−−	−−	125
10	90:2:5:3	++	+−	−−	−−	125
Lac La Bich	0:Tr:100:0	−−	−−	−−	−−	NT
Pigeon Lake						
1	7:Tr:0:93	−−	−−	−−	−−	NT
2	8:15:0:78	−−	−−	−−	−−	NT
3	0:41:0:59	−−	−−	−−	−−	NT
Lac La Nonne						
1	16:0:76:8	++	+−	−−	−−	2,000
2	0:0:81:19	++	−−	−−	−−	2,000
3	8:0:67:25	++	−−	−−	−−	2,000
Gull Lake						
1	7:0:0:93	−−	−−	−−	−−	NT
2	Tr:4:0:96	−−	−−	−−	−−	NT
Wizard Lake						
1	35:5:10:50	++	++	+−	−−	250
2	33:3:3:61	++	++	+−	−−	125
3	33:8:17:42	++	++	+−	−−	125

[a] *M. aeruginosa/Anabaena flos-aquae/Aphanizomenon flos-aquae*/other. Tr, Trace (<1%).
[b] Expressed as in Table 2.
[c] These samples also tested positive against human type AB and O blood.
[d] Mouse bioassay not done.

presence of toxic factors which can cause the acute toxicity; therefore, even if it is not directly responsible for acute toxicity, its presence is an indication of a toxic water bloom condition. The hemagglutinating peptides produced by these toxic cyanophytes are not the only known phytohemagglutinins, for many are produced by higher plants as well. These higher plant agglutinins are part of a broad group of sugar-containing proteins called lectins. Plant lectins are produced primarily by the legumes such as castor beans, navy beans, peanuts, and lentils (22). Plant lectins are also toxic, and some reports list effects on the liver similar to those seen with these cyanobacterial toxins (17, 18). In addition, many marine algae produce hemagglutinating compounds (12). Higher plant lectins, however, have molecular weights in the range of 100,000, whereas molecular weights of antx-c and related peptides are reported to be in the range of 500 to 1,700 (14). Survival times with acute dosages of plant lectin are typically much longer (2 to 4 days) than with these toxins of cyanobacteria. Similarity among the toxins is suggested, however, and the assay described here should help to determine the nature of these small peptide toxins, their role in aquatic communities, and their effect on animal and human health.

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